

Transient costimulatory blockade overcomes immune barriers to durable and redosable liver-directed gene therapy

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Transient costimulatory blockade overcomes immune barriers to durable and redosable liver-directed gene therapy

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ABSTRACT

In vivo liver-directed gene therapy (GT) is a therapeutic option for the permanent correction of monogenic diseases. Lentiviral vectors (LV), which integrate into the target cells' genome, represent a unique platform for intervention in childhood, with negligible limitations from pre-existing immunity to the parental virus. However, adaptive immunity to the encoded transgene and vector components may hamper the efficacy of the therapy and its potential re-administration. In a mouse model of hemophilia A, an inherited disease due to lack of coagulation factor VIII (FVIII) activity, we tested variants of the FVIII transgene with increased expression (codon-optimized) and half-life (XTENylated) to reconstitute *in vivo* therapeutic FVIII amounts with a minimally effective LV dose, revealing a direct correlation between FVIII productivity *per* integrated vector copy and immunogenicity. Maximizing the proportion of LV-engineered hepatocytes with low output *per* LV copy established immune tolerance to FVIII, thereby stably maintaining therapeutically relevant levels. Conversely, using LV with high FVIII output required co-administration of an immunomodulatory regimen to transiently block T cell costimulatory pathways and achieve stable reconstitution of FVIII and immune tolerance. Moreover, this regimen prevented the development of vector-neutralizing immune responses, enabling GT redosing and adjustment of the transgene output based on the outcome of the first dose. This strategy could also be extended to adeno-associated-vector-mediated liver gene transfer. Overall, this study reveals key determinants of GT-evoked immunity and provides new strategies to overcome immune-mediated limitations on efficacy and redosing of GT, which proved effective across two different monogenic diseases and viral vector platforms.

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INTRODUCTION

In vivo delivery of a transgene to hepatocytes using replication-defective viral vectors offers a valuable opportunity to treat monogenic inherited hematologic, lysosomal, and metabolic diseases ¹. Currently, the most advanced delivery system for *in vivo* liver-directed gene therapy (GT) is based on adeno-associated virus (AAV)-derived vectors. A single intravenous (i.v.) administration of AAV vectors expressing coagulation factor VIII (FVIII) or factor IX (FIX) from hepatocytes has shown multi-year efficacy and safety in adults with hemophilia A or B, two monogenic coagulation disorders caused by deficiencies in clotting FVIII or FIX, respectively. This milestone served as the foundation for subsequent commercial authorization ². However, while FIX expression did not decline over up to 13 years of follow-up, FVIII expression experienced a gradual yearly decline in long-term follow-up studies involving adult patients ³. For those who have lost FVIII expression, administering a second dose of an AAV vector would be ineffective due to neutralizing anti-vector immune responses triggered by the initial treatment.

We and others have proposed integration-competent lentiviral vectors (LV) as complementary delivery vehicles for liver-directed GT ⁴⁻⁸. Compared to AAV-vector-based GT, LV-based GT has the potential to provide lifelong correction of genetic defects in pediatric patients, as the therapeutic transgene would be maintained in the genome of target cells upon hepatocyte proliferation ⁹. A single i.v. administration of vesicular stomatitis virus G protein (VSV.G)-pseudotyped LV enabled stable transfer of therapeutic transgenes into the livers of mice, dogs, and non-human primates (NHP), and provided dose-dependent correction of disease manifestations ^{6,10-12}. Despite the low prevalence of pre-existing immunity that cross-reacts with VSV.G, systemic administration of LV induces the production of neutralizing antibodies (NAbs) through a T cell-dependent response, similar to that induced by AAV vectors. VSV.G envelope-specific LV-neutralizing responses do not affect the *in vivo* persistence of genetically modified hepatocytes but completely hinder the effectiveness of LV re-administration, which may be desirable if the first dose does not achieve full therapeutic efficacy.

In addition to anti-vector immunity, T cell-mediated adaptive immune responses against the transgene product, whether pre-existing from prior exposure to enzyme or protein replacement therapy or induced by GT, may lead to the clearance of transgene-expressing hepatocytes, thereby reducing GT effectiveness ¹³. We have reported several advances aimed at reducing the immunogenicity of LV-based GT. First, combining transcriptional and post-transcriptional control, we significantly improved the stringency of hepatocyte-specific transgene expression, promoting immunological tolerance rather than immunity to the transgene ¹⁴. This outcome was achieved through an expression cassette comprising the hepatocyte-specific enhanced transthyretin (ET) promoter and microRNA 142 target sequences (142T), which reduce off-target transgene expression in antigen-presenting cells (APC) ^{15,16}. More recently, we developed immune-shielded, major histocompatibility complex (MHC)-free, and CD47-high (CD47hi) LV particles to minimize carryover of allogeneic antigens from the producer cell line ¹⁷ and to significantly reduce phagocytic uptake of LV particles, thus lowering innate immune responses ⁵. Nevertheless, despite these improvements, *in vivo* LV GT delivering a transgene encoding a highly immunogenic secreted neoantigen (Ag) may still trigger a wave of unwanted adaptive immune responses, conceivably through Ag uptake by professional APC. Examples of highly immunogenic proteins include FVIII and lysosomal enzymes, such as alpha-L-iduronidase (IDUA), the enzyme defective in mucopolysaccharidosis type I (MPS-I). Transgene-specific immune responses can be exacerbated by genetic factors such as the host's MHC haplotype and by the failure to produce cross-reacting immunological material (CRIM) from the defective locus, which, when present, may confer a degree of central tolerance. Indeed, seroconversion to NAb positive occurs in approximately 30% of people with hemophilia A treated with recombinant FVIII

products, and this proportion is higher in the CRIM-negative subgroup¹⁸. Notably, up to 90% of MPS-I patients receiving IDUA enzyme replacement therapy develop humoral anti-IDUA immune responses^{19,20}.

Similar to humans, hemophilia A mice (HemoA) that express a truncated form of murine FVIII²¹ (mFVIII) with less than 1% of normal activity are prone to mounting FVIII-specific neutralizing responses. Conversely, HemoA transgenic mice expressing a mutated, inactive and non-secreted human FVIII (hFVIII), hFVIII.R593C, are tolerant to both hFVIII protein replacement therapy and LV-mediated gene transfer of functional hFVIII, which allows for stable transgene expression^{6,22}. In the context of *in vivo* liver-directed LV GT for hemophilia A, in the past we focused on maximizing the expression and half-life of FVIII transgenes and we tested them in newborn mice (which are immunologically immature), in immune-deficient or immune-tolerant adult HemoA mice, or in immunosuppressed non-human primates.

