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Rescue of infant progressive familial intrahepatic cholestasis type 3 mice by repeated dosing of AAV gene therapy

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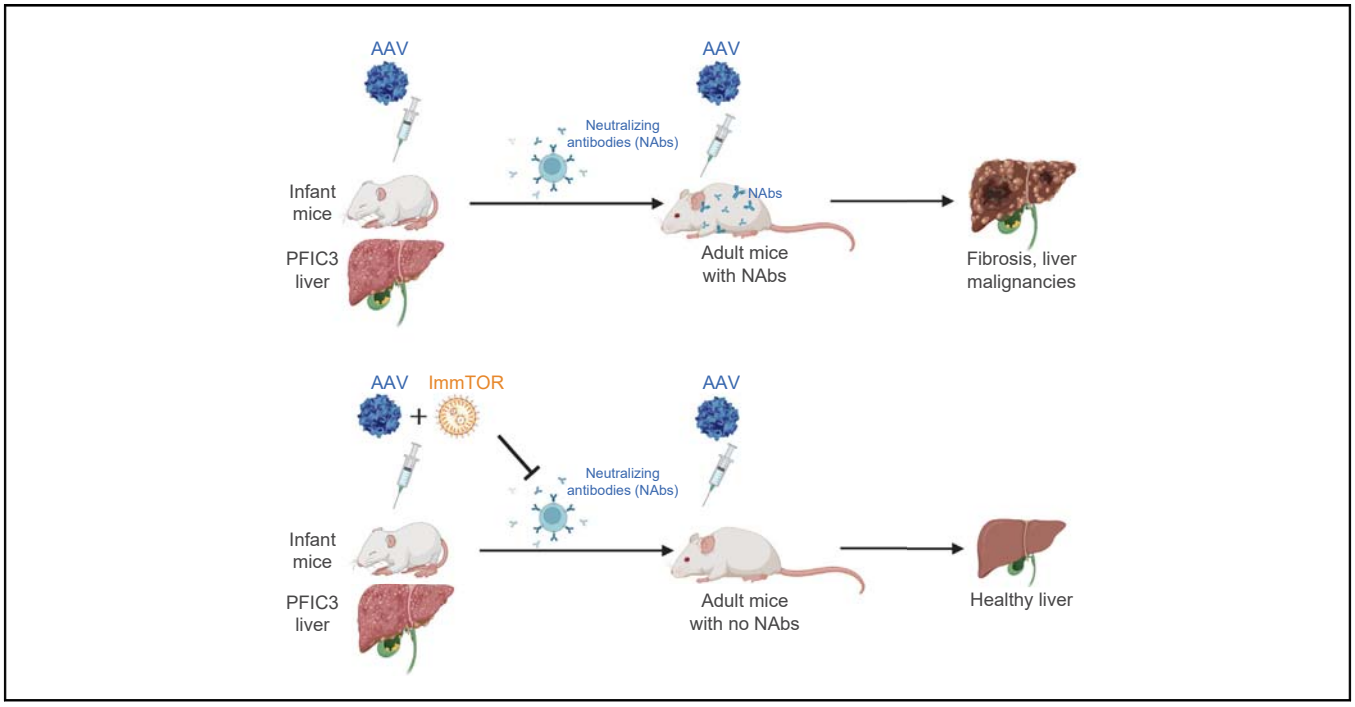
Graphical abstract



Highlights

- Gene therapy redosing may be necessary to maintain efficacy in paediatric patients with inborn hepatobiliary disorders.
- Co-administration with tolerogenic nanoparticles carrying rapamycin enabled an efficacious second administration of AAV.
- Only following a second AAV dose after treatment with AAV plus ImmTOR did infant PFIC3 mice exhibit a therapeutic effect.
- Repeat administration also prevented the appearance of liver malignancies in mice prone to hepatocellular carcinoma.

Impact and implications

Redosing of gene therapy for inborn hepatobiliary disorders may be essential as effect wanes during hepatocyte division and renewal, particularly in paediatric patients, but the approach may carry long-term risks of liver cancer. Viral vectors carrying a therapeutic gene exerted a durable cure of progressive familial intrahepatic cholestasis type 3 in infant mice and reduced the risk of liver cancer only following a second administration.

https://doi.org/10.1016/j.jhepr.2023.100713

Research article

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Rescue of infant progressive familial intrahepatic cholestasis type 3 mice by repeated dosing of AAV gene therapy

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JHEP Reports **2023.** https://doi.org/10.1016/j.jhepr.2023.100713

Background & Aims: Gene therapy using recombinant adeno-associated virus (rAAV) vector carrying multidrug resistance protein 3 (MDR3) coding sequence (AAV8-MDR3) represents a potential curative treatment for progressive familial intrahepatic cholestasis type 3 (PFIC3), which presents in early childhood. However, patients with the severest form of PFIC3 should receive treatment early after detection to prevent irreversible hepatic fibrosis leading ultimately to liver transplantation or death. This represents a challenge for rAAV-based gene therapy because therapeutic efficacy is expected to wane as rAAV genomes are lost owing to hepatocyte division, and the formation of AAV-specific neutralising antibodies precludes re-administration. Here, we tested a strategy of vector re-administration in infant PFIC3 mice with careful evaluation of its oncogenicity – a particular concern surrounding rAAV treatment.

Methods: AAV8-MDR3 was re-administered to infant *Abcb4*$^{-/-}$ mice 2 weeks after a first dose co-administered with tolerogenic nanoparticles carrying rapamycin (ImmTOR) given at 2 weeks of age. Eight months later, long-term therapeutic efficacy and safety were assessed with special attention paid to the potential oncogenicity of rAAV treatment.

Results: Co-administration with ImmTOR mitigated the formation of rAAV-specific neutralising antibodies and enabled an efficacious second administration of AAV8-MDR3, resulting in stable correction of the disease phenotype, including a restoration of bile phospholipid content and healthy liver function, as well as the prevention of liver fibrosis, hepatosplenomegaly, and gallstones. Furthermore, efficacious repeat rAAV administration prevented the appearance of liver malignancies in an animal model highly prone to developing hepatocellular carcinoma.

Conclusions: These outcomes provide strong evidence for rAAV redosing through co-administration with ImmTOR, as it resulted in a long-term therapeutic effect in a paediatric liver metabolic disorder, including the prevention of oncogenesis.

Impact and implications: Redosing of gene therapy for inborn hepatobiliary disorders may be essential as effect wanes during hepatocyte division and renewal, particularly in paediatric patients, but the approach may carry long-term risks of liver cancer. Viral vectors carrying a therapeutic gene exerted a durable cure of progressive familial intrahepatic cholestasis type 3 in infant mice and reduced the risk of liver cancer only following a second administration.

