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

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Liver gene therapy with adeno-associated viral (AAV) vectors delivering clotting factor transgenes into hepatocytes has shown multiyear therapeutic benefit in adults with hemophilia. However, the mostly episomal nature of AAV vectors challenges their application to young pediatric patients. We developed lentiviral vectors, which integrate in the host cell genome, that achieve efficient liver gene transfer in mice, dogs and non-human primates, by intravenous delivery. Here we first compare engineered coagulation factor VIII transgenes and show that codon-usage optimization improved expression 10-20-fold in hemophilia A mice and that inclusion of an unstructured XTEN peptide, known to increase the half-life of the payload protein, provided an additional >10-fold increase in overall factor VIII output in mice and non-human primates. Stable nearly life-long normal and above-normal factor VIII activity was achieved in hemophilia A mouse models. Overall, we show long-term factor VIII activity and restoration of hemostasis, by lentiviral gene therapy to hemophilia A mice and normal-range factor VIII activity in non-human primate, paving the way for potential clinical application.
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 treated. 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 subcutaneously. 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 hemophilia. Advanced-phase clinical studies have highlighted the potential of gene therapy to fulfill this promise, by showing multiyear 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. However, a decreasing trend in FVIII gene expression has been reported, for reasons that are not fully understood and might be related to the challenge of stably maintaining functional episomal vector genomes reaching up to their packaging limit. These studies represent milestones for gene therapy and provided the first evidence 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-administration; (iii) larger packaging capacity may make LV better suited for transferring FVIII expression cassettes. Absence of prior clinical testing of systemic administration, manufacturing hurdles and concerns about insertional mutagenesis have until now hindered pre-clinical development of in vivo LV gene therapy directed to the liver. It has been shown that the natural source of most FVIII production is endothelial cells. 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, however, the stability and turnover of these cells, both in post-natal liver growth and homeostasis in adulthood remain not fully understood. Indeed, currently, the most clinically advanced AAV-based gene therapy strategies and the LV-based strategy described in this work exploit hepatocytes to produce transgenic FVIII.

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. 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. 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 activity: 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). 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 setting. We then cloned these transgenes (FVIII, coFVIII, coFVIII.XTEN) into a LV construct carrying a hematocyte-specific enhancer promoter (Enhanced Transthyretin, ET) and target sequences for the hematopoietic-specific microRNA 142 (142 T). This LV design was previously shown to provide highly specific and robust transgene expression in hepatocytes. 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 x 10^10 transducing units (TU)/kg of LV, average VCN in Kupffer cells was between 0.2 and 0.54 (Fig. 1e). Average VCN in Kupffer cells was between 0.2 and 0.54 (Fig. 1e). Average VCN in Kupffer cells (KC) and plasmacytoid dendritic cells (PDLC) 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 reported and show afterward in this work. LV output normalized on VCN in hepatocytes confirmed higher expression by coFVIII (32-fold compared to FVIII) with an additional nearly 2-fold increase by

ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-30102-3

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-30102-3

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NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-30102-3

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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 and followed them for 1.5 years. Note that we reduced the dose of LV.coFVIII to 1 × 10^10 TU/kg, to obtain physiological-range FVIII activity. We showed FVIII blood concentration and activity that remained stable throughout the near lifetime follow-up at around 5–10% of normal on average (Fig. 2a, b). All these mice had low amounts of anti-FVIII Abs (<1 µg/mL, Fig. 2g). We also performed an LV-dose–response of LV.coFVIII.XTEN in neonatal hemophilia 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. 2h, i). The coF-VIII.XTEN steady-state amounts were approximately between 2- and 7-fold higher than coFVIII at similar LV doses in these experiments (Fig. 2j). A smaller difference in FVIII amounts between the 2 transgenes was observed at the highest LV doses, in line with the previous experiments (see Fig. 1a, b and Supplementary Fig. 2a). 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. 2k). 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. 2l). To confirm increased half-life by the Xten-containing FVIII, we administered recombinant B-domain deleted FVIII protein with or without Xten i.v. to adult hemophilia A mice. We observed a slower decay of the XTENylated FVIII which remained 10-fold higher than FVIII at 3.5 × 10^10 TU/kg LV-dose (Fig. 2e, f). Note the almost 20-fold increase in FVIII transgene output despite the 2-fold increase in LV dose, in line with the data shown above. All treated mice but 1 had barely detectable anti-FVIII Abs (<1 µg/mL, Fig. 2g).
2 days after administration (Supplementary Fig. 2d). Taken together, these data show stable long-term FVIII transgene expression and activity by LV gene therapy from mice treated as newborns and improved steady-state FVIII output by the XTEN-carrying transgene, particularly at lower LV doses. 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 $8 \times 10^{10}$ 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). Higher variability in FVIII output in adult compared to newborn mice may be due to lower permissiveness to transduction of hepatocytes in the former. The average VCN in purified hepatocytes at the end of the experiment was 0.37 (Fig. 3c). Notably, the average VCN in KC was 15, in line with our
previous data from mice treated as adults. The lower VCN in KC observed at 6 months post-LV in mice treated with low doses of LV.coFVIII. We also evaluated LV gene therapy in an immune-competent hemophilia A mouse model, both F8 KO and transgenic for a F8 transgene (Supplemental Table 1), by a good manufacturing practice like process with a 2-step chromatography purification. 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 reported (17). We then treated NHP with either LV at 2 different doses each: coFVIII at 3 or 6 × 10^9 TU/kg, coFVIII.XTEN at 3 × 10^9 TU/kg. Doses were