Here, we selected several worst-case-scenario experimental settings, such as mice with active, fully functional immune systems, in the absence of transgene-specific central tolerance, and tested LV expressing highly immunogenic transgenes like FVIII and IDUA. Our first goal was to identify factors influencing the immunological outcome of *in vivo* LV GT, and we found that expressing high levels of FVIII from few hepatocytes is more immunogenic than expressing lower levels of FVIII *per* cell from a larger proportion of hepatocytes. We then tested possible ways to counteract anti-transgene immune responses and promote transgene-specific immune tolerance, identifying a transient immunomodulatory regimen that enhances transduction efficiency and inhibits T-cell-mediated immune responses. This approach enabled stable transgene expression and activity. Interestingly, in addition to inducing durable immune tolerance to the transgene product, this approach also prevented anti-vector immunity, enabling effective vector re-administration, a long-sought goal in the field of *in vivo* GT.

RESULTS

Improving hFVIII translation and extending its half-life favors anti-hFVIII immune responses in HemoA mice.

To assess the immunogenicity of LV-mediated *in vivo* delivery of FVIII transgenes, we employed variants of the human B domain-deleted (BDD) FVIII coding sequence, engineered from the human wild-type (*wt*) sequence to enhance protein translation and stability. We combined codon optimization (*co*) to increase the translation rate with XTENylation to extend the half-life of the transgenic product, generating 4 transgenes (hFVIII, *co*.hFVIII, hFVIII.XTEN, *co*.hFVIII.XTEN)⁶, which were then cloned into LV constructs bearing the previously described and validated hepatocyte-specific expression cassette (ET.142T). First, LVs encoding these hFVIII variants were administered *i.v.* to 2-week-old HemoA immunodeficient (HemoA-Rag) mice⁶ to assess hFVIII output without the confounding effects of immune responses. We measured the ratio of plasma hFVIII activity (at the latest in-life follow-up) and liver VCN, and observed the expected exponential increase in hFVIII output with the introduction of XTENylation and codon optimization (**Fig. 1A and S1A-B**). Following the initial characterization in immune-deficient HemoA-Rag mice, the same LVs were then administered *i.v.* to 2-week-old immunocompetent HemoA mice as an intermediate immunological setting to evaluate FVIII reconstitution and immunogenicity, before testing them in fully immunocompetent adult mice. Administration of LV encoding hFVIII resulted, on average, in normalization of hFVIII plasma concentrations (190 ng/mL, 18 weeks post LV) (**Fig. 1B**). These hFVIII amounts were produced by multiple vector copies *per* diploid genome (5.3 vector copy number, VCN) in the liver of LV-treated mice (**Fig. 1C**). Anti-hFVIII IgG and cellular responses remained comparable to those in untreated HemoA mice during an 18-week follow-up and did not affect the persistence of hFVIII activity *in vivo* (**Fig. S1C-F**). Administration of LV encoding *co*.hFVIII led to circulating hFVIII amounts approximately 2-fold higher than those observed with the hFVIII transgene (276 ng/mL, 12 weeks post-LV), despite a lower liver VCN (**Fig. 1D, E**). A significant anti-hFVIII IgG titer

was detected in 4/10 mice, accompanied by a lack of hFVIII expression and by hFVIII-specific cellular immune responses that remained detectable 12 weeks post LV in LV-treated HemoA mice (**Fig. S1G-J**). Administration of LV encoding XTENylated hFVIII resulted in stable hFVIII output in 12/13 treated HemoA mice (average 61 ng/mL) (**Fig. 1F, G**). Only 1/13 mice displayed high immunoglobulin G (IgG) amounts in the circulation (15.5 μ g/mL), while the titer of anti-FVIII IgG remained low (<5 μ g/mL) in the other 12 mice during a 12-week follow-up post LV (**Fig. S1K, L**). Human FVIII-specific cytotoxic T lymphocyte (CTL) reactivity was detectable in the spleen of 2/12 LV-treated HemoA mice (**Fig. S1M, N**). Finally, administration of LV encoding co.hFVIII.XTEN led to detectable hFVIII expression in 3/4 LV-treated HemoA mice, as reflected by the measured VCN at the end of the 12-week follow-up period (0.92 VCN) (**Fig. 1H, I**). A high proportion of mice developed an anti-hFVIII immune response: 3/4 mice developed a significant anti-hFVIII IgG titer accompanied by an hFVIII-driven T cell proliferative response (**Fig. 1I, Fig. S1P-R**). We then collectively analyzed the kinetics of appearance and amounts of anti-hFVIII IgG across the 4 hFVIII-tested transgenes in the experiments described above in LV-treated, immunocompetent, 2-week-old HemoA mice. We observed early seroconversion in most mice treated with the LV-co.hFVIII.XTEN, whereas the LV-hFVIII-treated mice remained seronegative throughout the follow-up (18 weeks post-LV) (**Fig. 1K**). Mice treated with either LV-co.hFVIII or LV-hFVIII.XTEN displayed intermediate kinetics of anti-hFVIII IgG development (see Fig. 1K). Mice treated with LV-co.hFVIII.XTEN developed the highest levels of anti-FVIII IgG, followed by those treated with LV-co.hFVIII, whereas anti-hFVIII IgG remained low or undetectable in most mice treated with LV-hFVIII.XTEN or LV-hFVIII (**Fig. 1J**). Finally, we correlated the different experimental outcomes on a transgene-by-transgene basis, rescaling each measured value to the highest within its category (**Fig. 1L**). Interestingly, the LV expressing the co.hFVIII.XTEN transgene yielded the highest hFVIII output at the lowest VCN, making it the most optimized for expression and stability (see Fig. 1A); however, in immunocompetent hosts, it induced the strongest anti-hFVIII immune responses (**Fig. 1L**). By contrast, LV expressing hFVIII was not immunogenic but required the highest VCN to achieve therapeutically relevant hFVIII levels. Overall, these data suggest that codon optimization and low liver transduction favored anti-transgene immune responses.

Costimulatory blockade regimen enables durable reconstitution of transgene activity in HemoA and MPS-I mice treated with LV GT by inhibiting T cell-mediated transgene-specific immunity.