Introduction

Progressive familial intrahepatic cholestasis type 3 (PFIC3) is a rare metabolic disorder that arises from genetic alterations in the ATP binding cassette subfamily B member 4 (*ABCB4*) gene,[1] which encodes multidrug resistance protein 3 (MDR3), a flippase involved in the translocation of phosphatidylcholine (PC) from hepatocytes into the bile.[2] PC is an important component of bile that is necessary to form mixed micelles with bile salts and cholesterol (CHOL).[3] Without sufficient PC transport into bile, the concentration of free bile acids is highly elevated, increasing the detergent properties of the bile and causing toxic injury to the canalicular membrane of hepatocytes and cholangiocytes, resulting in hepatic parenchyma and bile duct damage. Biliary obstruction may result from long-term damage of biliary epithelium and from formation of gallstones as a result of reduced phospholipid concentration in the bile. This pathology leads to cholestasis, cirrhosis, and ultimately end-stage liver disease, which is fatal in the absence of liver transplantation.[4]

PFIC3, like many inborn errors of metabolism, poses a prime target for curative treatment approaches based on gene therapy.[5] Indeed, preclinical and proof-of-concept studies have shown the

Keywords: Metabolic disorders; Child; Liver cancer; Immunological tolerance.
Received 22 August 2022; received in revised form 1 February 2023; accepted 3 February 2023; available online 24 February 2023

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validity of gene supplementation strategies using mRNA[6] and adeno-associated virus (AAV) vectors.[7,8] We previously developed and tested a therapeutic AAV vector for PFIC3 via a series of *in vitro* and mouse model studies.[9] This vector comprises a codon-optimised version of the *ABCB4* gene encoding human MDR3 isoform A protein under control of the liver-specific alpha-1 antitrypsin promoter flanked by AAV serotype 2 inverted terminal repeats housed in an AAV serotype 8 (AAV8) capsid. The therapeutic efficacy of AAV8-MDR3 was tested in an FVB background strain mouse model for PFIC3 that lacks the first two exons of the *Abcb4* gene, resulting in a total lack of multidrug resistance protein 2 (MDR2) protein (the mouse orthologue of human MDR3) expression and activity.[10] This model, in the absence of treatment, reliably presents many of the same phenotypic manifestations of MDR3 deficiency observed in children, such as elevated serum liver enzymes, hepatosplenomegaly, reduced biliary phosphatidylcholine, cholelithiasis,[11] and progression to severe liver fibrosis.[12] FVB.*Abcb4*$^{-/-}$ mice treated with AAV8-MDR3 showed a complete reversal of this pathology when administered at 2 or 5 weeks of age, normalising levels of disease biomarkers to those observed in wild-type (WT) controls. However, a non-inconsequential shortcoming of the therapy was the loss over time of the therapeutic effect. This appeared to be driven by the loss of MDR3 expression, resulting in an increase in liver damage and hepatocyte regeneration and the creation of a positive feedback loop. The appearance of PFIC3 disease manifestations in a subset of the study animals was accompanied by hepatosplenomegaly, a complete reversion of bile PC to untreated levels and the appearance of liver fibrosis. Thus, evidence from these first studies supported a common assumption in gene therapy approaches for paediatric and degenerative liver diseases that re-administration may be essential to maintain the phenotypic correction of the disorder.[13–16]

Despite its potential in achieving long-term therapeutic benefit, re-administration of an AAV-based gene therapy vector would have to circumvent AAV-specific neutralising antibodies (NAbs)[17] and immunological memory[18] produced as a consequence of the first administration. One approach is the use of vectors with different capsid serotypes for subsequent administrations,[19–21] which has the drawbacks of potentially reduced transduction efficiency and the required development of multiple vectors. The use of techniques to remove antibodies from circulation such as plasmapheresis, immunoadsorption, or immunoglobulin G proteases has shown promising results but only for low antibody titres.[22–25] Alternatively, an approach that prevents the immune response during the initial vector administration could allow for subsequent rounds of the same AAV treatment. Paramount to this approach is the prevention of AAV-specific NAbs.[26]

Tolerogenic nanoparticles containing rapamycin (ImmTOR) have demonstrated the ability to induce durable immunological antigenic tolerance when co-administered with biologic drugs, including enzymes, monoclonal antibodies, and AAV.[27–32] ImmTOR nanoparticles, when injected intravenously, are taken up by antigen-presenting cells such as dendritic cells or macrophages in the spleen and liver.[33,34] Following endocytosis, the rapamycin is released from the biodegradable nanoparticle and induces cell programming towards a tolerogenic state.[35] Immunological tolerance results from the induction of antigen-specific regulatory T cells, lower activation of B cells, and, therefore, an inhibition of the production of antibodies.[29] This status allows for repeat administration of a therapeutic drug with similar activity as a result of an absence of neutralisation.[30] Previous studies have demonstrated the ability of ImmTOR to enable redosing of AAV vectors in adult animals.[29,30] Therefore, it was hypothesised that co-administration of ImmTOR nanoparticles with AAV8-MDR3 might mitigate formation of specific NAbs against the vector and allow for its re-administration to rescue infant PFIC3 mice.

Here, we evaluated this hypothesis with AAV8-MDR3 co-administered with ImmTOR. FVB.*Abcb4*$^{-/-}$ mice that received re-administration following a first dose co-administered with ImmTOR were unique in showing a robust and durable therapeutic effect as shown by the correction of all disease markers and evidence of the prevention of NAb formation from the first AAV treatment. Of particular importance, the AAV8-MDR3 plus ImmTOR treatment was administered to mice at 2 weeks of age, demonstrating to our knowledge the first instance of a successful immunotolerogenic effect and vector redosing in study animals that are representative of young paediatric patients. Moreover, the treatment rather than inducing oncogenicity showed a clear inhibitory effect in the formation of liver malignancies.

Materials and methods

A detailed description of the materials and methods used in this study is included in the Supplementary information. The graphical abstract was created with Biorender.com.

Results

Identification of subtherapeutic dose of AAV8-MDR3

We first performed a dose range finding study of AAV8-MDR3 in 2-week-old infant FVB.*Abcb4*$^{-/-}$ mice of both sexes to identify a subtherapeutic dose that would transiently delay the appearance of cholestasis symptoms. It was predicted that a transient treatment-induced normalisation of cholestatic serum biomarkers would indicate a delay in the appearance of liver fibrosis, allowing for a second AAV treatment to be tested for the ability to provide long-term correction of the disease without transduction efficiency being impaired in a fibrotic liver. Mice were treated intravenously (IV) with AAV8-MDR3 at doses of 2×10^{13}, 4×10^{13}, and 8×10^{13} vector genomes (vg)/kg and then followed up weekly for levels of cholestasis biomarkers in the serum. Although the two lower doses were ineffective at normalising biomarker levels, the 8×10^{13} vg/kg dose was transiently effective in reducing serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), bile acids (BA), and bilirubin (BIL) (Fig. 1A–D and Fig. S1A and B). After 11 days of AAV8-MDR3 administration, any evidence of a therapeutic effect was lost. Upon sacrifice 31 days post injection (p.i.), none of the treated mice showed any reduction in hepatosplenomegaly (Fig. 1E and F), a characteristic feature of diseased animals, or any improvement in biliary PC levels (Fig. 1G). Animal body weights were unaffected by treatment ($p = 0.67$, two-way analysis of variance [ANOVA]; Fig. S2). The 8×10^{13} vg/kg dose was thus selected for the next studies.

Re-administration of AAV8-MDR3

Next, we investigated the immunomodulatory properties of ImmTOR when co-administered with the sub-therapeutic dose of 8×10^{13} vg/kg AAV8-MDR3. Two-week-old male and female mice were treated IV. with AAV8-MDR3 with or without co-administration of ImmTOR (equivalent of 15 mg/kg rapamycin).

