

Liver-directed lentiviral gene therapy corrects hemophilia A mice and achieves normal-range factor VIII activity in non-human primates

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Abstract

Liver gene therapy with adeno-associated viral (AAV) vectors delivering a clotting factor transgene into hepatocytes has shown multi-year therapeutic benefit in adults with hemophilia. However, anti-AAV pre-existing immunity and the mostly episomal nature of AAV vectors, currently challenges application of AAV-vector mediated liver gene therapy to people with anti-AAV neutralizing antibodies and young pediatric patients. We have developed lentiviral vectors (LV), which integrate in the host cell genome, which achieve stable and efficient liver gene transfer in mice, dogs and non-human primates (NHP), by intravenous (i.v.) delivery. Here we show long-term coagulation factor VIII (FVIII) activity and restoration of hemostasis, by LV i.v. gene therapy to newborn and adult hemophilia A mice and normal-range FVIII activity in NHP, paving the way for potential clinical application.

Introduction

Hemophilia is an inherited bleeding disorder due to mutations in *F8* or *F9* genes encoding for factor VIII (FVIII) or factor IX (FIX) protein, respectively, which are necessary factors for proper blood coagulation and hemostasis. People with severe hemophilia A have FVIII activity below 1% of normal and experience spontaneous and uncontrolled bleedings that progressively cause arthropathies and may be fatal, if not properly treated¹. Patient treatment is based on life-long prophylactic administration either of recombinant FVIII products that require at least weekly intravenous (i.v.) infusion to prevent hemorrhages, or of a recently approved activated-FVIII mimetic antibody that can be administered subcutaneously². Despite the success of these drugs in improving clinical management and quality of life of people with hemophilia in high-income countries, gene therapy has long been considered a potentially definitive cure for hemophilia³. Advanced-phase clinical studies have highlighted the potential of gene therapy to fulfil this promise, by showing multi-year therapeutic benefit following a single i.v. administration of an adeno-associated virus (AAV) vector delivering a functional FIX or FVIII transgene to the liver, in adults affected by severe hemophilia B or A, respectively^{4, 5, 6}. However, a decreasing trend in FVIII transgene expression has been reported, for reasons that are not fully understood⁶. These studies represent milestones for gene therapy and provided among the first evidences for safe and effective genetic modification of the human liver. However, AAV-vector gene therapy remains affected by some limitations: i) the widespread pre-existing immunity to the parental virus, which precludes access to 20-30% of patients and imposes an immune-suppression regimen for a period of time following gene therapy to maintain AAV-transduced hepatocytes; ii) dilution of episomal AAV vectors following liver growth; iii) cargo capacity limited to 5 kb, particularly challenging for incorporating FVIII transgenes. HIV-derived lentiviral vectors (LV) may represent a complementary strategy for liver-directed gene therapy, for the following reasons: i) low occurrence or feasible overcoming of immune barriers in humans, since worldwide prevalence of HIV infection is estimated at 0.8% and prior exposure to the vesicular stomatitis virus, whose surface glycoprotein (VSV-G) is used for pseudotyping LV, is rare, although natural low-titer antibodies (Abs) cross-reacting with the VSV-G protein are often present in human plasma; ii) efficient integration of LV in the host cells' genome may be preferred for life-long maintenance of the therapeutic transgene and

potentially allows treatment of pediatric patients without the need for vector re-administration⁷; iii) larger packaging capacity may make LV better suited for transferring FVIII expression cassettes. It has been shown that the natural source of most FVIII production is endothelial cells⁸, however, currently the most advanced gene therapy strategies exploit hepatocytes to produce transgenic FVIII⁹. Gene transfer of LV expressing FVIII from liver endothelial cells has been proposed and some encouraging results have been reported in hemophilia A mice treated as adults^{10, 11}, however the stability and turnover of these cells, both in post-natal liver growth and homeostasis in adulthood remain not fully understood¹².

We have previously shown that i.v. administration of LV results in efficient and long-term gene transfer to the liver and achieves phenotypic correction of hemophilia B in mice and dogs^{13, 14, 15}. More recently, we generated allo-antigen free and phagocytosis-shielded (CD47hi) LV that allowed supra-normal activity of a human coagulation FIX transgene in non-human primates (NHP), without evidence of acute toxicity and clonal expansion of transduced cells^{16, 17}. Here we evaluated LV-mediated gene delivery of engineered versions of FVIII transgene in hemophilia A mice and in NHP, showing long-term FVIII activity and restoration of hemostasis, following i.v. administration to newborn and adult mice and normal-range human FVIII activity in NHP.

Results

LV expressing engineered FVIII transgenes allow phenotypic correction of hemophilia A mice

We generated 3 different versions of human FVIII transgenes, all lacking the B-domain, previously reported to be non-essential for clotting activity¹⁸: a wild-type (wt) sequence, a codon-usage optimized (co) and an engineered co version also containing an unstructured XTEN polypeptide in place of the B domain (Supplementary Fig. 1a and Supplementary methods)¹⁹. Inclusion of this polypeptide has been shown to increase protein stability and prolong circulating half-life of FVIII and other proteins, but, to our knowledge, has not been tested in a gene therapy setting²⁰. We then cloned these transgenes (FVIII, coFVIII, coFVIII.XTEN) into a LV construct carrying a hepatocyte-specific enhancer promoter (Enhanced Transthyretin, ET) and target sequences for the hematopoietic-specific microRNA 142 (142T). This LV design was previously shown to provide highly specific and robust transgene expression in hepatocytes^{13, 15, 17, 21}. Transduced mouse and human hepatic cell lines showed LV dose dependent FVIII production in the supernatant. The coFVIII and coFVIII.XTEN transgenes were expressed 18- and 22-fold higher than wt FVIII, respectively in the mouse cell line and 47- and 89-fold higher than wt FVIII, respectively in the human cell line, at matched LV DNA copies *per* cell (vector copy number, VCN; Supplementary Fig. 1b-g). We treated newborn (2-day old) hemophilia A (*F8* knock out, KO) mice by i.v. administration of 2.5×10^{10} transducing units (TU)/kg of LV (n=5-9 mice *per* LV) and measured FVIII blood concentration and activity overtime. We observed steady-state 19 ng/mL and 0.25 U/mL of FVIII corresponding to approximately 19 and 25 % of normal in mice treated with LV.FVIII, while mice treated with LV.coFVIII or LV.coFVIII.XTEN produced 10-20 fold more FVIII corresponding to approximately 197 and 225 % of normal FVIII activity (Fig. 1a, b). Some of these mice developed anti-FVIII Abs starting 12

weeks post LV (Fig. 1c), however still maintaining circulating FVIII activity at the end of experiment, despite a decreasing trend, likely due to Abs formation. All LV-treated mice, untreated hemophilia A and wt mice were then subjected to hemostatic challenge, by transecting the tip of the tail at the end of experiment, 5-6 months post LV and collecting the blood from the wound. Blood loss was then determined by measuring hemoglobin absorbance in the blood-containing solution. All the hemophilia A mice treated with LV.coFVIII or coFVIII.XTEN showed improved hemostasis, comparable to the range observed for wt mice, while hemostasis remained defective in mice treated with LV.FVIII that only expressed approximately 20 % of normal FVIII on average (Fig. 1d). We measured LV VCN in different liver cell types at the end of experiment, as previously described¹⁷. Average LV VCN in purified hepatocytes was between 0.2 and 0.54 (Fig. 1e). Average VCN in Kupffer cells (KC) and plasmacytoid dendritic cells was between 1 and 1.5, much lower than the VCN observed in mice treated by LV i.v. administration as adults, as we have previously reported¹⁷ and show afterward in this work. FVIII output normalized on VCN in hepatocytes confirmed higher expression by coFVIII (32-fold compared to FVIII) with an additional nearly 2-fold increase by FVIII.XTEN (Supplementary Fig. 2a). FVIII expression at steady state well correlated with VCN in hepatocytes (Supplementary Fig. 2b), which was similar in both Abs-positive and Abs-negative mice (Supplementary Fig. 2c). Taken together, these data show improved protein output and therapeutic efficacy by the engineered FVIII transgenes.