We thus set out to identify a regimen capable of counteracting anti-hFVIII immune responses. To this end, we selected the LV encoding co.hFVIII based on its high *in vivo* hFVIII expression, while acknowledging its limited therapeutic efficacy due to immunogenicity toward hFVIII elicited in immunocompetent HemoA mice following GT. To overcome T cell-dependent adaptive immunity to hFVIII, we applied several strategies in 2-week-old HemoA mice. First, an anti-interferon alpha receptor 1 (IFN α R1) monoclonal antibody (mAb) was systemically administered 3 hours before LV to block IFN α signaling upon its release by innate immune cells²³ that sense the vector genome, supporting APC activation and the induction of adaptive immunity to hFVIII. However, IFN α R1 blockade could not prevent the clearance of hFVIII-expressing hepatocytes by hFVIII-specific T cells and seroconversion in this setting (**Fig. S2**). As an alternative approach, we combined with IFN α R1 blockade a five-dose anti-CD3F(ab')₂ regimen that depletes and inactivates T cells²⁴⁻²⁶ (murine analog of Teplizumab²⁷) after LV-co.hFVIII GT. We detected effective modulation of T cell responses, but this was insufficient to inhibit the formation of anti-hFVIII IgG or to prevent the immune-mediated clearance of hFVIII-expressing hepatocytes in the liver (**Fig. S3**). We then tested a short B-cell-depleting regimen comprising 2 doses of an anti-CD20 mAb (murine analog of Rituximab²⁸; **Fig. S4**), in combination with IFN α R1 blockade, which resulted in elevated hFVIII plasmatic levels and activity (**Fig. S4A-D**), higher VCN in the liver, and milder hFVIII-specific humoral and cellular responses compared to control LV-only treated mice (**Fig. S4E-I**). To assess whether anti-

hFVIII immune responses could be controlled in fully immunocompetent mice, we next applied the short B-cell-depleting regimen in adult HemoA mice treated with LV-co.hFVIII. However, only limited and transient hFVIII expression was achieved. Transduced hepatocytes were cleared within less than 4 weeks by hFVIII-specific CTLs (**Fig. S5**). These results suggested that, as soon as hepatocytes release transgene-derived Ags, priming of Ag-reactive T cell clones proceeds properly, supporting the initiation of adaptive immunity, independently of transient lymphocyte depletion or inactivation. Therefore, to maintain the beneficial effects of IFN α R1 blockade, we supplemented it with a costimulatory blockade (CoB) regimen (anti-CD154 and CTLA4-Ig) to interfere with the full set of activating signals required for T cell priming. First, a cohort of 2-week-old HemoA mice pretreated with anti-IFN α R1 mAb was injected with 2×10^{10} TU/kg of LV-co.hFVIII, and half of them received 5 doses of CoB 2-3 days apart. All CoB-treated mice maintained hFVIII expression and activity, with a consistent VCN in the liver (0.8 ± 0.1), whereas transgene expression was lost in 4/5 control mice not receiving CoB, mirrored by the loss of VCN (**Fig. S6**). Based on these results, CoB regimen was administered to adult 8-week-old HemoA mice pretreated with anti-IFN α R1 mAb and administered 2×10^{10} TU/kg of LV-co.hFVIII. CoB administration allowed stable hFVIII activity and expression (**Fig. 2A, B**), sustained by a consistent liver VCN (0.32 ± 0.06) during the first 12 weeks of follow-up (**Fig. 2C**). hFVIII was never detected in the plasma of control mice treated with anti-IFN α R1 mAb alone, whose hFVIII-expressing hepatocytes (VCN 0.09 ± 0.2) were cleared by severe humoral and T cell-mediated anti-hFVIII immune responses, which were instead abrogated in CoB-treated adult HemoA mice. Furthermore, at 12 weeks post-LV, we tested whether CoB-treated HemoA mice had developed immune tolerance to hFVIII by i.v. injection of hFVIII protein (4×10 units, U, recombinant hFVIII-BDD, Re-facto, 7 days apart), which induces a hFVIII-specific response in naïve age-matched HemoA mice (**Figure 2D**). hFVIII antigenic re-challenge induced seroconversion in only 1/4 of CoB-treated mice, without inducing significant T cell responses (**Fig. 2E, F**) or loss of liver VCN (0.4 ± 0.16 , see Fig. 2C). Furthermore, the tolerogenic activity of the CoB regimen was tested in adult HemoA mice pretreated with anti-IFN α R1 mAb and receiving the highly immunogenic LV-co.hFVIII.XTEN. Results confirmed the efficacy of CoB in overcoming immune-mediated limitations to achieve stable reconstitution of hFVIII activity *in vivo* (**Fig. S7A-G**) and to maintain hFVIII-expressing hepatocytes. At this point, we investigated the impact of CoB regimen on the state of activation of circulating T cells phenotypically by flow cytometry at different time points from treated mice. Unsupervised analysis of cytofluorimetric data revealed dynamic changes of T cell subpopulations over time, showing that CoB administration inhibited the acquisition of an effector phenotype in both CD4 and CD8 T cells compared to controls treated without CoB, which showed significant T cell activation at early time points after LV. Interestingly, the FoxP3 regulatory T cell (Treg) population was severely reduced by the CoB regimen (**Fig. 3A-D, Fig. S8**), possibly favoring renewal of this compartment crucial for tolerance. All T cell subsets returned to steady-state conditions between 8 and 12 weeks post LV.

Having established that the CoB regimen effectively prevented immune responses against hFVIII, we then tested the same protocol with another highly immunogenic transgene (IDUA), mutated in MPS-I. In mild forms of MPS-I, hepatocyte-mediated overexpression of the deficient enzyme IDUA can mitigate disease manifestations; however, anti-hIDUA immune responses have thus far limited the efficacy of this approach in mouse models. Adult *Idua*^{-/-} mice were pre-treated with IFN α R1 mAb, i.v. injected with LV encoding for hIDUA and then administered with CoB regimen. This protocol enabled reconstitution of hIDUA activity in the liver of 5 out of 9 *Idua*^{-/-} mice, with supraphysiological activity observed in 3 animals, thereby maintaining the population of transduced hepatocytes (**Fig. 4A, B**). This was associated with almost complete inhibition of anti-hIDUA IgG development in 8/9 GT-treated *Idua*^{-/-} mice and of anti-hIDUA-specific T cell responses (**Fig. 4C-F**).