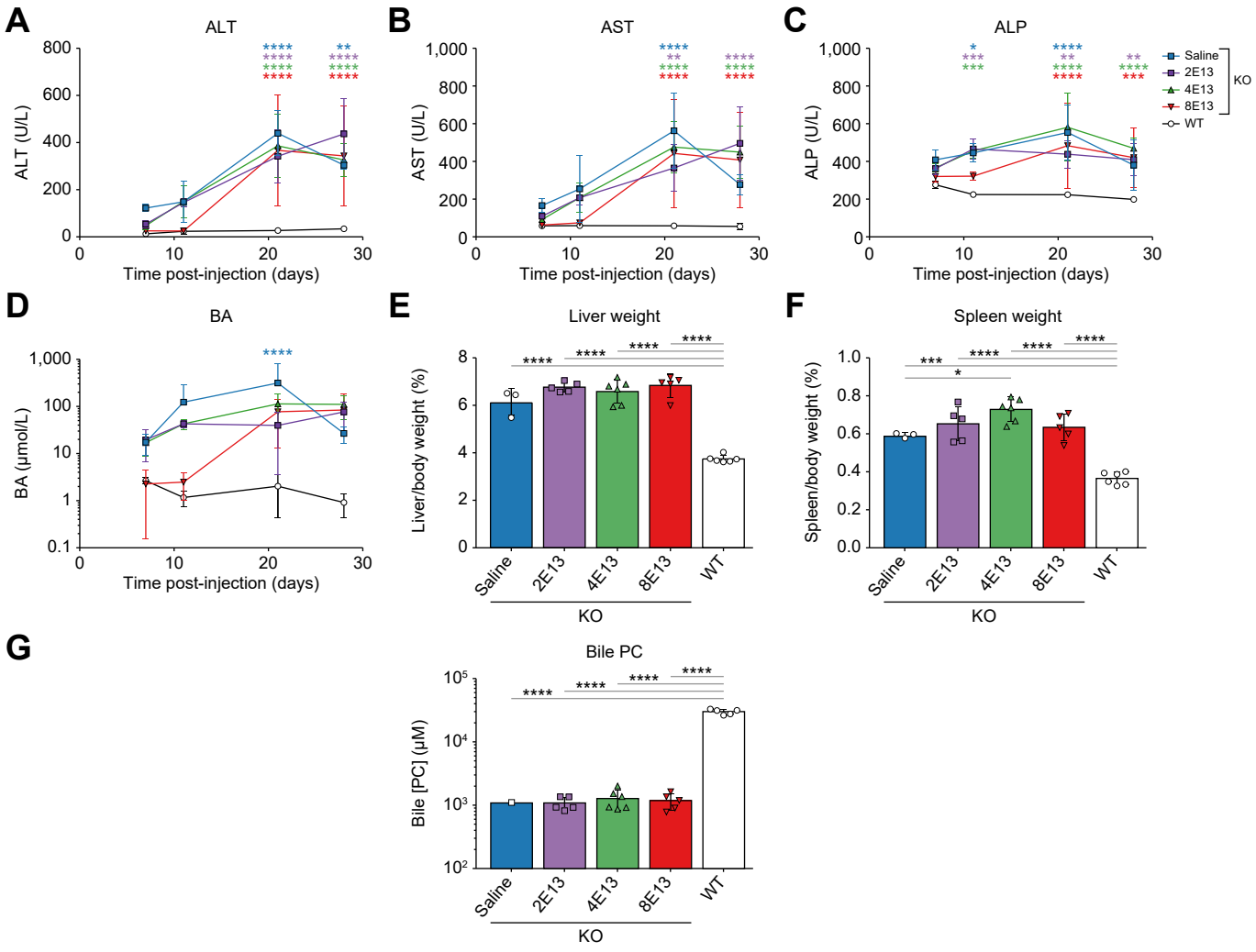

Fig. 1. Single administration of subtherapeutic doses of AAV8-MDR3 in infant FVB.*Abcb4*$^{-/-}$ mice can exert a transient therapeutic effect. FVB.*Abcb4*$^{-/-}$ mice (n = 3M/2–3F for each treated group; n = 2M/1F for saline) were treated IV with AAV8-MDR3 at 2 × 10^{13}, 4 × 10^{13}, or 8 × 10^{13} vg/kg at 2 weeks of age. (A) ALT, (B) AST, (C) ALP, and (D) BA in serum are indicated at 7 to 28 days post AAV treatment. Statistics: two-way ANOVA; colour of asterisks indicates significance of corresponding group *vs.* WT at indicated time point; **p* <0.05; ***p* <0.01; ****p* <0.001; ****p* <0.0001. (E) Liver and (F) spleen weights as percent of body weight and (G) bile PC levels at sacrifice, Day 31 post AAV treatment, are shown. Age-matched WT mice (n = 3M/3F) are shown. Statistics: one-way ANOVA; **p* <0.05; ****p* <0.001; ****p*<0.0001. AAV, adeno-associated virus; AAV8, adeno-associated virus serotype 8; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BA, bile acids; KO, knockout; MDR3, multidrug resistance protein 3; PC, phosphatidylcholine; WT, wild-type.

Then, a subset of the study animals received a second dose of AAV8-MDR3 (8 × 10^{13} vg/kg) alone 2 weeks later (Fig. S3). AAV co-administration with empty nanoparticles served as a vehicle control. Mice were followed up for 8 months with periodic analyses of serum biomarkers to determine the progression of the disease.

Only FVB.*Abcb4*$^{-/-}$ mice treated with AAV8-MDR3 co-administered with ImmTOR at the first dose and treated again 2 weeks later with a second dose of AAV8-MDR3 showed a durable therapeutic effect (Fig. 2A–C and Fig. S4). Mice treated with AAV8-MDR3 twice without ImmTOR lost any therapeutic effect after 2 weeks post treatment, as the second administration of AAV8-MDR3 had no apparent effect. Interestingly, mice given one treatment of AAV co-administered with ImmTOR showed a partial and transient therapeutic effect up through 4 weeks post treatment, but without a second AAV treatment, the effect was lost in all animals, and the serum liver enzyme levels of these mice rebounded to levels seen in other untreated and unsuccessfully treated PFIC3 mice (Fig. 2A–C and Fig. S4). Body weights of treated mice were statistically similar to those of WT mice (p = 0.92, two-way ANOVA; Fig. S5).