FVIII activity is maintained long-term following LV gene therapy in both newborn and adult hemophilia A mice

We then treated additional neonatal hemophilia A mice with a lower dose of LV.coFVIII (1×10^{10} TU/kg), to obtain physiological-range FVIII amounts, followed them for 1.5 years and showed FVIII blood concentration and activity that remained stable throughout the nearly lifetime follow-up at around 5-10% of normal (Fig. 2a, b). VCN in purified hepatocytes was 0.02 at the end of experiment (Fig. 2c), in line with FVIII expression. We also treated neonatal hemophilia A mice with increasing doses of LV.coFVIII.XTEN ranging from 1.5×10^9 to 1.3×10^{10} TU/kg and achieved between 5 and 82 ng/mL of circulating FVIII concentration and between 0.05 and 0.6 U/mL of FVIII activity (corresponding to 5 to 60-80 % of normal) with an increasing LV dose-response relationship (Fig. 2d-f). The coFVIII.XTEN steady-state amounts were about 10-fold higher than coFVIII at similar LV doses in these experiments (Supplementary Fig. 2d, e). A smaller difference in FVIII amounts between the 2 transgenes was observed in the previous experiment (see Fig. 1a, b and Supplementary Fig. 2a) likely due to the higher LV doses used and the non-linear LV-dose response above 2×10^{10} TU/kg. FVIII output was stable for 6 months (longest time analyzed) except for mice that developed anti-FVIII Abs, starting from week 10 *post* LV, in which circulating FVIII amounts declined as anti-FVIII Abs rose (Fig. 2g). Likely, the higher amounts of Abs in this experiment impaired FVIII detection whereas in the previous experiment lower amounts of Abs did not. Average VCN in the total liver ranged from 0.04 to 0.12 at the end of the experiment (Fig. 2h). Taken together, these data show stable long-term FVIII transgene expression and activity by LV gene therapy from mice treated as newborns. In order to evaluate FVIII output and stability in adult mice, without confounding factors due to

anti-FVIII immune responses, that are more likely to develop upon treatment at this age, we generated immune-deficient hemophilia A mice, by crossing *F8* KO with *Rag1* KO mice (RagHemoA). We confirmed that the double KO progeny was both devoid of circulating T and B lymphocytes and showed prolonged clotting times (Supplementary Fig. 3). We then treated adult (8-week-old) RagHemoA mice by i.v. administration of 8×10^{10} TU/kg of coFVIII.XTEN containing LV. We observed an average concentration of 134 ng/mL and 1.12 U/mL activity of circulating FVIII in treated mice (corresponding to 134 and 112 % of normal), which remained stable for 11 months following gene therapy (Fig. 3a, b). Average VCN in purified hepatocytes at the end of the experiment was 0.37 (Fig. 3c). Notably, average VCN in KC was 15, in line with our previous data from mice treated as adults¹⁷. The lower VCN in KC observed at 6 months post LV in mice treated when newborns (see Fig. 1e) suggest reduced KC transduction in newborn mice. We also evaluated LV gene therapy in an immune competent hemophilia A mouse model, both *F8* KO and transgenic for a human FVIII point-mutant (HemoA-R593C). This mouse model completely lacks circulating FVIII protein and activity, however it expresses a non-secreted human FVIII, thus it combines the hemophilia phenotype with immune tolerance to human FVIII epitopes, as previously described²². This mouse model better recapitulates hemophilia A patients previously treated with FVIII protein products and negative for anti-FVIII Abs, that would be the target population for a first clinical testing of this gene therapy. I.v. administration of LV.coFVIII.XTEN in adult HemoA-R593C allowed long-term stable reconstitution of FVIII expression and activity on average 65% of normal (Fig. 3d, e) for 6 months after gene therapy. Treated mice presented minimal anti-FVIII Abs, which did not affect FVIII stability or activity (Fig. 3f). These data show that LV gene therapy does not break immune tolerance in this immune competent mouse model. Overall, these data show efficient transduction of hepatocytes and long-term stable fully normal reconstitution of FVIII activity in adult hemophilia A mice, by using the engineered codon-optimized XTEN-carrying FVIII transgene.

***In vivo* LV gene therapy achieves normal-range human FVIII activity in NHP**

Collectively these data prompted us to evaluate LV-mediated human FVIII gene transfer in NHP. We thus produced large-scale purified batches of major-histocompatibility complex (MHC) free and CD47hi LV carrying either the coFVIII or coFVIII.XTEN transgene (Supplemental Table 1), by a good-manufacturing practice like process with a 2-step chromatography purification, as previously reported¹⁷. We then treated 10 NHP with either LV at 2 different doses each: coFVIII at 3 or 6×10^9 TU/kg, coFVIII.XTEN at 1 or 3×10^9 TU/kg. Doses were selected based on previous experience with FIX-expressing LV¹⁷. Note that the LV-dose response in NHP is more favorable than in mice, on a *per weight* basis, as previously described¹⁷. All LV-treated NHP received an immune suppressive regimen from 1-3 days before to 7-9 days after LV administration, to allow detection of human FVIII transgene in the absence of anti-FVIII Abs. We observed no major alteration of clinical or blood chemistry parameters, except for transient minor (1.2-2.6-fold higher than the upper limit) elevation of serum aspartate aminotransferases (AST) and transient minor (1.7-2.4-fold lower than the lower limit) decrease of circulating lymphocytes (Fig. 4a-e), in line with our

previous LV-FIX study. AST elevation positively correlated with the dose of LV particles infused (Supplementary Fig. 4a). The blood concentration and activity of human FVIII peaked at 131 ng/mL and 0.68 U/mL (corresponding to 131 and 68% of normal) in NHP treated with LV.coFVIII.XTEN at 3×10^9 TU/kg, 22- and 17-fold higher than in NHP treated with LV.coFVIII at the same LV dose (Fig. 4f-h). NHP treated with the coFVIII.XTEN-carrying LV at 1×10^9 TU/kg showed 29 ng/mL and 0.2 U/mL of peak circulating FVIII concentration and activity, corresponding to 29 and 20% of normal, respectively. As expected, upon withdrawal of immune suppression, all NHP developed anti-FVIII Abs, though to different amounts, which in most cases were also neutralizing FVIII activity (Fig. 4i, j). Moreover, these Abs were cross-reacting with the endogenous NHP FVIII, because clotting times were prolonged (Supplementary Fig. 4b). Progressive reduction of hemoglobin and hematocrit, accompanied by increased in circulating reticulocytes in some cases, suggested the occurrence of bleeding events (Supplementary Fig. 4c-e). All other blood chemistry and hematology parameters mostly remained in the normal range throughout the follow up (60 days *post* LV), except for few fluctuations (Supplementary Tables 2-11). LV DNA was only detectable in the liver and spleen among the organs analyzed at the end of the experiment and ranged between 0.02 and 0.4 in the liver (Fig. 5a). Gene expression analysis in the liver at necropsy showed that the NHP treated with LV.coFVIII lost LV RNA (above background in only 1/5 animals), while LV.coFVIII.XTEN-treated NHP (4/5 animals) maintained it. These data are in line with remaining FVIII transgene at the end of the follow up and suggest maintenance of at least some transduced hepatocytes, despite induction of inhibitory FVIII Abs (Fig. 5b). Average LV VCN in KC purified from liver necropsies of treated NHP ranged between 0.05 and 0.25, showing effective protection of LV from phagocytosis by KC, as expected by the high surface content of CD47 on LV particles, and in line with our previous study¹⁷ (Fig. 5c). Expression of LV RNA well correlated with both VCN in the total liver at endpoint and with VCN in hepatocytes, calculated from the measured VCN in total liver and purified liver non-parenchymal cells (Supplementary Fig. 4f). Measurement of LV particles in the serum of treated NHP showed a very short LV circulating half-life, with 0.02-0.09% of administered particles remaining 1 day after infusion and becoming undetectable by day 3 (Supplementary Fig. 4g). Pathology analysis of liver and spleen showed no macroscopic or microscopic lesions, except for minimal hepatocytes atrophy with dilated sinusoids in one animal and mild neutrophilic infiltrate in the red pulp of the spleen in a second animal.