Overall, these data show that a transient systemic block of the costimulatory pathways CD40-CD40L by the anti-CD154 mAb and CD28-CD80/86 by CTLA4-Ig during the time window of transgene Ag

presentation impedes complete priming of anti-transgene-reactive T cell clones, thereby abrogating T-helper functions that support B cell responses, the release of anti-transgene IgG, as well as CTL-mediated clearance of LV-modified hepatocytes.

In vivo fate of T cell receptor-engaged T cells following GT in the presence of CoB.

Following systemic LV administration, innate responses, likely evoked by viral genome sensing^{23,29}, activate professional APC, resulting in the upregulation of costimulatory molecules (signal 2) and the release of proinflammatory chemokines and cytokines (signal 3). These events are pivotal for priming T cell clones that recognize LV- or transgene-derived epitopes via their T cell receptor (TCR) in the context of MHC molecules (signal 1). To identify T cells stimulated via TCR by the recognition of epitopes from LV particles and transgene product, we generated HemoA mice with T cells expressing GFP under the control of Nuclear Factor of Activated T-cells (NFAT)³⁰ (HemoA^{nfat.GFP}, **Fig. S9A**) by transplanting autologous engineered lineage-uncommitted (Lin-) cells, enriched for hematopoietic stem and progenitor cells (HSPC^{nfat.GFP}). After hematopoietic reconstitution, circulating T cells of HemoA^{nfat.GFP} mice were analyzed, showing that Ag recognition via TCR induces the expected GFP expression in the expected costimulation-independent manner *in vitro*, whereas it is silent at steady state *in vivo* (**Fig. S9 B-D**). To clarify the impact of CoB on T cells responsive to vector- or hFVIII-derived epitopes, IFN α R1 mAb-pre-treated HemoA^{nfat.GFP} mice were i.v. injected with LV-ORFless (LV carrying an expression cassette lacking an open reading frame, ORF) or LV-co.hFVIII, with or without the CoB regimen. Then, GFP-expressing CD8, Treg, and CD4 T conventional cells (Tconv) were sorted from the spleen and liver (**Fig. S9E, F**) of LV-treated mice. GT-activated T cells, labeled by NFAT-driven GFP expression, were phenotypically characterized, showing that the CoB regimen altered the effector memory CD4 T cell compartment (EM, CD44⁺CD62L⁻), reducing the fraction of cells expressing the activation markers LAG3, PD1, and KLRG, while conserving those that did not upregulate these markers (**Fig. S10, 11**). Similarly, memory precursor effector (MPEC, KLRG⁻CD127⁺) CD8 T cells isolated from the livers of CoB-treated mice were reduced, further indicating impaired CD8 T cell memory formation (**Fig. S12, 13**).

A comparison of the transcriptome from GFP-labeled TCR-activated T cells isolated from splenocytes of LV-treated mice revealed that the CoB regimen downregulated biological processes related to the activation and proliferation of CD4 Tconv cells, specifically affecting effector functions and cytokine production, compared to mice treated with LV-co.hFVIII but not with the CoB regimen (**Fig. 5A**). Similarly, in activated Treg cells from mice receiving CoB, we detected a profound downregulation of processes involved in T cell division, DNA replication, and cell cycle regulation (**Fig. 5B**). Conversely, in activated CD8 T cells, CoB negatively regulated effector functions of the adaptive immune response, particularly cytokine and chemokine signaling and proliferation, compared with control mice treated with LV-co.hFVIII but not with the CoB regimen (**Fig. 5C**). Analysis of T cells isolated from the liver suggests that CoB interfered with the expansion of activated CD4 Tconv cells in tissues, with a significant impact on metabolic processes (**Fig. S14A**), whereas the transcriptome of activated CD8 T cells in the liver was less affected (**Fig. S14B**). Overall, these data shed light on how, in secondary lymphoid organs, CoB severely impairs the induction of adaptive responses to GT-derived Ags by blocking the expansion, selection, and migration of effector T-cell clones, thereby allowing the persistence of hFVIII-expressing hepatocytes.

Low transgene expression, combined with widespread transduction of liver parenchyma, allows persistent hFVIII expression and promotes immune tolerance.

An alternative strategy to control GT-driven immunogenicity may be to use an LV encoding the hFVIII sequence without any optimization. Although it displayed the lowest clotting activity *per* LV copy in the liver of HemoARag mice (see Fig. 1A), it was not immunogenic in 2-week-old HemoA immunocompetent mice (see Fig. 1A, B, K, L). To validate this hypothesis, adult HemoA mice were treated with an LV dose

~2.5-fold higher than that used for HemoA mice treated with LV-co.hFVIII, to achieve detectable and possibly therapeutic amounts of hFVIII *in vivo*. The elevated LV dose induced seroconversion and hFVIII-specific T cell-mediated responses, thereby hampering the establishment of a stable hFVIII level (**Fig. 6A-C**), suggesting that a higher LV dose was insufficient to establish transgene expression in the absence of an anti-hFVIII immune response. We thus tested additional previously described transduction enhancers, such as a 24-hour fasting period and anti-IFN α R1 mAb blockade before LV administration⁸, to significantly increase the percentage of LV-transduced hepatocytes. This strategy resulted in stable hFVIII reconstitution (**Fig. 6A-C**) in adult HemoA mice, without inducing inhibitory Abs or T-cell immune responses to hFVIII (**Fig. 6D-F**) over 16 weeks of follow-up. The induction of hFVIII-specific tolerance was challenged by hFVIII recombinant protein administration (4x10U of Re-facto) starting from 16 weeks post LV. We observed that 2/5 mice maintained stable hFVIII expression for 17 weeks post-protein re-challenge, whereas in 3/5 mice, a temporary hFVIII-specific IgG response transiently masked hFVIII in the plasma. The measured liver VCN (4.87 ± 1.38) and the undetectable hFVIII-specific CTL throughout the follow-up period indicated that hFVIII-releasing hepatocytes were not cleared from the liver parenchyma (see Fig. 6C, F). Collectively, the data indicate that instructing a high proportion of hepatocytes to express low levels of hFVIII *per cell* is well tolerated and disfavors the activation of adaptive immunity against transgene-expressing hepatocytes.

CoB inhibits anti-vector neutralizing immunity, allowing vector reinfusion.