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 reported (17). 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 3 × 10^9 TU/kg. Doses were...
selected based on previous experience with FIX-expressing LV\textsuperscript{17}.

Note that the LV-dose–response in NHP is more favorable than in mice, on a per weight basis, as previously described\textsuperscript{17}.

All LV-treated NHP received an immune suppressive regimen from 1 to 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 aminotransferase (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); however, it may also be related to muscle damage during animal handling for sample collection, since serum creatin phosphokinases were also elevated (Supplementary Fig. 4).
Tables 2–11). 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 3×10⁹ 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 × 10⁹ 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 in 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 a few fluctuations (see 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 the 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, pro-inflammatory cytokines, and chemokines, likely released from innate immune cells, significantly increased in the blood-stream 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), IFNγ 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 increasing CD47 signaling on professional phagocytes from high amounts of LV.coFVIII.XTEN) or range (Gray area represent the window of immune-suppression regimen in LV.coFVIII or LV.coFVIII.XTEN-treated NHP (see “methods” section for details).

k) Histogram reporting the frequency of FVIII-specific IFNγ producing T cells/10⁶ T cells in NHP treated as indicated. The 18 pools of FVIII peptides cover the entire transgenes encoded by the administered LV (see Supplementary Fig. 7 for ELISPOT wells). i Heatmap reporting the stimulation index (i.e. proliferation over unstimulated cells) of splenocytes kept in culture in presence of the indicated amounts of FVIII, as indicated. Source data are provided as a Source Data file.

**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. 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 molecule. A recombinant XTEN-containing FVIII product has shown 3-4-fold half-life extension compared to conventional FVIII in humans, without evidence of adverse events or increased incidence of development of neutralizing anti-FVIII Abs. 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 increased half-life of the protein in the circulation, as shown here, especially at physiological blood concentrations, and potentially to improved FVIII secretion by hepatocytes, 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 the absence of selective disadvantage of transduced hepatocytes, which may be due to toxicity from FVIII overexpression. On the same line, the absence of increase in transgene over time indicates a lack of expansion of transduced hepatocytes, which may potentially be triggered by LV insertional mutagenesis. These data also confirm that LV gene therapy allows maintenance of the therapeutic transgene following liver growth and homeostasis in mice. In humans, restoration of stable, therapeutic amounts of FVIII activity since early childhood may be preferred to avoid the accumulation of joint damage, by preventing bleeding as early in life as possible. 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 favoring in mice treated as newborns compared to mice treated in our previous study, which received a similar cytokine response observed in NHP treated in this study. The corticosteroid regimen did not apparently affect the innate immune response that 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-FIX administration without corticosteroid treatment. 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 adequate to mostly prevent joint hemorrhages and may thus be set as the minimal target for correction in gene therapy. 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. The 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 naive to FVIII. With this perspective, the inclusion of the XTEN polypeptide in the FVIII transgene might alleviate 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. The overall low frequency of anti-FVIII T-cell responses detected in this study suggests that more sensitive immune assays are needed to confirm these data and further assess anti-transgene immune responses in future studies carried out in similar experimental settings.