NHP treated with the FVIII.XTEN transgene show reduced anti-FVIII immune responses

We longitudinally monitored a panel of 26 cytokines and chemokines in treated NHP before and after LV administration to investigate acute innate response to LV particles and potentially detect delayed release related to developing adaptive responses against vector and/or transgene antigens (Ag). We observed that 10/26 analytes significantly increased in the first hours to days following LV administration and then returned to baseline values in most cases already at day 2 *post* LV (Fig. 6a-j). Similarly to the acute response previously reported following i.v. LV administration in murine models^{17, 23}, pro-inflammatory cytokines and chemokines, likely released from innate immune cells, significantly increased in the bloodstream of NHP after LV administration. We observed transient elevation of multiple signals for the

recruitment and activation of innate immune effectors, such as neutrophils, eosinophils, monocytes and dendritic cells, and in particular growth-regulated protein beta (GRO- β), interleukin (IL)-5, monocyte chemoattractant protein 1 (MCP1), IFN γ and interferon gamma-induced protein 10 (IP-10) within the first 1-2 days *post* LV, as well as interferon-inducible T cell alpha chemoattractant (I-TAC) and MCP1 chemokines for the recruitment and activation (IL-6) of T and B lymphocytes. By comparing the peak concentration of each of these cytokines among the different groups of NHP, we did not find any general increase at increasing LV doses (Supplementary Fig. 5a-j). Moreover, we repeated the measurement of these cytokines on serum samples from non-immune suppressed NHP treated with LV encoding human FIX from our previous study and observed that the peak concentration of these cytokines was similar to that observed in NHP treated in this study, suggesting that the administered immune suppression did not substantially alter the innate immune response to LV administration (see Supplementary Fig. 5a-j). Interestingly, we noted that the peak concentration of some of these cytokines seemed to inversely correlate with the administered LV particle dose (Supplementary Fig. 5k-m). This data might suggest that accumulation of CD47 signaling on professional phagocytes from high amounts of infused vector particles reduces cytokine release. We observed a rise followed by a decline of anti-VSV-G Abs, as expected due to i.v. administration of VSV-G pseudotyped LV (Supplementary Fig. 6a). However, in some NHP the concentration of these Abs was lower than in previous reports¹⁷, suggesting that the immune suppression regimen reduced the humoral immune response to this Ag that was only present at the time of LV administration. In parallel to humoral response to FVIII and VSV-G, anti-FVIII specific T lymphocytes were quantified over time in peripheral blood mononuclear cells (PBMC) and at the end of experiment in splenocytes, by enumerating IFN γ -producing T cells in response to 18 peptides pools covering the entire FVIII aminoacidic sequence. We observed a delayed transient anti-FVIII T cell response in PBMC which became detectable from day 21 *post* LV, mainly in NHP expressing the highest FVIII amounts, with the overall tendency to contract at undetectable level at the end of the follow up, 60 days *post* LV (in 3/5 of LV.coFVIII-treated NHP and 4/5 of LV.coFVIII.XTEN-treated NHP; Supplementary Fig. 6b). Analyses of secondary lymphoid organs revealed that FVIII-responsive T cells were not detectable in hepatic lymph nodes (Supplementary Fig. 6c). Conversely, IFN γ -producing T cells were detected in response to FVIII stimulation in splenocytes of NHP treated with LV.coFVIII (3/5), while only a weak response was present in 1/5 NHP treated with LV.coFVIII.XTEN (Fig. 6k). This rapid *in vitro* response upon FVIII peptide stimulation (24 hours of stimulation) correlates with the lower residual transgene expression in the liver (see Fig. 5b), therefore likely derived from memory FVIII-specific cytotoxic T cells that mediated elimination of FVIII-expressing hepatocytes. In line with these results, splenic T cells from NHP treated with LV.coFVIII displayed a higher proliferative response upon *in vitro* culture in the presence of increasing concentration of FVIII protein, which suggests that a more pronounced T-helper response was mounted in NHP to LV.coFVIII treatment (Fig. 6l). Splenocytes were also tested to evaluate the presence of IL-5 producing T helper cells, in response to FVIII peptides stimulation, which however were undetectable in all NHP at necropsy (Supplementary Fig. 6d). In order to better visualize and correlate data of innate and adaptive immune responses and data of FVIII expression at peak and endpoint, we generated a heatmap of these results (Supplementary Fig. 7). We noted that amounts of remaining FVIII transgene activity and RNA at endpoint inversely correlated with the presence of FVIII-reactive T cells in the spleen. Moreover,

higher cytokine response did not appear to predict higher amounts of FVIII-specific T cells at end point, rather correlates with higher initial FVIII output. Collectively, these data show that the applied immune suppressive regimen minimally affected the innate immune responses, which likely still recruited and activated subsets of antigen-presenting cells and lymphocytes in the spleen and in the liver, leading to the induction of adaptive anti-FVIII immune responses once the immunosuppression was withdrawn. Overall, these results indicate that the NHP treated with FVIII.XTEN encoding LV expressed the highest FVIII amounts despite the lowest administered LV doses and showed the lowest anti-FVIII cellular immune response, while maintaining FVIII-expressing hepatocytes at the end of the follow up.

Discussion

Here we show that liver-directed LV gene therapy allows establishing long-term FVIII activity in the blood and phenotypic correction of hemophilia A mice and achieves therapeutic-range human FVIII activity in NHP, albeit only during immunosuppression. The codon-optimization of the B-domain deleted FVIII transgene provided for 10–20 fold increase in expression both in human hepatic cell lines and in mice, in line with previous reports^{24, 25}. Incorporation of the unstructured XTEN polypeptide into the FVIII transgene further improved the steady-state level and activity of the protein *in vivo*. XTENylation of biologically active molecules has been shown to increase protein half-life in pre-clinical models and in humans, by increasing the hydrodynamic volume of the modified molecule²⁰. A recombinant XTEN-containing FVIII product has shown 3-4-fold half-life extension compared to conventional FVIII, without evidence of adverse events or increased incidence of development of neutralizing anti-FVIII Abs²⁶. Beyond these data, here we show a remarkable 10-fold higher and 20-fold higher FVIII transgene expression and activity in mice and NHP, respectively, by inclusion of the XTEN element, thus significantly reducing the LV dose required for therapeutic efficacy, decreasing manufacturing needs and alleviating concerns related to possible LV-dose dependent toxicities. These results may be due to both improved FVIII secretion by hepatocytes and increased half-life of the protein in the circulation, thus overall resulting in higher plateau blood FVIII concentration and activity. The difference between mice and NHP may be due to the different LV doses used and resulting FVIII amounts in the circulation of LV-treated animals. We consider unlikely that the anti-human FVIII immune response observed in NHP upon corticosteroid removal, influenced peak amounts of circulating human FVIII in the NHP, since the animals were still immune suppressed at that time.

Long-term stability of FVIII-XTEN transgene expression and activity in both mice treated as newborns and adults indicates absence of selective disadvantage, or advantage, of transduced hepatocytes, which may potentially be due, the former to toxicity from FVIII overexpression, the latter to LV insertional mutagenesis. These data also confirm that LV gene therapy allows maintenance of the therapeutic transgene following liver growth and homeostasis in mice. Interestingly, LV-dose response was more favorable in mice treated as newborns compared to mice treated as adults, suggesting better access and/or permissiveness to LV transduction in the former, a finding that warrants further investigation. We also report the generation of novel double KO mice that combine the hemophilia and immune-deficient

phenotypes, a useful mouse model for pre-clinical investigation of gene transfer and editing strategies or FVIII protein products.

In agreement with our previous LV-FIX study, the present study confirms minor and self-resolving acute toxicities and pro-inflammatory response, following i.v. administration of LV in NHP, across different LV doses and transgenes. The corticosteroid regimen administered for a few days around LV infusion temporarily inhibited the adaptive arm of the immune system, thus allowing determination of peak FVIII transgene expression and activity for a window of time, before the development of anti-FVIII humoral and cellular immune responses, which occurred upon corticosteroid removal. However, this immune suppression regimen did not apparently affect the innate immune response to LV administration, as shown from the similar cytokine response observed in NHP treated in this study compared to NHP treated in our previous study, which received i.v. LV administration without corticosteroid treatment¹⁷. The lack of increase in cytokine release when increasing the amount of infused LV particles suggests that the high surface content of CD47 *per* vector particle conferred shielding from uptake and innate immune sensing also in a dose-dependent non-particle autonomous manner. We also confirm selective targeting of liver and spleen of CD47hi LV and protection from phagocytosis by KC in NHP, following peripheral vein infusion.

Restoration of FVIII activity above 12% of normal in people with severe hemophilia A is considered sufficient to prevent joint hemorrhages and is being set as the minimal desirable target for correction in gene therapy¹⁷. The favorable acute toxicity profile observed, combined with therapeutic-range LV dose dependent FVIII activity, provide a comfortable therapeutic window and encourage further development of LV-mediated liver gene therapy for hemophilia A. Because of the high immunogenicity of the FVIII protein, anti-human FVIII immune responses are expected in NHP and have been previously observed in pre-clinical studies of both FVIII protein and AAV-vector mediated gene replacement^{25,27}. Absence of anti-FVIII Abs development following AAV-vector gene therapy in human trials in Abs-negative people previously treated with FVIII protein replacement therapy suggests that this population is at low risk of mounting immune responses to FVIII following gene therapy and that the value of the NHP model is limited in predicting this outcome. Maintenance of FVIII activity following LV gene therapy in immune-competent hemophilia A mice transgenic for the R593C human FVIII mutant supports this hypothesis. However, further investigation and possible modulation of anti-FVIII immune responses in pre-clinical models is anticipated to further reduce the risk of inducing such responses in humans and in view of future application of LV gene therapy to previously untreated hemophilia patients that are naïve to FVIII. With this perspective, inclusion of the XTEN polypeptide in the FVIII transgene might have alleviated some of its immunogenicity, as the NHP treated with FVIII.XTEN LV showed the highest FVIII transgene RNA in the liver and circulating activity at the end of the follow up accompanied by a reduced FVIII-specific T cell response compared to NHP treated with LV expressing the non-modified transgene.