Administration of VSV.G-pseudotyped LV particles to immunocompetent hosts induces an anti-VSV.G neutralizing IgG response (**Fig. 7A**) that does not affect the persistence of LV-modified cells but efficiently neutralizes a subsequently infused LV dose, precluding additive correction of a not fully therapeutic GT. We evaluated anti-VSV.G IgG titers in mice receiving the CoB regimen and found that the anti-VSV.G response was suppressed. Therefore, we administered 9×10^9 TU/kg of LV-GFP to anti-IFN α R1 mAb-pretreated *wt* C57Bl/6 adult mice (n=12). Half of them (n=6) received the CoB regimen. We then monitored anti-VSV.G IgG titers in the plasma of all LV-treated mice. Anti-VSV.G IgG remained undetectable in CoB-treated mice, and 10 weeks after LV-GFP administration (**Fig. 7B**), all the mice received 7×10^9 TU/kg of LV-hFIX, encoding human clotting Factor IX (hFIX), together with naïve C57Bl/6 control mice (n=6) receiving only LV-hFIX, as controls. Notably, further follow-up of these mice revealed that CoB-treated mice did not develop an anti-VSV.G IgG response, even after a second LV dose, administered in the absence of the CoB regimen. On the other hand, mice treated with LV.GFP, but not CoB, showed an amplified secondary immune response to VSV.G exposure (see Fig. 7B). Quantification of total and LV-GFP-specific VCN showed that transduction by hFIX-encoding LV occurred in CoB-treated as well as in naïve mice, while the induced anti-VSV.G IgG immune response completely neutralized LV-hFIX particles in mice previously treated with LV-GFP alone (**Fig. 7C**), preventing successful liver hFIX gene transfer. Furthermore, hFIX expression confirmed that LV-GFP CoB-treated and naïve mice were equally permissive to LV transduction, leading to comparable levels of hFIX expression *in vivo* (**Fig. 7D**).

To assess whether the effect of CoB extended beyond LV-mediated GT, we applied this regimen in the context of AAV8 liver-directed gene transfer. CoB treatment effectively prevented the development of neutralizing anti-AAV8 capsid IgG, indicating that the control of anti-vector humoral immunity by CoB is not restricted to the LV platform and may be applicable across distinct gene transfer modalities. This immune modulation allowed the administration of an initial AAV8 vector encoding GFP (AAV8-GFP), followed by a second AAV8 vector encoding hFIX (AAV8-hFIX), resulting in detectable hFIX levels in the plasma of both naïve controls and CoB-treated mice previously exposed to AAV8-GFP (**Fig. 7E-G**).

Therefore, to explore the possibility of LV re-administration in a disease model, a cohort of HemoA mice (n=8) received transduction enhancers before 7×10^{10} TU/kg of LV-hFVIII, followed by the CoB regimen. Then, 8 weeks after the first LV infusion, half of the GT-treated (n=4) and naïve HemoA mice (n=3)

received transduction enhancers followed by 3.5×10^{10} TU/kg of LV-hFVIII. Plasma concentrations of hFVIII Ag and activity were monitored over time, revealing that CoB-treated mice were permissive to further adjustment of hFVIII levels with a second LV dose (**Fig. 8A, B**). VCN evaluation confirmed that additional copies were integrated in the liver parenchyma, as in naïve HemoA, by the second LV infusion (**Fig. 8C**). Although administration of the second half-dose of LV-hFVIII in 16-week-old naïve HemoA mice was preceded by transduction enhancers, it did not achieve transduction efficiency sufficient to establish full tolerance, thereby inducing low-titer Abs against both LV particles and hFVIII. By contrast, CoB-treated mice remained tolerant to both LV particles and transgene-derived Ags presented after the second LV infusion (**Fig. 8D-G**). Overall, these data show that the CoB regimen prevented vector-neutralizing immune responses, thereby enabling effective re-dosing.

DISCUSSION

Here, we show that transient T cell costimulation blockade during the presentation of GT-related Ags generates robust active tolerance to therapeutic transgene products and vector particles, preventing the induction of immune responses and opening the possibility of improving the therapy by effectively readministering a second dose to adjust the transgene output based on the outcome of the first dose.

Immune barriers in viral vector-based *in vivo* hepatocyte-directed GT have been thoroughly investigated, and several strategies have been attempted to evade them^{13,31}. For example, AAV capsids have been engineered to improve immune evasion from pre-existing Abs³², while in the case of the LV platform, virtually not limited by neutralizing pre-existing immunity, the immunogenicity of vector- and transgene-derived Ags has been extensively restrained by previous engineering strategies^{5,14,15}. However, in the setting of highly immunogenic secreted transgenes, it emerged that additional interventions are required to maintain long-term transgene activity. The present study shows that few hepatocytes expressing high transgene amounts *per cell* favor anti-transgene immunity, whereas a larger proportion of hepatocytes expressing lower transgene amounts *per cell* promotes immune tolerance to the transgene product. Additionally, extensive codon optimization may contribute to anti-transgene immunity by increasing the presentation of misfolded protein due to an accelerated translation rate. Recently, we identified liver transduction enhancers (24 hours of fasting, combined with transient IFN α R1 blockade⁸) that were successfully applied here to immune-competent HemoA mice, achieving a transduction efficiency high enough to reach therapeutically relevant hFVIII amounts, even with a non-codon-optimized transgene variant that was poorly expressed yet less immunogenic. These results support the concept that the extent of liver transduction is a key determinant of therapeutic efficacy and of the development of transgene-specific immune tolerance, which is favored by the pro-tolerogenic liver environment. Alternatively, durable and therapeutically relevant transgene activity can be achieved with a 2.5-fold lower dose of LV encoding a codon-optimized transgene by altering T cell priming soon after GT administration. A transient CoB regimen, comprising anti-CD154 and CTLA4-Ig, promoted abortive adaptive immune responses to transgene- and vector particle-derived epitopes, allowing stable expression of the therapeutic transgenes by hepatocytes and inducing active transgene-specific tolerance, as shown by resistance to Ag rechallenge.