The mice receiving AAV8-MDR3 twice with ImmTOR on the first treatment maintained a therapeutic effect up through 32 weeks post treatment, with serum ALT, AST, ALP, BA, CHOL, and BIL levels fully normalised to WT controls. Upon study termination, these animals showed liver and spleen sizes equivalent to those of WT mice, increased bile PC concentration, and a total absence of liver fibrosis, whereas all untreated and unsuccessfully treated PFIC3 animals showed clear manifestations of cholestasis (Fig. 3 and Figs. S6–S10). Notably, the livers of the cured PFIC3 mice were indistinguishable from WT liver tissue in terms of histology and degree of fibrosis (haematoxylin and eosin staining and Sirius Red staining), whereas all other animals showed extensive bridging fibrosis to severe cirrhosis, enlarged bile ducts, and cell

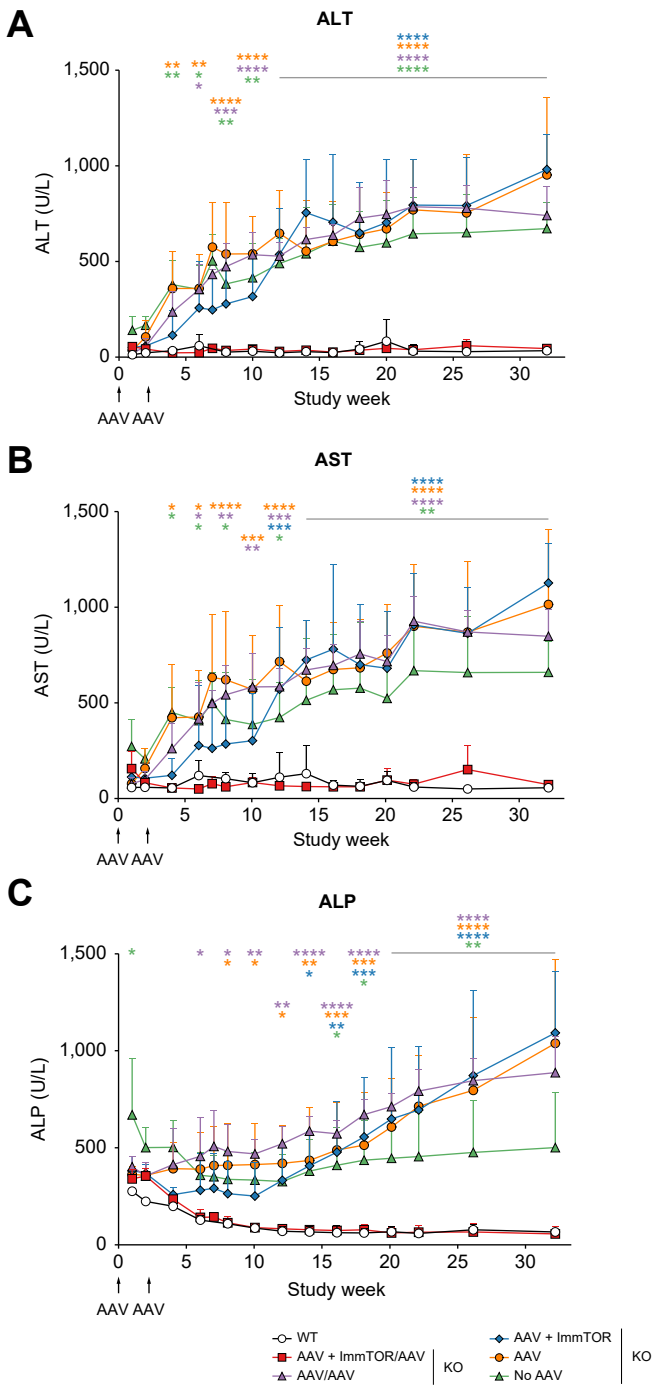

Fig. 2. Re-administration of AAV8-MDR3 when admixed with ImmTOR in infant FVB.*Abcb4*$^{-/-}$ mice induces a durable therapeutic effect. FVB.*Abcb4*$^{-/-}$ mice were injected IV at 2 weeks of age with AAV8-MDR3 admixed with ImmTOR and then again 2 weeks later with AAV8-MDR3 alone. (A) ALT, (B) AST, and (C) ALP in serum are indicated up to 32 weeks post AAV treatment. Group size: AAV8-MDR3 + ImmTOR/AAV8-MDR3, n = 3M/2F; AAV8-MDR3/AAV8-MDR3 and AAV8-MDR3 + ImmTOR, n = 5M/2F; AAV8-MDR3, n = 7M/5F; no AAV, 5M/3F; WT, 3M/3F. Statistics: two-way ANOVA; colour of asterisks indicates significance of corresponding group *vs.* AAV + ImmTOR/AAV at indicated time point; **p* <0.05; ***p* <0.01; ****p* <0.001; ****p* <0.0001. AAV + ImmTOR/AAV *vs.* WT, not significant at any point. AAV, adeno-associated virus; AAV8, adeno-associated virus serotype 8; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; KO, knockout; MDR3, multidrug resistance protein 3; WT, wild-type.

infiltrates (Fig. 3E and Fig. S6). Quantification of the total amount of collagen in the liver tissue of treated mice (Sirius Red staining), levels of which indicate the extent of fibrosis, was on average 14.8 ± 4.6% that of diseased animals (a 6.8-fold reduction), which was the same as that of WT mice (14.8 ± 5.0%) (p = 2E-5, two-tailed t test: treatment *vs.* all unsuccessfully treated mice). Liver fibrosis was also assessed by quantifying expression of the fibrosis markers collagen type 1 alpha 1 (*Col1a1*), lysyl oxidase 1 (*Lox*), and tissue inhibitor of metalloproteinase 1 (*Timp1*) (Fig. S7), showing significantly lower levels in successfully treated mice, which were similar to WT levels for all three markers (p = 0.002, p = 0.015, p = 0.008, respectively; two-tailed t test: treatment *vs.* all unsuccessfully treated mice).

Mean bile PC concentration reached 42.6 ± 10.3% WT levels in mice treated with 2 × AAV treatment with ImmTOR, whereas unsuccessfully treated PFIC3 mice had on average only 1.8 ± 3.0% WT levels (p = 6E-15) (Fig. 3C). Although diseased animals had livers and spleens that were on average twofold larger than those of WT animals (201 ± 37% and 198 ± 53% WT values for liver and spleen sizes, respectively), the mice that received 2 × AAV treatment with ImmTOR showed no increase in liver and only slight increase in spleen sizes (104 ± 4% and 119 ± 24% WT values, respectively; p = 2E-5 and 8E-3 *vs.* all unsuccessfully treated, respectively) (Fig. 3A and B). Diseased animals also showed extensively enlarged bile ducts in the liver (Fig. S6) and presented gallstones in the gall bladder and bile duct (39% of unsuccessfully treated PFIC3 mice) (Figs. S8 and S9), whereas none of the mice given 2 × AAV treatment with ImmTOR did. Bile duct proliferation was analysed by cytokeratin 19 (CK19) immunohistochemistry (IHC). Unsuccessfully treated PFIC3 mice showed a clear proliferation of bile ducts that was significantly reduced by 2 × AAV treatment with ImmTOR (p = 0.023, two-tailed t test: treatment *vs.* no AAV control) showing levels similar to those observed in healthy WT mice (Fig. S10). Double immunofluorescence analysis with anti-CK19 and epithelial cadherin (E-cadherin) or vimentin showed in all cases that CK19 is E-cadherin positive and vimentin negative. Vimentin-positive cells surrounded CK19-positive cells. However, we observed a very different organisation of the morphological structures depending on the treatment. Although WT mice and mice treated with 2 × AAV with ImmTOR showed a clear and well-organised bile duct structure surrounded by vimentin-positive cells, in untreated and unsuccessfully treated PFIC3 mice, we observed very disorganised cellular distributions (Figs. S11 and S12). Thus, the reactive cholangiocyte phenotype appeared not to be affected by successful treatment and prevention of cholestasis-related sequelae, as CK19-positive cells exhibited an epithelial phenotype and no epithelial–mesenchymal transition regardless of treatment or disease severity. This phenotype of cholangiocytes in the PFIC3 mice was not different from that in WT mice.