Integration of LV in the genome of target cells is required to maintain the therapeutic transgene in dividing cells, while raising concerns of inducing insertional mutagenesis. However, accumulating evidence supports the low genotoxicity of LV, as no insertional oncogenesis have been published so far in

> 300 patients treated by LV-transduced hematopoietic stem cells across different doses and transgenes, with multiyear follow up and mostly at high VCN²⁸. Moreover, we have previously shown undetectable genotoxicity in the liver by LV in sensitive mouse models and NHP^{15,17}. Whereas efforts are underway to continue modelling and possibly reducing LV genotoxic potential, the safety records of current LV design in *ex vivo* gene therapy in humans reassures about their potential use for *in vivo* gene therapy.

In conclusion, surface-engineered MHC-free and CD47hi LV have demonstrated efficient gene transfer into hepatocytes in NHP. By engineering the FVIII transgene we further enhanced the therapeutic index of *in vivo* LV gene therapy for hemophilia A, paving the way for potential clinical application.

Methods

Study design

Sample size in experiments with mice was chosen according to previous experience with experimental models and assays. Sample size in the NHP study was limited by ethical and feasibility reasons. No sample or animal was excluded from the analyses. Mice and NHP were randomly assigned to each experimental group. Investigators were not blinded.

Vector production

For the NHP study, we used large-scale purified CD47hi and MHCfree LV batches, produced by MolMed S.p.A. (now AGC Biologics), on 24 L scale of supernatant, as previously described^{15,17} and formulated in PBS 0.2% human serum albumin. Results of selected quality control assays performed on these batches are reported in Supplemental Table 1. For all the other experiments *in vitro* and with mice, we used lab-grade LV. Lab-grade third-generation self-inactivating (SIN) LV were produced by calcium phosphate transient transfection into 293T cells. 293T cells were transfected with a solution containing a mix of the selected LV genome transfer plasmid, the packaging plasmids pMDLg/pRRE and pCMV.REV, pMD2.VSV-G and pAdvantage, as previously described¹⁷. Medium was changed 14-16 hours after transfection and supernatant was collected 30 hours after medium change. LV-containing supernatants were sterilized through a 0.22µm filter (Millipore) and, when needed, transferred into sterile poliallomer tubes (Beckman) and centrifuged at 20,000 g for 120 min at 20° C (Beckman Optima XL-100K Ultracentrifuge). LV pellet was dissolved in the appropriate volume of PBS to allow 500-1000X concentration.

Mice experiments

Founder B6;129S-*F8*^{tm1Kaz}/J mice (referred to as HemoA or *F8* KO²⁹) were obtained from The Jackson Laboratories (stock #004424). Founder B6.129S7-Rag.1^{tm1Mom}/J mice (referred to as *Rag1* KO³⁰) were

purchased from The Jackson Laboratory (stock # 002216). *F8-Rag1* double KO (referred to as RagHemoA) mice were obtained by crossing *F8* KO homozygous mice with *Rag1* KO homozygous mice. Founder *F8*^{tm1Kaz} Tg(Alb-F8*R593C)T4Mcal/J mice (referred to as HemoA-R593C²²) were obtained from The Jackson Laboratories (stock #017706). For mice genotyping, DNA was extracted from tail biopsies using Maxwell 16 Mouse Tail DNA Purification Kit (Promega), following manufacturer's instructions. The genotype was then assessed following the protocols available on The Jackson Laboratory website. All mice were maintained in specific pathogen-free conditions. Vector administration was carried out in males and females adult (7-10-week-old) mice by tail-vein injection (250-500 μ L/mouse), while in newborns by temporal vein injection (25-30 μ L/mouse). Mice were bled from the retro-orbital plexus using capillary tubes and blood was collected into 0.38% sodium citrate buffer, pH 7.4. Mice were deeply anesthetized with tribromoethanol (Avertin) and euthanized by CO₂ inhalation at the scheduled times. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee.

Coagulation assays

Tail-clipping assay was performed as described¹⁴. Mice were anesthetized and tail was placed in pre-warmed 37° C water for 2 minutes and subsequently cut at 2.5-3 mm diameter. Tail was then immediately placed in 37° C PBS with calcium and magnesium and monitored for bleeding or clotting for 15 minutes. Blood-containing PBS was centrifuged at 520 g for 10 min at 4° C to collect erythrocytes and resuspended in 6 ml of lysis buffer (10mM KHCO₃, 150mM NH₄Cl, 0.1mM EDTA). Lysis proceeded for 10 minutes at room temperature and samples were centrifuged as above. OD at 595 nm of supernatants was measured.

To confirm RagHemoA phenotype, the clotting time of mouse plasma was determined by activated partial thromboplastin time (aPTT) using a semiautomated coagulometer (BioMerieux, France).

FVIII assays

The concentration of human FVIII was determined in mouse plasma by an enzyme-linked immunosorbent assay (ELISA) specific for human FVIII antigen. Microtiter plates were coated with anti-hFVIII binding Ab (Green Mountain Antibodies #GMA8016, 0.2 μ g/well in 0.1 M carbonate buffer, pH 9.6) over night at 4° C and then blocked 1 hour at room temperature with blocking buffer (PBS 0.05% Tween-20, 1M NaCl, 10% heat inactivated horse serum, Gibco). Plasma samples are diluted as needed starting from 1:10 in blocking buffer, added to wells (100 μ L/well) and incubated 2 hours at 37° C. hFVIII was detected by adding detection Ab (Affinity Biologicals, F8C-EIC-D) 1 hour at 37° C, followed by 5-10 minutes incubation with 100 μ L/well of TMB substrate (Surmodics). Reaction was blocked with HCl 1N (50 μ L/well) and absorbance of each sample was determined spectrophotometrically at 450nm, using a Multiskan GO

microplate reader (Thermo Fisher Scientific) and normalized to antigen standard curve (ReFACTO, Pfizer, from 25ng/mL to 0.39ng/mL serially diluted 1:2 in blocking buffer; dilution was corrected with 10% HemoA murine plasma). hFVIII activity in mouse plasma was measured using Coatest SP FVIII (Chromogenix) following manufacturer's instructions.

Anti-hFVIII Abs were measured in mouse plasma by ELISA. Microtiter plates were coated with ReFACTO (Pfizer, 0.1 µg/well in 0.1 M carbonate buffer, pH 9.6) over night at 4°C and then blocked 1 hour at room temperature with blocking buffer (PBS 0.05% Tween-20, 10% heat inactivated horse serum, Gibco). Samples are heat inactivated for 30 minutes at 56°C, diluted as needed starting from 1:100 in blocking buffer, added to wells (100µL/well) and incubated 2 hours at 37°C on orbital shaker. Anti-hFVIII Abs were detected by adding detection Ab (goat anti-mouse IgG-HRP, Sigma, 1:10,000 in blocking buffer) 1 hour at 37°C on orbital shaker, followed by 5-10 minutes incubation with 100µL/well of TMB substrate (Surmodics). Reaction was blocked with HCl 1N (50µL/well) and absorbance of each sample was determined spectrophotometrically at 450nm, using a Multiskan GO microplate reader (Thermo Fisher Scientific) and normalized to standard curve. The standard curve is a pool of 7 different commercial anti-human FVIII Abs raised against different FVIII domains (Green Mountain Antibodies #GMA8002, #GMA8005, #GMA8008, #GMA8011, #GMA8015, #GMA8016, #QED10104) serially diluted 1:2 from 100ng/mL to 0.78ng/mL in blocking buffer; dilution was corrected with 1% HemoA murine plasma.