This study suggests that the administration of CoB regimen is a potent modulator of T cell-mediated responses that are concomitantly induced. A significant downregulation of effector functions, differentiation, clonal expansion, and memory formation of T cells recognizing GT-related Ags *in vivo* is induced by blocking fundamental crosstalk pathways between T cells and APCs. In particular, up-regulation of CD154 (CD40L) within a few hours after recognition of the cognate Ag in the context of MHC-II molecules by CD4 T cells provides a signal to complete activation of APCs, likely initiated by Ag capture and IFN- α signaling from innate recognition of the vector genome²⁹. CD40 engagement further increases co-stimulation and promotes the release of active IL12 p70³³, driving Th1 differentiation,

supporting Ag cross-presentation and CD8 T cell priming^{34,35}, B cell maturation and IgG switch. Therefore, activated APC are equipped to support T cell activation via CD80/86-CD28 pathways, and the programmed up-regulation of CTLA-4, which binds CD80/86 with higher affinity than CD28, acts as a negative regulator. Therefore, anti-CD154 and CTLA4-Ig effectively target CD4 T cells, which play a pivotal role in supporting adaptive responses to the transgene product and vector particles, with CTLs and NAb serving as the final immune mediators.

CoB is the latest evolution of immune-modulatory strategies in kidney transplantation to prevent graft rejection without the nephrotoxicity typical of calcineurin inhibitors (CNIs), such as Cyclosporin A and Tacrolimus. Moreover, the specificity of CoB's molecular targeting of the CD40-CD40L and CD80/86-CD28 pathways also implies specific targeting of naïve T cells, which depend on co-stimulation for their activation, in contrast to CNI immunosuppression, which interferes with T cell activation independently of the stage of differentiation and also suppresses the activity of protective memory T cells³⁶. Therefore, CoB-based immunosuppression, which spares the memory compartment from anergy and clonal deletion³⁷, allows regulation of T cell responses at the time of exposure to allogeneic tissues or, as shown here, to GT-related neo-Ags. Although tolerance to therapeutic transgenes and vector particles is essential for GT efficacy and re-dosing, the use of CoB in association with GT may raise concerns. While tolerance to the transgene product also allows adjustment of protein-based therapy, the lack of immune reactivity to vector particles may reduce future responsiveness to parental viruses. However, cross-tolerance induced by the CoB regimen in the context of VSV.G-pseudotyped LV-based GT is unlikely to be relevant, since VSV has poor human tropism and may only sub-clinically infect people in contact with livestock³⁸. Conversely, the use of the CoB regimen in AAV-based GT may result in a lack of reactivity against parental AAVs, which are themselves not pathogenic in humans but are commonly encountered and have elevated seroprevalence. CoB was less efficient at inhibiting the formation of anti-AAV capsid than anti-VSV.G NAb IgG. Mice treated with AAV8-GFP in combination with CoB were less permissive to the second AAV8-hFIX infusion, compared to mice treated with two subsequent LV infusions, showing reduced efficiency of the second transduction, which is compatible with partial vector inactivation. The incomplete protection against vector-specific NAb development by CoB in the context of AAV-mediated GT may be due to prolonged Ag availability *in vivo*, suggesting that optimizing the CoB immunoregulatory approach may be required to fully inhibit NAb induction. On the contrary, CoB-mediated tolerization toward LV particles was more effective in inhibiting NAb induction, at least in mice that were NAb negative even after the second LV infusion.

Preventing anti-FVIII transgene immune responses after *in vivo* GT in previously untreated individuals may be particularly crucial for pediatric patients who have never been exposed to FVIII recombinant products and, until now, have not been eligible for GT. This patient population is progressively increasing due to the adoption of Emicizumab, a bispecific Ab that bridges activated Factor IX and Factor X. This drug represents a subcutaneous alternative to i.v. recombinant FVIII products, indicated for both anti-FVIII Abs-positive and- negative patients³⁹. In this scenario, an effective immunotherapy to prevent immune responses to FVIII will be crucial to the safe application of GT, particularly for pediatric patients not exposed to FVIII protein products seeking a lifelong cure for hemophilia A.

The CoB immune-modulatory approach described here in the context of hepatocyte-driven FVIII expression may also be applied to favor tolerization when FVIII is expressed by endothelial cells, the physiologic source of the endogenous FVIII^{40,41}. Similarly, the application of the CoB may also be applied to control anti-drug immunity for all the available therapeutic options for lysosomal storage disorders, such as MPS-I. The administration of enzyme replacement therapy (ERT) at diagnosis represents the conventional intervention⁴² that may induce T cell-dependent anti-drug immunity^{20,43,44}. This outcome may represent a complication for future treatment of stably seroconverted patients, either remaining under ERT or potentially eligible for *in vivo* liver-directed GT, if affected by the milder forms of MPS-I not

involving the central nervous system. Effective LV re-dosing enabled by the CoB regimen may also be useful in the context of *in vivo* gene therapy to hematopoietic stem/progenitor cells, where a lower initial efficiency may be compensated by a second vector re-administration⁴⁵. Notably, the CoB regimen does not contemplate prolonged steroid use, which is instead typical for controlling anti-AAV-capsid immune responses in humans but is associated with substantial side effects and induces a more general immunosuppression rather than immune modulation.

This study revealed elusive critical factors to be considered for the optimization and safe application of *in vivo* GT, particularly when dealing with highly immunogenic secreted transgene proteins, such as hFVIII and lysosomal enzymes. Deliberate optimization of transgene expression might increase misfolded product levels and immunogenicity, particularly when only a minimal proportion of the liver parenchyma is transduced, resulting in a small subset of hepatocytes expressing high transgene amounts *per* cell that is rapidly cleared by CTLs. By contrast, more limited transgene expression *per* cell, when accompanied by a large proportion of transduced hepatocytes, promotes a tolerogenic response. The proportions of hepatocytes and transgene expression rates required for liver-mediated immune tolerance induction may not be easily predictable and may vary with the type of transgene, underlying disease, and host conditions. Therefore, without well-defined dose thresholds and transduction/expression ratios for tolerance induction, the CoB regimen proposed here robustly and selectively regulates detrimental immune responses, enabling tolerance to GT-related Ags without affecting the already established memory compartment, and with the added benefit of allowing administration of further GT doses.

Overall, we present a viable targeted immune-modulatory strategy that overcomes critical immune barriers to durable and redosable *in vivo* liver-directed GT across different disease settings and vector platforms, which, following further refinements and pre-clinical evaluation, may substantially improve the clinical outcomes and uptake of *in vivo* GT applications.