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 has been published so far in >300 patients treated by LV-transduced hematopoietic stem cells across different doses and transgenes with multi-year follow-up and mostly at high VCN, although clonal expansions leading in 2 patients to myelodysplastic syndrome were recently reported in a single trial using LV with strong viral enhancer/promoter (https://doi.org/10.1089/hum.2021.29180.abstracts). Moreover, we have previously shown undetectable genotoxicity in the liver by our LV design in sensitive mouse models, dogs, and NHP. Whereas efforts are underway to continue modeling and possibly reducing LV genotoxic potential, the safety track 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.** The sample size in experiments with mice was chosen according to the power analysis with experimental models and assays. The 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 MHC-free LV batches, produced by MolMed S.p.A. (now AGC Biologics), on 24 L scale of supernatant and formulated in PBS 0.2% human serum albumin. The vector batches were produced by using a large-scale validated process and following pre-GMP guidelines. Briefly, LV is produced by transient 4 plasmid transfection of CD47hi MHC-negative 293T cells in 10-tray cell factories by calcium phosphate precipitation. Twenty-four hours after removal of the transfection medium, the cell supernatant is harvested and stored at 4 °C. The culture medium is replaced and after a further 24 h a second harvest is performed. The medium collected from the two pools is pooled and filtered through 5/0.45 filters to discard cell debris. The downstream purification process includes a benzonase treatment overnight at 4 °C, followed by a DigestAmino Anion Exchange (DEAE) chromatography step, concentration, and gel filtration in PBS or PBS 5% dimethyl sulfoxide (DMSO). The resulting LV preparation undergoes 0.2 or 0.45 μm filtration and aseptic filling. The purified vector preparation is stored at −80 °C. 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 transfection into 293T cells (from ATCC). 293T cells were transfected with a solution containing a mix of the selected LV genome transfer plasmid, the packaging plasmids pMD1/gP-RRE and pCMV.REV, pMD2.G and pAdvantage (Promega). Calcium phosphate-mediated transfection: 9 x 10^9 293T cells are seeded 24 h before transfection in 15-cm dishes. Two hours before transfection culture medium is replaced with a fresh medium. For each dish, a solution containing a mix of the selected transfer plasmid, the packaging plasmids pMD1/gP-RRE and pCMV.REV, pMD2.G and the pAdvantage plasmid is prepared using 35, 12.5, 6.25, 9, and 15 μg of plasmid DNA, respectively. A 0.1X TE solution.
for 20–30 min, and then diluted 1:2 in blocking buffer (PBS 0.05% Tween-20, 0.1% heat inactivated horse serum, Gibco). Samples were diluted as needed starting from 1:20 in blocking buffer, added to wells (100 µL/well) and incubated 2 h at 37 °C. Anti-FVIII Abs were detected by adding detection Ab (goat anti-mouse IgG-HRP, Sigma #A2054, 1 U/mL in blocking buffer) 1 h at 37 °C on orbital shaker, followed by washing and absorbance of each sample was determined spectrophotometrically at 450 nm, using a Multiskan GO microplate reader (Thermo Fisher Scientific) and normalized to the standard curve (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. The cutoff value is 1 BU/mL, calculated on mean ± 2 standard deviations on 10 pre-gene therapy samples.

**NHP study.** Ten adults (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 and felt enrichment devices at the Boisbonnand Center (Nantes, France), under protocol APAFS#4302-2015122314563838 that was approved by the Institutional Animal Care and Use Committee of the Pays de Loire. The study complies to all relevant ethical regulations related to the use of research animals.

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 #GMA8032, 0.2 µg/well in 0.1 M carbonate buffer, pH 9.6) overnight at 4 °C. Plates were blocked 1 h at room temperature with blocking buffer (PBS 0.05% Tween-20, 1% heat inactivated horse serum, Gibco). Plasma samples were diluted as needed starting from 1:10 in blocking buffer, added to 100 µL/well and incubated 2 h at 37 °C. Anti-FVIII Abs were detected by adding detection Ab (goat anti-mouse IgG-HRP, Sigma, 1 L.U./mL in blocking buffer) 1 h at 37 °C on orbital shaker, followed by washing and absorbance of each sample was determined spectrophotometrically at 450 nm, using a Multiskan GO microplate reader (Thermo Fisher Scientific) and normalized to the standard curve (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. The cutoff value is 1 BU/mL, calculated on mean ± 2 standard deviations on 10 pre-gene therapy samples.