AAV re-administration prevents liver malignancies in PFIC3 mice

In prior studies with FVB.*Abcb4*$^{-/-}$ mice,[9] shorter study durations did not allow for an analysis of whether AAV8-MDR3 treatment could prevent or induce the appearance of liver tumours. After 8 months of follow-up in the current study, none of the mice receiving AAV8-MDR3 twice with ImmTOR showed any liver pathology indicative of hepatocellular carcinoma (HCC). Conversely, almost all unsuccessfully treated animals presented

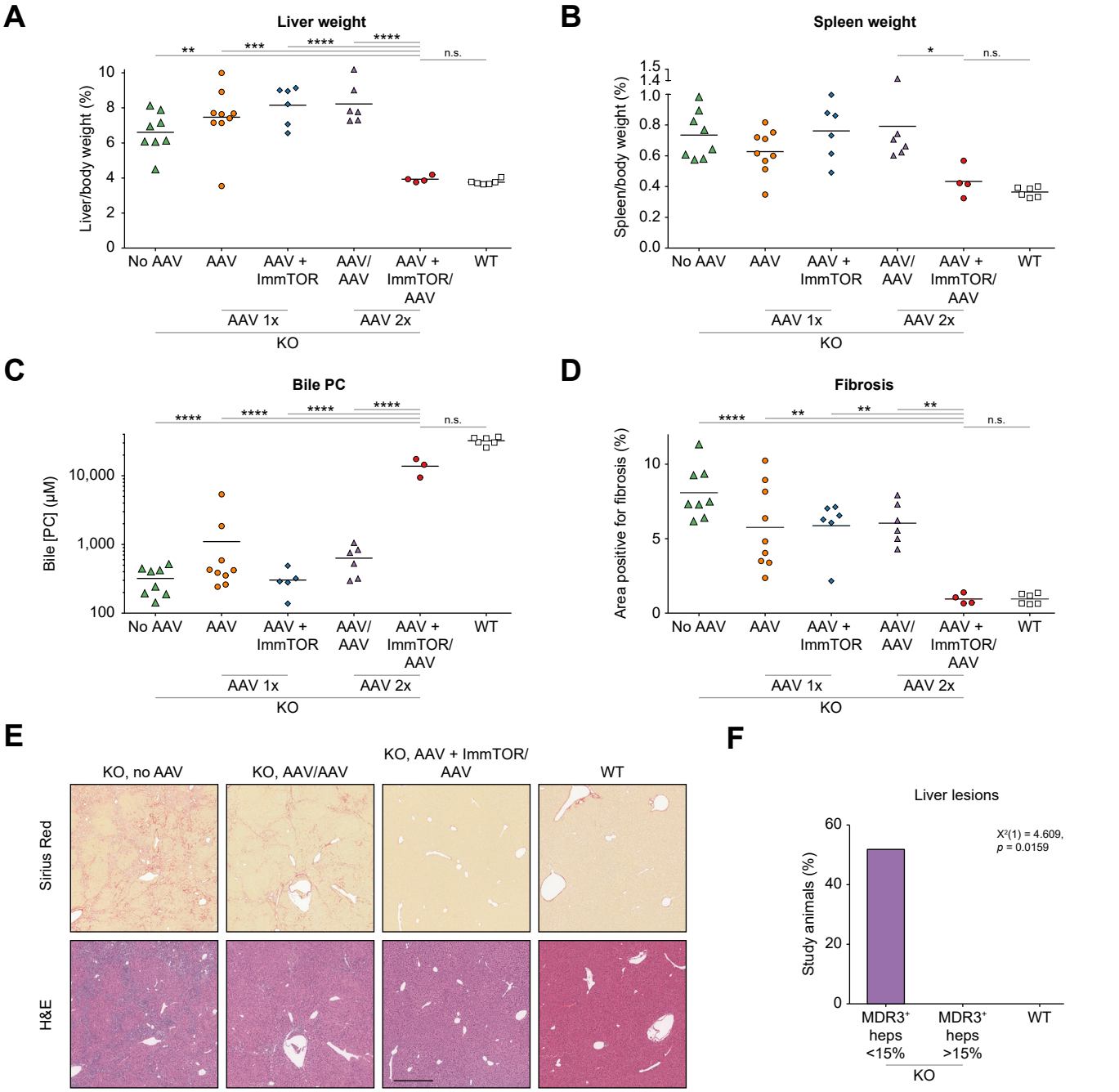

Fig. 3. Phenotypic correction in infant FVB.*Abcb4*$^{-/-}$ mice following re-administration of AAV8-MDR3 admixed with ImmTOR. (A) Liver and (B) spleen weights as percent of body weight and (C) bile PC levels at sacrifice, 32 weeks post the first AAV treatment, are shown. (D) Quantification of liver fibrosis as indicated by Sirius Red staining of liver sections of study animals at sacrifice are shown. Statistics: one-way ANOVA, *p <0.05; **p <0.01; ***p<0.001; ****p <0.0001. (E) Representative images of Sirius Red and H&E stained liver sections from study animals as indicated. Scale bar, 0.5 mm. (F) Hepatocellular neoplasms in KO mice according to percentage of hepatocytes with MDR3 protein expression via IHC or WT mice. Statistics: chi-square test. 1× , one time; 2× , two times; AAV, adeno-associated virus; AAV8, adeno-associated virus serotype 8; IHC, immunohistochemistry; H&E, haematoxylin and eosin; KO, knockout; MDR3, multidrug resistance protein 3; PC, phosphatidylcholine; WT, wild-type.

significant liver damage as expected, and half developed atypical hepatocellular neoplasms or dysplastic nodules detected via histopathological analysis (Figs. S9A and S13–S15). To assess a correlation between MDR3 expression and the appearance of hepatocellular neoplasms, the mice were binned according to percentage of hepatocytes with MDR3 expression as detected by IHC with a threshold of 15% positivity serving as the cut-off. Neoplasms were detected in 51.9% of animals below this threshold but in none of the animals with MDR3-positive hepatocytes above this threshold (Fig. 3F) ($\chi^2(1)$ = 4.61, p = 0.0159). Survival rates were similar for all study groups (Fig. S8C) (p = 0.55, Mantel–Cox log-rank test). We performed an analysis of hepatocyte proliferation in the liver tissue by detecting Ki-67, a nuclear marker for cell proliferation, via IHC

and reverse transcription quantitative PCR (RT-qPCR). Unsuccessfully treated animals had 0.38 ± 0.47% nuclei that stained positive for Ki-67 *vs*. 0.054 ± 0.049% for 2 × AAV treatment with ImmTOR (p = 0.19; Fig. S16), a sevenfold reduction. *Mki67* mRNA in unsuccessfully treated PFIC3 mice were 1.9E-3 ± 1.1E-3 copies per transcript copy of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) *vs*. 2.4E-4 ± 1.7E-4 in mice receiving 2 × AAV treatment with ImmTOR (p = 4.4E-3; Fig. S16G), an eightfold reduction. Reduced hepatocyte proliferation is evidence of a lower risk of developing HCC. The phosphorylated histone H2ax (γH2ax) was also quantified via IHC. γH2ax is an indicator of DNA double-strand breaks that has been reported to be a good preneoplasia marker of HCC in these mice.[36] Results showed 0.93 ± 0.60% γH2ax-positive nuclei in unsuccessfully treated mice compared with 0.20 ± 0.15% in mice given 2 × AAV treatment with ImmTOR (p = 0.026; Fig. S17), whereas WT mice had 0.18 ± 0.26% (p = 0.94). These results all support the conclusion that AAV8-MDR3 redosing via the use of ImmTOR successfully prevented the development of HCC in PFIC3 mice.