MATERIALS AND METHODS

Experimental design

Sample size in experiments with mice was chosen according to previous experience with experimental models and assays. Mice were randomly assigned to each experimental group. Investigators were not blinded.

Plasmid construction

Plasmid pCCLsin.cPPT.ET.FVIII-BDD.142T (ET.hFVIII.142T), pCCLsin.cPPT.ET.FVIII-BDD-XTEN.142T (ET.hFVIII-XTEN.142T) pCCLsin.cPPT.ET.coFVIII.142T (ET.cohFVIII.142T) and pCCLsin.cPPT.ET.coFIX.XTEN.142T (ET.cohFVIII-XTEN.142T), pCCLsin.cPPT.ET.coIDUA.142T (ET.cohIDUA.142T) were constructed by standard cloning techniques, by exchanging the synthesized different versions of the human FVIII cDNA (GeneScript) with FIX into the previously described pCCLsin.cPPT.ET.canineFIX.142T (NheI-SalI)¹⁶. ET.hFIXR338L.4x142T and ET.GFP.4x142T cassettes were cloned (GeneScript) in AAV backbone plasmid (XhoI-NotI). AAV8 encoding for hFIX and GFP were produced and titrated by InnovaVector.

Vector production

Lab-grade VSV.G-pseudotyped 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.G and pAdvantage, as previously described⁴⁶. 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 x g for 120' at 20° C (Beckman Optima XL-100K Ultracentrifuge). LV pellet was dissolved in the appropriate volume of PBS to allow 500-1000X concentration.

LV titration

For LV titration, 1×10^5 293T cells were transduced with serial LV dilutions in the presence of polybrene (8 µg/ml). Genomic DNA (gDNA) was extracted 14 days after transduction, using Maxwell 16 Cell DNA Purification Kit (Promega), following manufacturer's instructions. VCN was determined by ddPCR, starting from 5-20ng of template gDNA using primers (HIV fw: 5'-T ACTGACGCTCTCGCACC-3'; HIV rv: 5'-TCTCGACGCAGGACTCG-3') and a probe (FAM 5'-ATCTCTCTCCTTCTAGCCTC-3') designed on the primer binding site region of LV. The amount of endogenous DNA was quantified by a primers/probe set designed on the human telomerase gene (Telo fw: 5'-GGCACACGTGGCTTTTCG-3'; Telo rv: 5'-GGTGAACCTCGTAAGTTTATGCAA-3'; Telo probe: VIC 5'-TCAGGACGTCGAGTGGACACGGTG-3' TAMRA) or the human GAPDH gene (Applied Biosystems HS00483111_cm). The PCR reaction was performed with each primer (900 nM) and the probe (250 nM, 500nM for Telo) following manufacturer's instructions (Biorad), read with QX200 reader and analyzed with QuantaSoft software (Biorad). Infectious titer, expressed as TU/mL, was calculated using the formula $TU/mL = (VCN \times 100,000 \times (1/\text{dilution factor}))$. LV physical particles were measured by HIV-1 Gag p24 antigen immunocapture assay (Perkin Elmer) following manufacturer's instructions. LV specific infectivity was calculated as the ratio between infectious titer and physical particles.

Vector copy number (VCN) determination

DNA was extracted from whole liver samples using Maxwell® 16 Tissue DNA Purification Kit (Promega), DNA was extracted from fractionated/sorted liver cells using DNeasy Blood & Tissue Kit (Qiagen) or QIAamp DNA Micro Kit (Qiagen), according to cell number. VCN was determined by ddPCR, starting from 5-20 ng of template gDNA using a primers/probe set designed on the primer binding site region of LV (HIV fw: 5'-T ACTGACGCTCTCGCACC-3'; HIV rv: 5'-TCTCGACGCAGGACTCG-3') and a probe (FAM 5'-ATCTCTCTCCTTCTAGCCTC-3') or alternatively binding site region of GFP (GFP fw: 5'-CAGCTCGCCGACCACTA-3'; GFP rv: 5'-GGGCCGTCGCCGAT-3') and a probe (FAM 5'-CCAGCAGAACACCCCC-3'). The amount of endogenous murine DNA was quantified by a primers/probe set designed on the murine sema3a gene (Sema3A fw: 5'-ACCGATTCCAGATGATTGGC-3'; Sema3A rv: 5'-TCCATATTAATGCAGTGCTTGC-3'; Sema3A probe: HEX 5'-AGAGGCCTGTCCTGCAGCTCATGG-3' BHQ1). The PCR reaction was performed with each primer (900 nM, 150nM for RT-LV primers) and the probe (250 nM) following manufacturer's instructions (Biorad), read with QX200 reader and analyzed with QuantaSoft software (Biorad).

Mice experiments

Founder B6;129S-F8tm1Kaz/J mice ²¹ (referred to as HemoA) were obtained from The Jackson Laboratories (stock #004424). Founder B6.129S7-Rag.1t1Mom/J mice ⁴⁷ (referred to as Rag1 KO) were purchased from The Jackson Laboratory (stock # 002216). HemoA-Rag1 double KO (referred to as HemoA-Rag) mice were obtained by crossing HemoA homozygous mice with Rag1 KO homozygous mice ⁶. For mice genotyping, DNA was extracted from tail biopsies using Maxwell 48 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 adult (7-10 week old) mice by tail-vein injection, while in 2-week-old by retro-orbital injection. Mice were bled from the retro-orbital plexus using

capillary tubes and blood was collected into 0.38% sodium citrate buffer, pH 7.4. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee.

Anti-IFN α R1 mAb (clone MARI-5A3, BioXcell) was administered always as 1 dose of 1 mg at -3 hours from LV administration, alone (blockade innate signaling) or in conjunction with: 24 hours of fasting (transduction enhancers); anti-CD3 F(ab')₂ (clone 145-2C11, BioXcell) 5 doses x 0.05mg 2-3 days apart from LV injection (T cell depleting / inactivating regimen); anti-CD20 (clone MB20-11, BioXcell) 2 doses X 0.3mg 7 days apart from day 3 post LV injection (B cell depleting regimen); anti-CD154 (0.5 mg/ dose clone MR1 BioXcell), CTLA4-Ig (0.5 mg/ dose Orenicia, Bristol Myers Squibb) 5 doses 2-3 days apart from LV injection (costimulatory blockade (CoB) regimen). Anti-KLH IgG was provided as an isotype control in control mice. For fasting experiments, the weight used to calculate LV dose/kg was measured before starting the fasting procedure. During fasting, the mice were injected i.p. twice with saline.