The concentration of hFVIII was determined in NHP plasma by an ELISA specific for hFVIII antigen. Microtiter plates were coated with anti-hFVIII binding Ab (Green Mountain Antibodies #GMA8023, 0.2µg/well in 0.05 M carbonate buffer, Sigma) over night at 4°C and then blocked 1 hour at room temperature with blocking buffer (PBS 0.05% Tween-20, 0.5M NaCl, 10% heat inactivated horse serum, Gibco). Plasma samples are diluted as needed starting from 1:10 in blocking buffer, added to wells (100µL/well) and incubated 2 hours at 37°C. hFVIII was detected by adding detection Ab (Affinity Biologicals, F8C-EIA-D) 1 hour at 37°C, followed by addition of 100µL/well of TMB substrate (Surmodics). Reaction was blocked with HCl 1N (50µL/well) and absorbance of each sample was determined spectrophotometrically at 450nm, using a Multiskan GO microplate reader (Thermo Fisher Scientific) and normalized to antigen standard curve (ReFACTO, Pfizer or recombinant hFVIII-XTEN, from 50ng/mL to 0.39ng/mL serially diluted 1:2 in blocking buffer; dilution was corrected with 10% NHP plasma). hFVIII activity was quantified in NHP plasma by a modified FVIII chromogenic assay: hFVIII in plasma samples was first captured by a hFVIII specific Ab immobilized onto a 96-well plate (Green Mountain Antibodies, #GMA8023, 0.1µg/well in 0.05 M carbonate buffer, Sigma), then chromogenic activity of hFVIII was measured using Coatest SP FVIII kit (Diapharma, K824086) following manufacturer's instructions. Standard curves were obtained by diluting recombinant human FVIII (ReFACTO, Pfizer) into untreated NHP plasma. Total anti-human FVIII Abs were quantified in heat inactivated NHP serum (1 hour at 56°C) by ELISA. Microtiter plates were coated with ReFACTO (Pfizer, 0.1 µg/well in 0.1 M carbonate buffer, pH 9.6) over night at 4°C and then blocked 1 hour at room temperature with blocking buffer (PBS 0.05% Tween-20, 0.5M NaCl, 10% heat inactivated horse serum, Gibco). Samples were diluted as needed starting from 1:20 in blocking buffer, added to wells (100µL/well) and incubated 2 hours at 37°C on orbital shaker. Anti-hFVIII Abs were detected by adding detection Ab (rabbit anti-monkey IgG-HRP, Sigma #A2054,

1:10,000 in blocking buffer) 1 hour at 37°C on orbital shaker, followed by 5-10 minutes incubation with 100µL/well of TMB substrate (SurModics). Reaction was blocked with HCl 1N (50µL/well) and absorbance of each sample was determined spectrophotometrically at 450nm, using a Multiskan GO microplate reader (Thermo Fisher Scientific) and normalized to standard curve (Monkey IgG, MyBioSource #MBS679190, from 125ng/mL to 1.9ng/mL serially diluted 1:2 in coating buffer).

Neutralizing anti-human FVIII Abs were determined in heat inactivated NHP plasma samples (1 hour at 56°C) by Bethesda assay. The test sample was incubated for 2 hours at 37° C with recombinant human FVIII (ReFACTO, Pfizer, 1 U/mL) with FVIII activity of 100%. Residual FVIII activity was measured using Coatest FVIII SP kit (Diapharma, K824086) and converted into Bethesda Units (BU)/mL, where one BU is defined as the inverse of the dilution factor of the test sample that yields 50% residual FVIII activity.

NHP study

Ten adult (3-5 kg body weight) males *Macaca Leonina* (Northern pig-tailed macaques) were purchased by Bioprim (Baziège, France). Macaques were housed in an enriched environment with access to toys, fresh fruits, and vegetables at the Boisbonne Center (Nantes, France), under protocol APAFIS#4302-2015122314563838 that was approved by the Institutional Animal Care and Use Committee of the Pays De Loire. For LV administration, animals were anesthetized with Demetomidine (Domitor) 30 µg/kg and Ketamine 7 mg/kg and maintained under gas anesthesia, 1-2% Isoflurane (Vetflurane). The LV-containing solution was administered using a syringe with controlled flow rate fixed at 1.5 mL/min via a catheter in the saphenous vein (31-100 total mL according to LV type and dose, 6.6-23.5 mL/kg). Each NHP was treated with pools coming from different LV batches (see Table S1). Solu-Medrol (methylprednisolone, 10 mg/kg/day) was administered intramuscularly as immune suppression regimen from day -1 to day 7 for high dose LV.coFVIII and LV.coFVIII.XTEN treated NHP, while it was extended from day -3 to day 9 for low dose treated NHP (LV administration occurred at day 0). In addition, an antihistamine pre-medication regimen was administered: dexchlorpheniramine (Polaramine, 4 mg/kg) i.v. 30 minutes before LV. Blood samples were taken at different time points from the femoral vein upon anesthesia with 10 mg/kg ketamine (Imalgene) intramuscularly. For hematology, 1 mL of total blood samples was collected on EDTA-coated tubes. For clinical biochemistry, 2 mL of total blood samples was collected on heparin-coated tubes and for hemostasis, 1.8 mL of total blood was collected in citrate-coated tubes. Blood tests were performed on fresh samples at the Veterinary School of Nantes (LDHvet, Oniris). Biochemistry parameters were analyzed by automatons and analyzers based on spectrometry, reflectometry, potentiometry and enzyme immunoassays. Hemostasis was analyzed by a hemostasis analyzer based on electro-mechanical clot detection (viscosity-based detection system). Tissue samples were collected following euthanasia, performed by i.v. injection of pentobarbital sodium (Dolethal).

ELISPOT splenocytes / PBMC

PBMC and splenocytes were isolated by density gradient (Ficoll, Sigma-Aldrich). Multiscreen filter plate (Millipore-Merck) were coated overnight 4°C with capture Ab for interferon-g (IFN γ , MT126L 10 μ g/mL 50 μ L/well, Mabtech) or IL5 (TRFK5 10 μ g/mL 50 μ L/well, Mabtech) and blocked with PBS 1% BSA for 2 hours at 37°C. Plates were equilibrated with culture medium for 10 minutes at room temperature before seeding cells. Total NHP splenocytes or PBMC (2.5x10⁵ cells/well) were plated in X-vivo-15 (Lonza) at least in duplicates without antigenic stimulation (DMSO alone) or stimulated with 18 different peptide pools covering the entire aminoacidic sequence FVIII.BDD (each peptide at 1 μ M final concentration, FVIII.BDD peptide library 15aa long, 5aa offset, Sigma-Aldrich) for 24 hours at 37C 5% CO₂. At the end of the culture detection Ab for IFN γ (7-B6-1 1 μ g/mL 50 μ L/well, Mabtech) or IL-5 (5A10 1 μ g/mL 50 μ L/well, Mabtech) was added and incubated for 2 hours at room temperature. Avidin-POD solution (Roche, 1:5,000, 50 μ L/well) was then added and incubated for 1 hours at room temperature and spot were developed by AEC solution (Sigma-Aldrich) for 15 minutes at room temperature in the dark. Plate image was acquired and spots were counted by Immunospot S6-Ultra (Cellular Technology Limited). Mean number of spots+2SD from the unstimulated condition was subtracted to stimulated condition and reported as IFN γ or IL-5 producing cells in 1x10⁶ splenocytes or PBMC.

Splenocyte proliferation

Total NHP splenocytes were plated in flat-bottom 96 well plate (3x10⁵ cells/well) in X-vivo-15 (Lonza). Cells were left unstimulated or stimulated with increasing dose of FVIII.BDD protein (ReFACTO, Pfizer) in triplicates. After 5 days of culture, 1 μ Ci/well of ³H-Thymidine was added and incubated for additional 16 hours. Cell proliferation was indirectly quantified by measuring ³H-Thymidine incorporation. Stimulation index (SI) was obtained as ratio between the mean counts *per minutes* (cpm) in each stimulated condition and the mean counts of the unstimulated cell.

Statistical analysis

Statistical analyses were performed upon consulting with professional statisticians at the San Raffaele University Center for Statistics in the Biomedical Sciences (CUSSB). When normality assumptions were not met, non-parametric statistical tests were performed. Two-tailed Mann-Whitney test was performed to compare two independent groups, while in presence of more than two independent groups Kruskal-Wallis test followed by post-hoc analysis (Dunn's test for multiple comparisons against the reference control group along with Bonferroni's correction) was applied. Strength of the relationship between two quantitative variables was assessed by Spearman's rank-order test. Expression of FVIII in different treatment groups and over time (starting from the steady-state) was modelled using Linear Mixed-Effects (LME) models. Group/dosage indicator variable and time variable were included in the model, both as main effects as well as in interaction. This modelling approach allows to properly capture the

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Declarations

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Author Contributions:

M.M. designed and performed experiments, analyzed and interpreted data and wrote the manuscript. C.C. and T.L. designed and performed experiments, analyzed data and edited the manuscript. M.B., F.R., T.P., R.C., S. P-W., D.D., I.V. performed experiments and analyzed data. C.B. performed statistical analysis. P.A. supervised I.V. work. E.A. coordinated experiments with NHP. C.M. supervised T.L. research. A.A. performed experiments, analyzed and interpreted data related to immune responses and edited the manuscript. L.N. supervised research, interpreted data and edited the manuscript. A.C supervised and coordinated research, interpreted data and wrote the manuscript.

Conflict-of-interest-disclosures: L.N., A.C., A.A., M.M., T.L., S.P.W. are inventors on patent applications submitted by Foundation Telethon and San Raffaele Scientific Institute or Bioverativ/Sanofi on LV technology related to the work presented in this manuscript. FT and SRSI, through SR-Tiget, have established a research collaboration on liver-directed lentiviral gene therapy of hemophilia with Bioverativ/Sanofi.