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coated overnight 4 °C with capture Ab for interferon-γ (IFNγ, MTI-126, 10 μg/mL, 30 μL/well, Mabtech) or IL-5 (TRFK5 10 μg/mL, 30 μL/well, Mabtech) and blocked with PBS + 5% FCS at 37 °C. Plates were incubated for 4 h at 37 °C for temperature before seeding cells. Total NHP splenocytes (3 × 10^5 cells/well), LN cells or PBMC (2.5 × 10^5 cells/well) were plated in X-vivo-15 (Lonza) at least in duplicates without antigen stimulus (DSMO alone) or stimulated with 18 different peptide pools covering the entire aminoacidic sequence of FVIII-XTEN (each peptide at 1 μM final concentration, peptide library 15aa long, 5aa offset, Sigma-Aldrich) for 24 h at 37 °C. Plates were incubated and for 2 h at room temperature. Avidin-POD solution (Roche, 15,000, 50 μL/well) was then added and incubated for 1 h at room temperature and spot were developed by AEC solution (Sigma-Aldrich) for 15 min at room temperature in the dark. A plate image was acquired and spots were counted by Immunospot S6-Ultra (Cellular Technology Limited). The mean number of spots ± 2 SD from the unstimulated condition was subtracted to each stimulated condition and reported as IFNγ or IL-5 producing cells in 1 × 10^6 splenic T cells or PBMC or LN cells. The number of splenic T cells/well has been calculated based on cytofluorimetric analysis of cell before seeding (anti-human CD4 clone MT477, and anti-human CD8 clone SK1 cross-reacting with Macaca Leonina).

Splenocyte proliferation. Total NHP splenocytes were plated in flat-bottom 96-well plate (3 × 10^5 cells/well) in X-vivo-15 (Lonza). Cells were left unstimulated or stimulated with increasing dose of FVIII-BDD (protein (RefActo, Pfizer) in triplicates (NHP, cell clone CD1-1) and served as positive control as polyclonal inducer of T-cell proliferation. After 5 days of culture, 1 μCi/well of 3H-Thymidine was added and incubated for additional 16 h. Cell proliferation was indirectly quantified by measuring 3H-Thymidine incorporation. The stimulation index (SI) was obtained as the ratio between the mean counts per minutes (cpm) in each stimulated condition and the mean counts of the unstimulated cell.

Transgene expression. Codon-optimization of human BDD-FVIII transgene was performed by taking into consideration the codon-adaptation index, G/C content, Matrix Attachment Region-like sequences, destabilizing sequences, potential promoter binding sites, other acting negative regulatory elements, as described in the patent WO 2019/152692 under the name of SEQ ID 71 and SEQ ID 72, respectively. XTEN 144 was inserted in the place of the B domain. The aminoacidic sequence of the XTEN polypeptide is: GAPTSESATPESGPSEPATSGSETPGTSESATPEGSPTGSEPSTGSATPTGSESATPTGSESATPEGSPTGSEPSTGPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGSEPSTGA...
Hylocine, penicillin and streptomycin 100 IU/mL (Lonza) and digested with 5 μg/mL of digestion solution (EBSS, Hylocine, collagenase A 2.5 mg/mL, Sigma, dispase II 2.5 mg/mL). Thermolysin for 10 min at 37 °C. Then tumbling was added for an additional 2 h with 5 μg/mL of digestion solution at 37 °C on a shaker (135–150 rpm). At the end of the digestion, cells were washed in cold washing medium (4 min at 120 g) and then the nCP-containing supernatant was processed as described in the murine liver. After Percoll (Sigma) gradient separation, cells were washed twice and incubated with the following monoclonal antibodies: APC-conjugated anti-CD45 (D058-1283, BD phar- mingen) and APC-H7-conjugated anti-HLA-DR (G46-6, BD pharmingen). nCP subpopulations (LSEC, KC) were sorted by FACS, BD FACSAria II Cell Sorter (BD Biosciences). LSEC were sorted as CD34-negative/CD31-positive, while KCs were sorted as CD45-positive/HLA-DR-positive.