AAV transduction and transgene expression

To gain a measure of the effectiveness in transduction and transgene expression of AAV redosing following co-administration with ImmTOR, AAV genome copy numbers and MDR3 transcripts in liver tissue of sacrificed mice were quantified via quantitative PCR (qPCR) and RT-qPCR, respectively (Fig. 4). FVB.*Abcb4*$^{-/-}$ mice treated with AAV8-MDR3 only once (either with or without ImmTOR) or twice without ImmTOR showed low AAV transduction at 32 weeks post treatment with on average 0.0015 ± 0.0013 AAV genome copies per genomic copy of the housekeeping gene *Gapdh*. In contrast, mice treated with AAV8-MDR3 twice with ImmTOR at the first treatment showed 1.8-log higher AAV genome copies (0.091 ± 0.016 copies; p = 2E-19). This transduction efficiency, achieved owing to a successful repeat administration of the AAV vector, led to a similar 1.8-log higher level of *MDR3* mRNA transcripts in 2 × AAV treatment with ImmTOR in comparison with the groups receiving one dose of AAV (with or without ImmTOR) or two doses of AAV without ImmTOR. There were on average 0.54 ± 0.21 *MDR3* mRNA copies per *Gapdh* transcript, whereas the mice treated with other AAV regimens had only 0.0095 ± 0.0203 copies (p = 5E-11). Interestingly, when murine *Mdr2* mRNA was quantified in livers of WT mice, 0.053 ± 0.013 copies per *Gapdh* transcript were determined, 10-fold less than the successfully re-administered FVB.*Abcb4*$^{-/-}$ mice (p = 9E-4) (data not shown).

Essential for strategies of gene supplementation via gene therapy, in addition to successful transgene delivery, is the correct translation and post-translational processing of the therapeutic protein. Detection of successful MDR3 protein translation and transport to the biliary canaliculi membrane was made via IHC staining of liver sections with an antibody specific for human MDR3. All animals re-administered with AAV8-MDR3 following AAV8-MDR3 + ImmTOR showed diffuse MDR3 staining throughout the whole liver localised to the biliary canaliculi of hepatocytes (Fig. 4C), whereas unsuccessfully treated animals showed little to no MDR3 protein. Quantification of the amount of staining revealed a 0.9-log increase as a result of re-administration following AAV8-MDR3 + ImmTOR (35.8 ± 17.6% *vs*. 4.4 ± 8.7% hepatocytes with adjacent properly localised MDR3 in treated *vs*. AAV-treated control, respectively; p = 2E-5; Fig. 4D).

Inhibition of AAV-specific NAbs

AAV-specific NAbs are known to be generated following AAV administration. We thus investigated here the impact of the tolerogenic ImmTOR nanoparticles on NAb formation when co-administered with AAV. NAb levels were measured in serum 2, 4, 6, and 8 weeks after the first AAV8-MDR3 administration, with the first samples being taken before the second administration. PFIC3 mice administered the vector alone rapidly developed AAV8-specific NAbs after the first administration, which increased after a second administration of the vector up to titres of 1,086 ± 399 on average (Fig. 5). In contrast, animals that received ImmTOR in the first injection presented undetectable NAb levels 2 weeks after administration. These levels slowly increased in two of seven animals that only received AAV vector once together with ImmTOR throughout the experiment albeit at very low levels (final titres, 97 and 105); the remaining five animals showed undetectable NAbs. In contrast, AAV8-MDR3 + ImmTOR animals that received a second administration of vector developed NAbs afterwards at levels similar to those in animals that received the vector twice without ImmTOR (p = 0.94 and 0.98 for 6 and 8 weeks p.i., respectively).

Discussion

AAV-mediated gene therapy has been developed with the goal of providing durable correction of inherited genetic disorders for which there are no satisfactory therapies. Sustainability of the therapeutic effect still represents a challenge for AAV-mediated gene therapy for paediatric indications involving liver pathology,[37] as is the case for PFIC3, a devastating monogenic inherited disease only curable by liver transplantation. PFIC3 manifestations occur early in life with one-third of patients showing clinical signs of cholestasis within the first year of life;[2] thus, early intervention is necessary to prevent the development of irreversible pathological sequelae such as liver cirrhosis. Because it is a monogenic disorder of a gene that is expressed exclusively in the liver,[38,46] PFIC3 is a strong candidate for liver-directed AAV-based gene therapy. Previously, we reported a sustained and significant reversal of PFIC3 disease biomarkers following treatment using an AAV vector harbouring a codon-optimised sequence of MDR3 isoform A (AAV8-MDR3) in young and adult PFIC3 mice.[9] However, this strategy has proven to have some important drawbacks. First, MDR3 is not a secreted protein but a membrane-bound transporter protein, which requires productive transduction of a sufficient percentage of hepatocytes to exceed a threshold in transgene expression necessary to exert a therapeutic benefit. Second, paediatric patients risk experiencing a wane in effect owing to the episomal nature of recombinant AAV genomes, which are progressively lost through cell division during liver growth[13–16] and may necessitate redosing. Furthermore, recent data showing continual hepatocyte renewal in human livers regardless of age[39] suggest that this issue may not be restricted only to paediatrics.

Our previous results obtained in the PFIC3 FVB.*Abcb4*$^{-/-}$ mouse model (in infant prefibrotic 2-week-old and juvenile fibrotic 5-week-old mice) treated with AAV8-MDR3 showed a significant therapeutic effect; however, several weeks after administration, this effect was lost in a proportion of study animals, indicating the value of a second administration of the vector. One major hurdle to vector re-administration is the humoral immune response against the first exposure of AAV that results in high