Figures

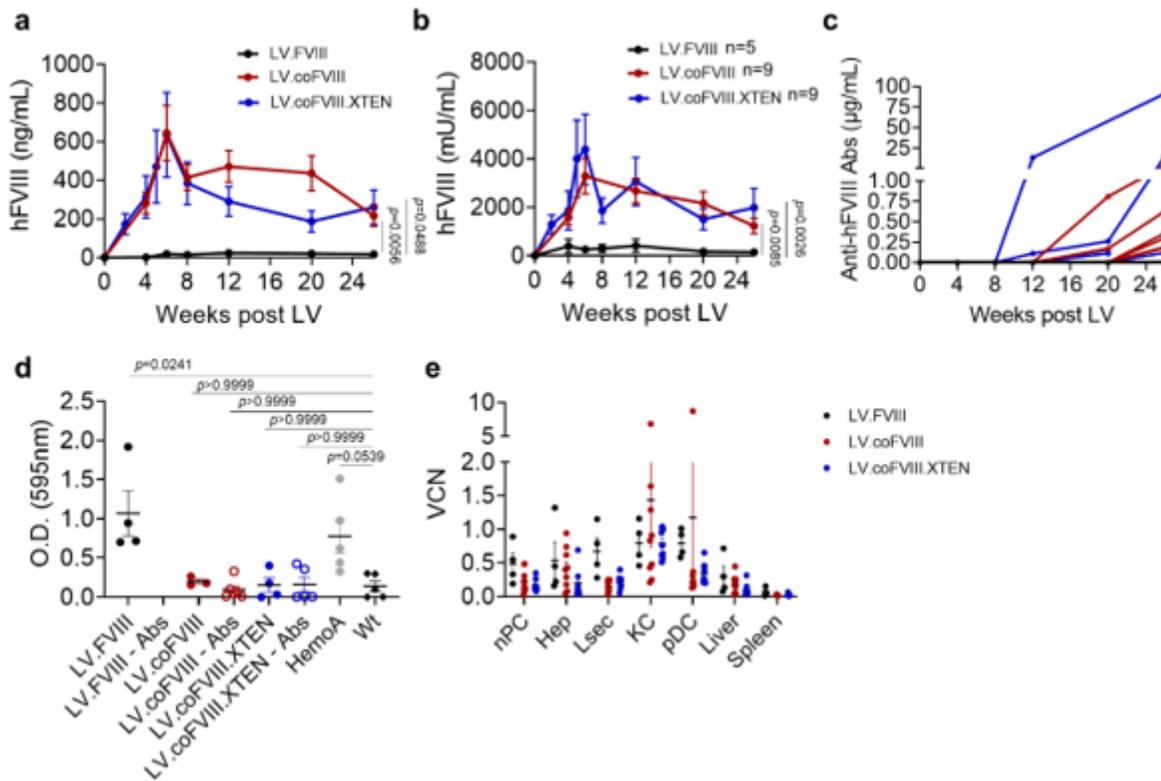


Figure 1

FVIII transgene selection in hemophilia A mice. a, b Mean with standard error of the mean (SEM) of human FVIII (hFVIII) antigen (a) or activity (b) measured in the plasma of Hemophilia A (HemoA) mice treated as newborns by i.v. injection of 2.5×10^{10} TU/kg of the indicated LV (n=5, for LV.FVIII in black; n=9 for LV.coFVIII in red; n=9 for LV.coFVIII.XTEN in blue). Linear mixed model (LME) followed by post-hoc analysis (difference between experimental groups and the reference control group is evaluated at the last time point). Bonferroni's adjusted p-values from the post hoc analysis are shown (b). c Single values of anti-hFVIII antibodies (Abs) measured in the plasma of mice in (a, b). d Single values and mean with SEM of hemoglobin absorbance at 595nm collected during tail-clipping assay (see methods section) from LV-treated mice, HemoA untreated mice and wild type (wt) age-matched controls, as indicated. Abs-positive mice (empty dots) show hemostasis restoration similar to Abs-negative and wt mice. Kruskal-Wallis test followed by post hoc analysis. e Single values and mean with SEM of vector copies per diploid genome (VCN) measured in fractionated and FACS-sorted liver subpopulations (nPC = non-parenchymal cells; Hep = hepatocytes; Lsec = liver sinusoidal endothelial cells; KC = Kupffer cells; pDC = plasmacytoid dendritic cells) or in total liver and spleen of mice 5-6 months after LV administration. O.D. = optical density; co = codon-optimized; U: units.

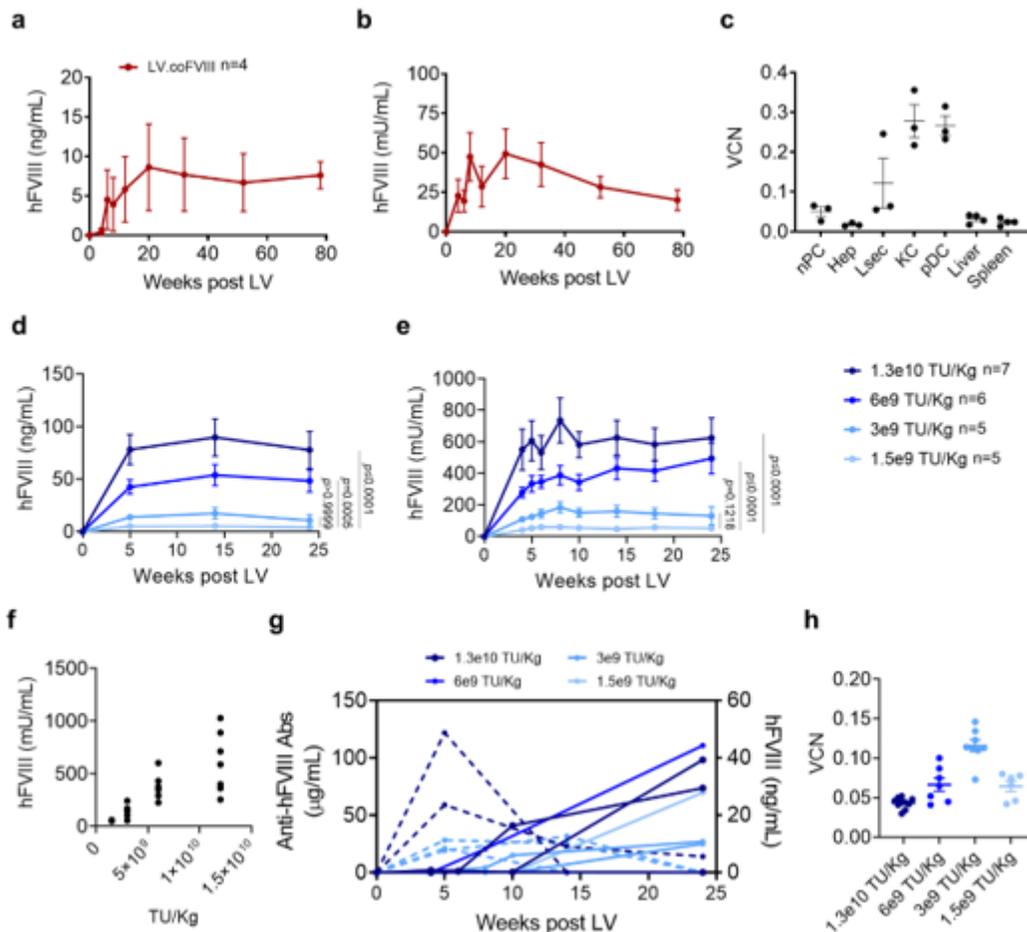


Figure 2

Dose-response of LV expressing selected FVIII transgenes in newborn-treated HemoA mice monitored long-term. a, b Mean with SEM of hFVIII antigen (a) or activity (b) measured in the plasma of HemoA mice treated as newborns (n=4) by i.v. injection of 1×10^{10} TU/kg of LV.coFVIII. c Single values and mean with SEM of VCN measured in fractionated and FACS-sorted liver subpopulations or in total liver and spleen of mice 20 months after LV administration. d, e Mean with SEM of hFVIII antigen (d) or activity (e) measured in the plasma of Abs-negative HemoA mice treated as newborns by i.v. injection of the indicated doses of LV.coFVIII.XTEN (n=7, for 1.3×10^{10} TU/kg; n=6, for 6×10^9 TU/kg; n=5, for 3×10^9 TU/kg; n=5, for 1.5×10^9 TU/kg). Linear mixed model (LME) followed by post hoc analysis (difference between experimental groups and the reference control group is evaluated at the last time point). Bonferroni's adjusted p-values from the post hoc analysis are shown. Due to the lower number of time points, to improve model fit, in panel (d), time variable has been entered in the model as categorical variable. In panel (e), four extreme outlier observations have been removed from the analysis. f Single values of plateau circulating hFVIII in mice shown in (d, e) at the indicated LV doses. g Single values of anti-hFVIII abs (full lines, plotted on left Y axis) and hFVIII antigen (dashed lines, plotted on right Y axis) measured in the plasma of Abs-positive HemoA mice (3/10 mice treated at 1.3×10^{10} TU/Kg; 2/7 mice treated at 6×10^9 TU/kg; 3/8 mice treated at 3×10^9 TU/kg; 1/6 mice treated at 1.5×10^9 TU/kg). h Single

values and mean with SEM of VCN measured in the liver of mice 6 months post LV administration at the indicated doses.