Cell cultures and in vitro experiments. 293T and HuH7 cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM, Sigma) supplemented with 10% fetal bovine serum (FBS, Hylocine), 4 mM glutamine (Lonza), penicillin, and streptomycin. 100 IU/mL (Lonza). Hepa1.6 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Hylocine), 4 mM glutamine (Lonza), penicillin and strepto- mycin 100 IU/mL (Lonza). All cells were maintained in a 5% CO2 humid environment and 20% oxygen atmosphere at 37 °C. All cell lines were routinely tested for mycoplasma contamination. HuH7 or Hepa1.6 cell lines were transduced for 24 h then cultured for 14 days before qDNA extraction and VCN determination (see "VCN determina- tion"). 293T cells were transduced and after 7 days the supernatant was collected and hFVIII concentration was assayed by ELISA (see "hFVIII assays"). Cell lines were originally obtained from ATCC.

Flow cytometry. Flow cytometry analyses were performed using a FACSCanTo analyzer (BD Biosciences), equipped with DIVA Software. Blood was collected from mice from retro-orbital plexus and 20 μL were directly stained with 20 μL of antibody mix (see below for details) for 20 min at 4 °C in the dark. Cells were then fixed with 500 μL of Lyse/Fix Buffer 1X (BD Biosciences, Phos STOP) and re-suspended in 1X PBS. 2 × 10^6 cells per sample were stained with the following monoclonal antibodies: APC-conjugated anti-CD45 (BDS8-812, BD phar- mingen), FITC-conjugated anti-CD31 (WM59, BD phar- mingen), and PE-Cy7-conjugated anti-CD11b (M1/70, BD phar- mingen). Taf7 was used as reference gene (see below for details). The PCR reaction was performed with each primer (900 nM) and the probe (250 nM) following the manufacturer’s instructions (Biorad), read with xGen® LNA Probe Detection System or Human cytokine A Premixed Magnetic Luminex Performance Assay, 26 analytes (NHP XL Cytokine Luminex Performance Premixed Panel, R&D Systems). Chemokines were determined in NHP serum by a magnetic-based multiplex ELISA (Milliplex, Merck). The Whitney test was applied for multiple comparisons against the reference control group along with Bonferroni’s correction (see "VCN determi- nation"). This modeling approach allows to properly capture the dependency structure on mouse ID, hence random intercept models were estimated. Cubic and square root transformations of the outcome were also considered to satisfy underlying model assumptions. After model estimation, post-hoc analyses have been imple- mented to compare experimental treatment groups to the reference control group at a fixed time point (Table S12). LMEs were estimated in R (version 4.0.3) by means of the lme4 package (Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team 2020). nlme. Linear and Nonlinear Mixed Effects Models. R package version 3.1-148, https:// CRAN.R-project.org/package=nlme; Russell V. Lenth (2021). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.5.4, https://CRAN.R- project.org/package=emmeans.

Data availability

The LV and reagents described in this manuscript are available to interested scientists upon signing a MTA with standard provisions. There are some restrictions on the use of the provided materials in research involving LV-based gene therapy of hemophilia, except for research aimed at reproducing the findings reported in this manuscript, according to the collaboration agreement between Fondazione Telethon, San Raffaele Scientific Institute and Bioverativ/Sanofi. All data associated with this study are available in the main text or the in the Supplementary Information/Source data file. Source data are provided with this paper.

Received: 30 August 2021; Accepted: 4 April 2022; Published online: 04 May 2022

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Acknowledgements
This work was supported by Telethon (SR-Tiget Core Grant 2011-2016) and Bioverativ/Sanofi sponsored research agreement. We thank CUSSB for statistical consulting, MolMed S.P.A. for large-scale production and purification of the LV batches used in the NHP study, and all members of the Cantore and Naldini laboratory for helpful discussions. C.C. conducted this study as partial fulfillment of his International Ph.D. Course in Molecular Medicine at San Raffaele University, Milan.

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. A.F. provided crucial reagents and intellectual input. 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.

Competing interests
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 (WO2019/152692; WO2016009326). FT and SRSJ, through SR-Tiget, have established a research collaboration on liver-directed lentiviral gene therapy of hemophilia with Bioverativ/Sanofi. The remaining authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-30102-3.

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Peer review information Nature Communications thanks Glenn Pierce, Steven W. Pipe and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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