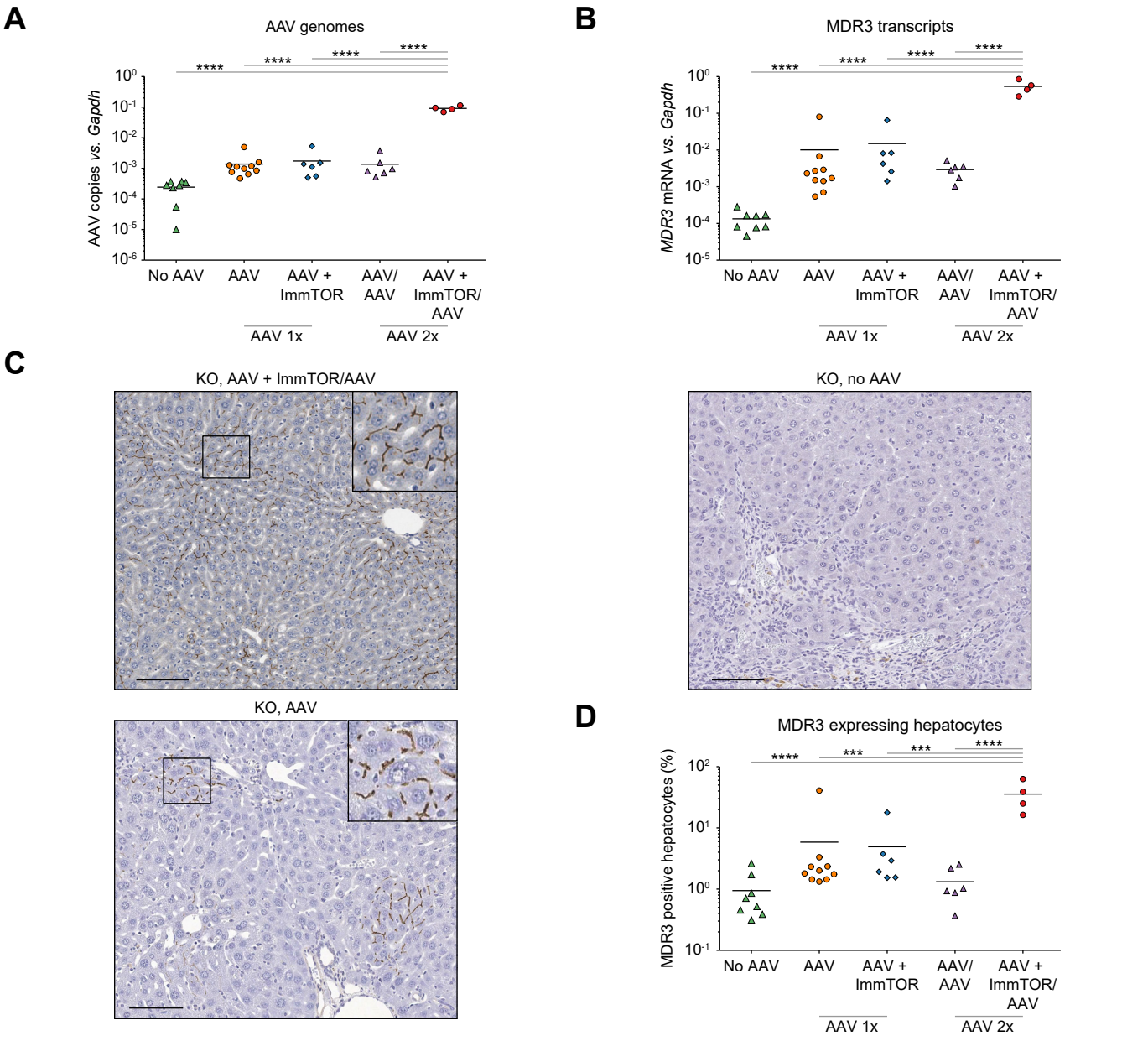

Fig. 4. AAV transduction and MDR3 expression after re-administration of AAV8-MDR3 admixed with ImmTOR in infant FVB.*Abcb4*$^{-/-}$ mice. (A) AAV vector genome copy numbers and (B) human MDR3 transcript levels in mouse liver were quantified by qPCR and RT-qPCR, respectively, at sacrifice, 32 weeks post the first AAV treatment. Values are presented relative to genomic copies or mRNA copies of the housekeeping gene *Gapgh*. (C) IHC staining of MDR3 protein in mouse liver sections at sacrifice. Scale bar, 100 μm. (D) Quantification of percent hepatocytes adjacent to positive MDR3 protein stain. Statistics: one-way ANOVA, $^{***}p$ <0.001; $^{****}p$ <0.0001. 1×, one time; 2×, two times; AAV, adeno-associated virus; AAV8, adeno-associated virus serotype 8; *Gapgh*, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemistry; KO, knockout; MDR3, multidrug resistance protein 3; qPCR, quantitative PCR; RT-qPCR, reverse transcription quantitative PCR; WT, wild-type.

titres of NAbs. The formation of NAbs is a host defence mechanism to prevent viral infection, and consequently, NAbs are an impediment for AAV-based gene therapy.[17,40,41] For indications that may require repeated treatments, avoidance of this immune response is essential to enable the possibility of re-administering AAV gene therapy vectors.

As a method to prevent NAb formation against the therapeutic vector, we tested co-administration with the tolerogenic rapamycin-loaded nanoparticles, or ImmTOR. Two significant observations resulted from the study. First, only AAV8-MDR3 treatment with ImmTOR followed by a second dose of the vector successfully rescued infant PFIC3 mice and provided long-term therapeutic correction of the disease at this dose. The ability to successfully re-administer AAV8-MDR3 and to achieve a sustained therapeutic effect correlated with the absence of NAbs 2 weeks after the first administration (at the time of the second treatment). The degree of the therapeutic correction was striking. The spleen and liver sizes were nearly fully normalised to WT. There was no increase in serum biomarkers of cholestasis. Liver fibrosis and the appearance of malignant hepatocellular tumours or their precursors were completely prevented. These outcomes were indicative of complete amelioration of disease,

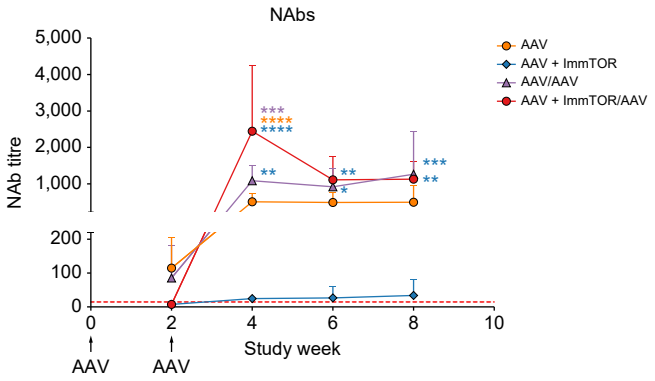

Fig. 5. AAV8-specific NAbs in infant FVB.*Abcb4*$^{-/-}$ mice treated with AAV8-MDR3 with and without ImmTOR. NAbs in serum of study animals were quantified via cell-based seroneutralisation assay. Lower limit of detection is indicated with a red dotted line. Statistics: two-way ANOVA, *p <0.05; **p <0.01; ***p <0.001; ***p <0.0001. AAV, adeno-associated virus; AAV8, adeno-associated virus serotype 8; MDR3, multidrug resistance protein 3; NAbs, neutralising antibodies.

and the length of time after treatment that they were observed points to the durability of the therapeutic approach. The disease correction occurred despite only achieving 42% WT levels of bile PC. However, previous results also pointed to a therapeutic threshold being substantially less than 100% normalisation of WT MDR2 functionality.[6,9,42,43] Second, animals that received ImmTOR with only one dose of AAV8-MDR3 showed improvement, albeit transiently, in cholestatic biomarkers compared with those that were given only AAV8-MDR3. This outcome points to a possible increase in transduction and/or expression when the vector is admixed with ImmTOR, which has been demonstrated elsewhere.[29,31] However, owing to the nature and the severity of the model, we have consistently observed that once any AAV8-MDR3-mediated therapeutic effect is lost, little to no evidence of transduction or transgene expression is present at sacrifice, especially not when such a long period has transpired since the loss of effect (5–6 months in this instance). Thus, a direct comparison of AAV genome copies or MDR3 expression between the study groups that lost the therapeutic effect in attempts to gauge the effect of ImmTOR on transduction and expression following a single administration is unreliable. Nonetheless, the 1.8-log difference in vector genome copy numbers and *MDR3* mRNA transcript levels between treatment with AAV8-MDR3 alone and a second AAV8-MDR3 after AAV8-MDR3 plus ImmTOR underscores the challenge of treating a rapidly progressing liver disorder in infant animals and the power of being able to re-administer gene therapy.