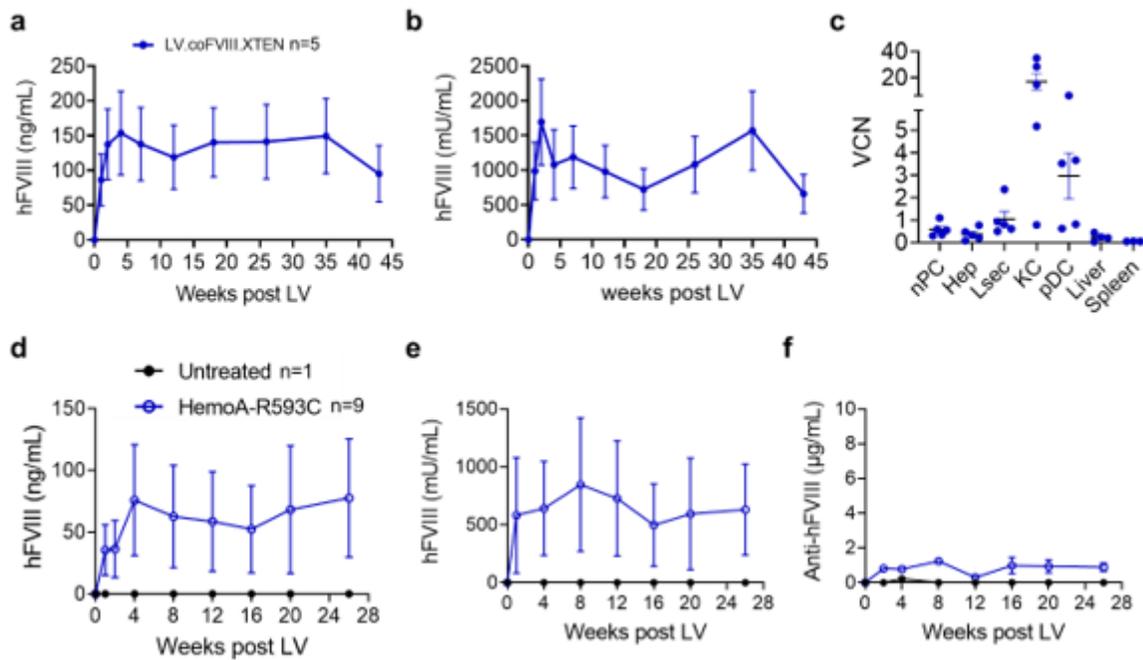


Figure 3

In vivo LV gene therapy in HemoA adult mice. a, b Mean with SEM of hFVIII antigen (a) or activity (b) measured in the plasma of immunodeficient RagHemoA mice treated at 8 weeks of age (n=5) by i.v. injection of 8×10^{10} TU/kg of LV.coFVIII.XTEN. c Single values and mean with SEM of VCN measured in fractionated and FACS-sorted liver subpopulations or in total liver and spleen of mice 11 months after LV administration. d-f Mean with SEM of hFVIII antigen (d), activity (e) or anti-hFVIII Abs (f) measured in the plasma of immune competent HemoA-R593C mice treated at 7 weeks of age (n=9) by i.v. injection of 4×10^{10} TU/kg of LV.coFVIII.XTEN.

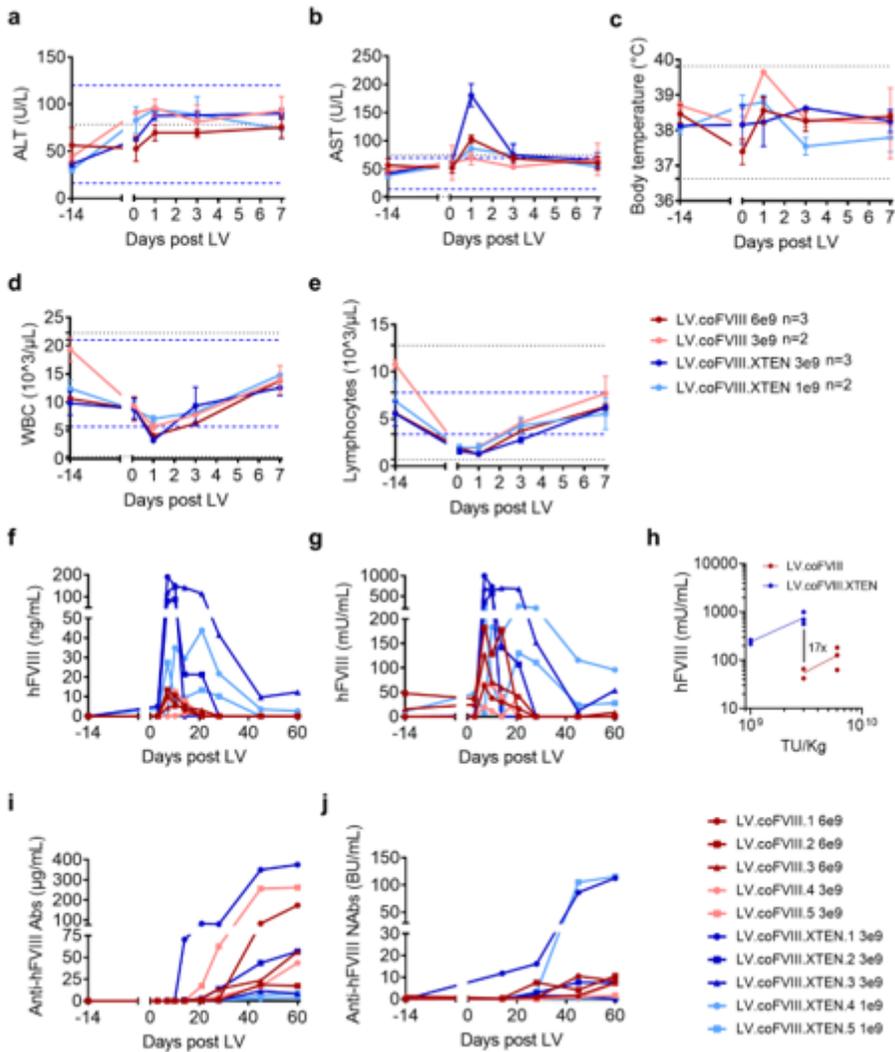


Figure 4

Tolerability and efficacy of in vivo LV gene therapy in NHP. a-e Mean with SEM (n=3) or range (n=2) of the serum concentration of alanine aminotransferase (ALT, a), aspartate aminotransferase (AST, b), body temperature (c), counts of white blood cells (WBC, d) and lymphocytes (e) of NHP treated with LV.coFVIII (n=3 6x10⁹ TU/kg; n=2 3x10⁹ TU/kg), or with LV.coFVIII.XTEN (n=3 3x10⁹ TU/kg; n=2 1x10⁹ TU/kg) at the indicated time after administration. The black dotted lines show the mean±3 standard deviations (SD) calculated on a pool of 38 pre-LV samples taken from 19 animals; the blue dashed lines show the normal reference values for *Macaca fascicularis*. f, g Single values of the concentration of hFVIII antigen (f) or activity (g) measured in the plasma of LV-treated NHP (as indicated) at the indicated time after administration. h Concentration of hFVIII activity at peak in NHP treated as indicated at the indicated dose. i, j Single values of the concentration of total anti-hFVIII Abs (i), or neutralizing anti-hFVIII Abs (j) in the serum of LV-treated NHP (as indicated) at the indicated time after administration. BU: Bethesda Units.