High titres of NAbs were observed in the sera of the animals that received one or two administrations of the vector alone, which corroborates previous results for similar doses of this vector (data not shown). In contrast, animals treated with ImmTOR at the first treatment instance presented no development of NAbs against the vector capsid at 2 weeks post administration, and five of seven animals treated with a single dose of AAV8-MDR3 with ImmTOR remained seronegative at the final time point analysed, that is, at 8 weeks, indicative of a sustained tolerogenic effect. However, high NAb titres developed rapidly in animals that received a second dose of the vector in the absence of ImmTOR, indicating that the tolerogenic effect was broken by the high dose of vector. Meliani *et al.*,[30] in contrast, showed partial blunting of the antibody response to a second, although lower (4×10^{12} vg/kg), dose of AAV8 vector alone. Our results differ from those observed combining ImmTOR with protein biologics, such as therapeutic enzymes, coagulation factors, monoclonal antibodies, and recombinant immunotoxin,[27,28,32] wherein immune tolerance was maintained even after multiple challenges of the biologic alone. However, in these cases, immune tolerance was first established by repeated administration of ImmTOR plus antigen. Repeated administration may reinforce immunological memory for immune tolerance, similar to the role of booster immunisation for effector immune memory.[44] In the case of AAV gene therapy, in contrast to other therapies, only a single dose is expected to be administered initially, with repeated administrations only coming months or years later, so the opportunity to develop durable immune tolerance capable of withstanding repeated challenge of vector alone may be reduced. Despite this, here we show that the administration of ImmTOR together with AAV8-MDR3 (8×10^{13} vg/kg) generates a tolerogenic state characterised by the complete inhibition of NAb production against the vector and that this state is enough to enable re-administration of the vector and effective liver transduction. The ability to redose in these infant animals was essential to provide a durable increase in transduction and transgene expression and amelioration of disease for at least 8 months, even after the induction of NAb production following the second administration. Nonetheless, given the anticipated low frequency of AAV re-administration in humans, it may be advantageous to co-administer ImmTOR with each dose of AAV gene therapy to maintain the tolerogenic effect with the goal of keeping patients eligible for further redosing if needed.[29,30]

In addition to ameliorating all disease manifestations, the re-administration of the vector following co-administration with ImmTOR resulted in the attenuation of the aberrant distribution and proliferation of bile ductular cells and, more importantly, in the prevention of the appearance of liver malignancies indicative of HCC. Although the clinical applicability of these findings is lessened because HCC is not a manifestation of PFIC3 in humans, it is a valuable finding for the field of AAV-based gene therapy where long-term safety, particularly risk of AAV-mediated insertional mutagenesis, is at the forefront of considerations in translation to the clinic. For example, Dalwadi *et al.*[45] observed that neonate mice with non-alcoholic fatty liver disease (NAFLD) treated with AAV designed to probe insertional mutagenesis consistently developed HCC, as did a proportion of NAFLD adult mice given a non-targeted control AAV. Hepatocyte regeneration, caused by liver growth or liver injury, was found to be important in the mechanism of AAV-induced oncogenesis. Our findings with repeat AAV treatment in 2-week-old infant mice with a liver damage phenotype and growing livers showed the opposite results, which is highly encouraging. Indeed, the correction of PFIC3 disease via MDR3 gene supplementation and the resulting reduction in hepatocyte proliferation is likely to have played a role in the protection from development of HCC.

To our knowledge, this is the first reported study of ImmTOR in study animals of such a young age. It is possible that the less mature immune system of 2-week-old mice played a role in the outcomes of the study, that is, that had the study been performed in adult animals the outcomes may have been different. However, the consistent loss of effect in all control animals that did not receive ImmTOR was indicative of an immune response sufficiently capable of inhibiting the second AAV8-MDR3 administration. Whether or not the tolerogenic state observed

with ImmTOR arose in part owing to the immature immune system of the young mice requires further investigation, although other studies support the ability of ImmTOR to mitigate the immune response to AAV vectors and enable redosing in adult animals.[29,30] The conclusion that a second AAV administration was effective only if the first treatment included ImmTOR is a promising sign for the further development of this strategy in paediatric diseases.

Abbreviations

AAV, adeno-associated virus; AAV8, adeno-associated virus serotype 8; *ABCB4*, ATP binding cassette subfamily B member 4; ALP, alkaline phosphatase; ALT, alanine aminotransaminase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BA, bile acids; BIL, bilirubin; CHOL, cholesterol; CK19, cytokeratin 19; *Col1a1*, collagen type 1 alpha 1; E-cadherin, epithelial cadherin; F, female; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; H&E, haematoxylin and eosin; IHC, immunohistochemistry; IV, intravenous; KO, knockout; *Lox*, lysyl oxidase 1; M, male; MDR2, multidrug resistance protein 2; MDR3, multidrug resistance protein 3; NAbs, neutralising antibodies; NAFLD, non-alcoholic fatty liver disease; p.i., post injection; PC, phosphatidylcholine; PFIC3, progressive familial intrahepatic cholestasis type 3; qPCR, quantitative PCR; rAAV, recombinant AAV; RT-qPCR, reverse transcription quantitative PCR; *Timp1*, tissue inhibitor of metalloproteinase 1; vg, vector genomes; WT, wild-type; γH2ax, phosphorylated histone H2ax.

Financial support

This study was funded by Vivet Therapeutics and Selecta Biosciences and via a Government of Navarra PhD grant 'Doctorados industriales 2019–2021'.

Conflicts of interest

NDW, IRG, JPC, and GGA are employees and stockholders of Vivet Therapeutics. TKK is an employee of Selecta Biosciences. All other authors declare no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study conception and design: NDW, JPC, TKK, GGA. Acquisition of data: NDW, LO, IRG, DS. Analysis and interpretation of data: NDW, LO, GGP, JA, GGA. Statistical analysis: NDW. Drafting of the manuscript: NDW, IRG, GGP, TKK, GGA. Important intellectual contributions during the study as well as critical revision of the manuscript: JPC, TKK, JA, GGA. Study supervision: NDW, GGA.

Data availability statement

The data generated from the studies are available from the corresponding author on reasonable request.

Acknowledgements

We would like to thank Dr Céline Bouquet for critical revisions of the manuscript, Dr Carlos Rodríguez-Ortigosa and Sara Arcelus for analysis of serum biomarkers, and Dr Matias A. Avila for help establishing the *Abcb4*$^{-/-}$ mouse colony. We also would like to thank all personnel at the Animal Facility and Laura Guembe and the Morphology and Image Departments of Cima Universidad de Navarra. IRG was supported financially by a Government of Navarra grant 'Ayudas para la contratación de doctorandos y doctorandas por empresas y organismos de investigación y difusión de conocimientos "doctorados industriales 2019-2021" expediente: 0011-1408-2018-000016'. Patent pending: WO 2020/074690.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100713.

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Author names in bold designate shared co-first authorship

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