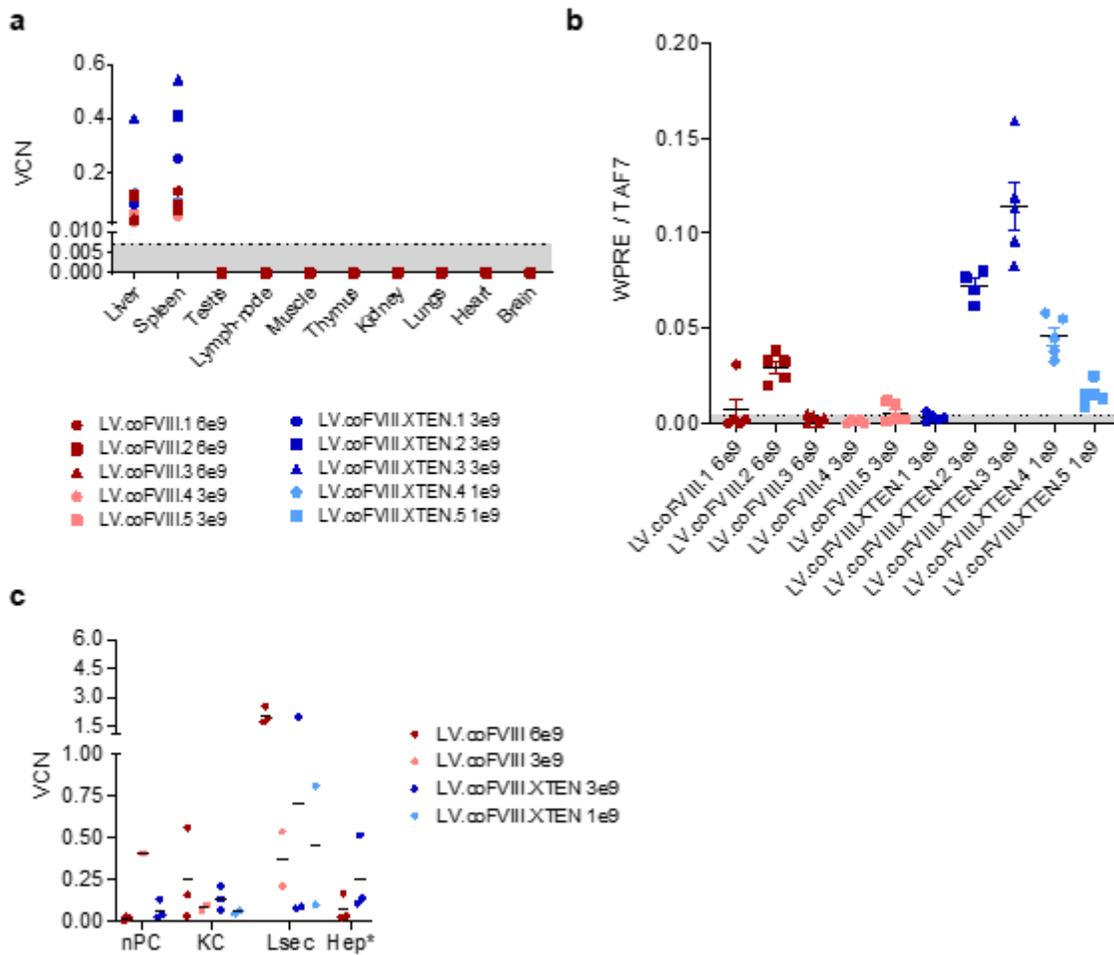


Figure 5

LV biodistribution in treated NHP. a Single values of VCN in the indicated organs of LV-treated NHP, as indicated, at necropsy (60 days post LV). The dashed lines defining the grey area represent the lower limit of detection (0.0003) and the lower limit of reliable quantification (0.007, see methods section). b Expression analysis by quantitative PCR of WPRE normalized on the endogenous TAF7 gene on RNA extracted from different liver lobes of LV-treated NHP, as indicated. The grey area represents mean background signal for WPRE expression in the spleen. c Single values of VCN measured or calculated (*) in the indicated liver subpopulation after gradient purification (nPC) or FACS-sorting (KC, Lsec). VCN in Hep is calculated according to the formula $VCN_{liver} = (VCN_{Hep} \times 0.7) + (VCN_{nPC} \times 0.3)$. This formula is based on average proportion of liver cell subpopulations in the liver.

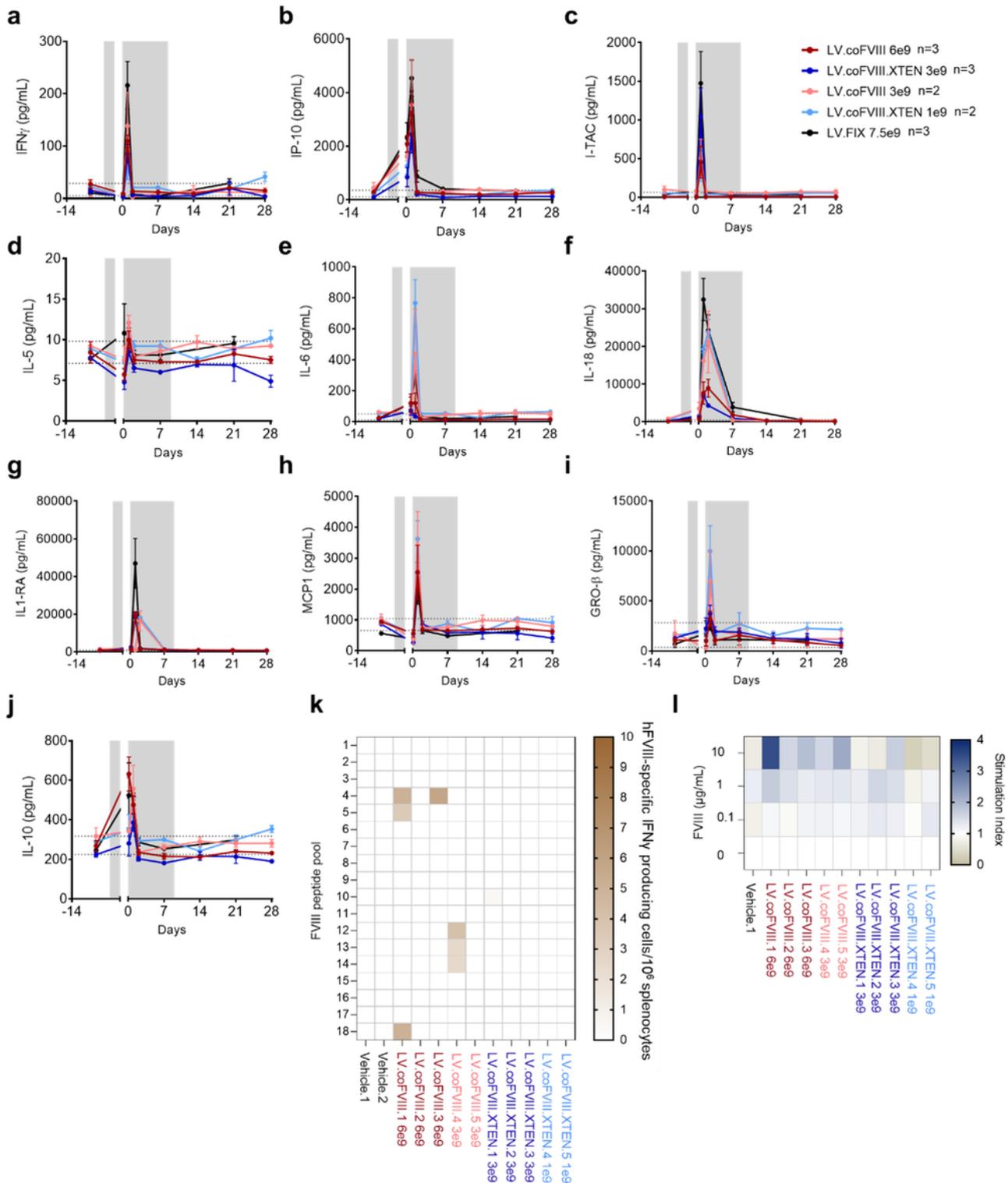


Figure 6

Immune responses following in vivo LV gene therapy in NHP. a-j Mean and SEM (n=3) or range (n=2) of the serum concentration of interferon- γ (IFN γ , a), interferon gamma-induced protein 10 (IP-10, b), interferon-inducible T cell alpha chemoattractant (I-TAC, c), interleukin-5 (IL-5, d), IL-6 (e), IL-18 (f), IL-1 receptor antagonist (IL-1RA, g), monocyte chemoattractant protein 1 (MCP1, h), growth-regulated protein beta (GRO- β , i) and IL-10 (j) in LV-treated NHP, as indicated. Black dotted lines are mean \pm 1SD calculated

on a pool of pre-LV samples taken from 20 animals. Grey area represent the window of immune-suppression regimen in LV.coFVIII or LV.coFVIII.XTEN treated NHP (see “methods” section for details). k Heatmap reporting the frequency of hFVIII-specific IFN γ producing cells/10⁶ splenocytes in NHP treated as indicated. The 18 pools of FVIII peptides cover the entire transgenes encoded by the administered LV. l Heatmap reporting the stimulation index (i.e. proliferation over unstimulated cells) of splenocytes kept in culture in presence of the indicated amounts of hFVIII, as indicated.

Supplementary Files

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