Flow cytometry

Flow cytometry analyses were performed using a FACSSymphony analyzer (BD Biosciences), equipped with DIVA Software, standard and unsupervised analyses were conducted with FlowJo 10.10. Blood was collected from mice from retro-orbital plexus and 20 μ L with or 0.35X10⁶ primary cells from spleen and liver infiltrates were directly stained 50 μ L of antibody mix (see below for details) for 20 minutes at 4°C in the dark. Cells were then fixed and permeabilized with 100 μ L of perm/fix buffer 1X (e-Bioscience) for 40 minutes at 4°C, washed 1X perm buffer (e-Bioscience). Foxp3 intracellular staining was conducted by diluting Ab 50 μ L 1X perm buffer and incubating for 20 minutes at RT. Cell were washed twice in 1X perm buffer and re-suspended PBS FACS. For T cell immunophenotyping, the following monoclonal antibodies were used: CD3 BUV737, clone 17A2 (BD Horizon, 1:200), CD45 BUV395, clone 30-F11 (BD Horizon, 1:200), CD4 BV605, clone RM4-4 (BD Horizon, 1:200), CD8 BV510, clone 53-6.7 (BD Horizon, 1:200), CD25 PerCP-Cy5.5, clone PC61 (BD Pharmingen, 1:200), CD127 PE, clone SB/199 (BD Pharmingen, 1:200), CD44 APC-Cy7, clone IM7 (BD Pharmingen, 1:200), CD62L BV786, clone MEL-14 (BD Horizon, 1:2500), PD1 BV421, clone J43 (BD Horizon, 1:250), LAG-3 BV711, clone C9B7W (BD Horizon, 1:200), TIM-3 APC, clone 8B.2C12 (Invitrogen, 1:2500), KLRG-1 PE-Cy7, clone 2F1 (Invitrogen, 1:200), Foxp3 FITC, clone JFjK-16s (Invitrogen, 1:200), CD19 APC, clone RA3-6B2 (BD Biosciences, 1:200), B220 PerCP, clone 1D3 (BD Biosciences, 1:200).

T cell-enriched splenocytes using an anti-mouse CD3 isolation kit (Miltenyi) and non-parenchymal hepatic cell isolation were stained with anti-CD4, anti-CD8, anti-CD25, and anti-CD127 to sort GFP-expressing from the indicated T cell subpopulation by BD FACSAria.

Transgene product quantification

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.

The concentration of human FIX was determined in mouse plasma by Affinity's Factor IX Paired Antibody Set (Affinity Biologicals FIX-EIA) consists of matched capture and detecting antibodies that have been titrated and optimized for use in sandwich style ELISA assays following the manufacturer's instructions. Absorbance of each sample was determined spectrophotometrically, using a Multiskan GO microplate reader (Thermo Fisher Scientific) and normalized to antigen standard curves.

The activity of hIDUA activity was measured fluorometrically in the liver as previously described⁴⁸. The area of GFP expression in the liver parenchyma was determined by Immunofluorescence imaging. Livers were harvested from mice, washed briefly in PBS and fixed 4 hours in PBS 4% paraformaldehyde (PFA), then washed again briefly in PBS before being stored at least 24 hours in 30% sucrose 0.02% sodium azide in H₂O. Livers were then frozen in OCT (optimal cutting temperature) compound (Killik, Bio Optica) and slices 5 to 20 µm thick were cut at cryostat (Histo-line MC5050), placed on Superfrost® Plus microscope slides (Thermo Scientific) and stored at -80°C. All the staining steps were performed protected from lights to preserve endogenous fluorescence. Slides were thawed at room temperature for at least 2 hours, then washed 3 times 5 minutes in PBS 0.1% X-Triton. Edges were drawn around the tissue using immunostaining pap pen (Sigma-Aldrich) to contain staining solutions. The blocking step was performed using PBS 0.1% X-Triton 1% bovine serum albumin (BSA) 5% FBS for 1 hour at room temperature in a humid chamber. The blocking solution was then substitute with mix containing primary antibodies in the blocking solution and incubation was carried on for 12-16 hours at 4°C in a humid chamber. The primary antibodies mix solution was removed and slides were washed 3 times 5 minutes in PBS 0.1% X-Triton. Secondary antibodies were diluted in the blocking solution together with Hoechst (Invitrogen, 1:20,000) and incubated for 1 hour at room temperature in a humid chamber. Slides were then washed in PBS and a coverslip was added using Fluoromount-G (Invitrogen) as mounting medium. Slides were dried for 16 hours at room temperature or for longer at 4°C, and stored at 4°C before acquisition. The following antibodies were used for immunofluorescence staining: Anti-GFP rabbit, polyclonal (Invitrogen, A11122, 1:1000), Anti-Rabbit IgG Alexa Fluor (AF)647 donkey, polyclonal (Invitrogen, 1:1000). Images were acquired using confocal microscope Leica TCS SP5, Leica TCS SP8, or Mavig Rs-G4 at 20X magnification. Images were analyzed using Image J.

Anti-transgene IgG quantification

Anti-hFVIII IgG 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 IgG 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 IgG 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.

Anti-hIDUA IgG titer was measured in mouse plasma by ELISA. Plates were coated with 2 µg/mL of recombinant hIDUA (Aldurazyme, Sanofi) in 1 M carbonate buffer overnight and blocked with 1% bovine serum albumin (BSA) + 0.05% Tween-20. Diluted serum (1:200, 1:500 and 1:1000) was added in duplicates and incubated for 2 h. The secondary antibody (anti-mouse IgG 1:10000, Sigma-Aldrich) was incubated for 2 h and revealed with o-phenylenediamine dihydrochloride (OPD) for 7 min. The reaction was stopped

with 1 M sulfuric acid (H₂SO₄), and the absorbance was read at 492 nm using a microplate reader Multiskan GO (Thermo Fisher, Waltham, MA, USA). IgG concentration using a standard curve, obtained by coating a known amount of mouse IgG in duplicate in a reference series of wells. The concentrations of *anti-VSV.G IgG* were similarly determined in mouse plasma by ELISA, coating with recombinant VSV.G (Alpha Diagnostic Intl.) 1 µg/mL and developing with a HRP-conjugated mouse anti-monkey IgG Ab (anti-mouse IgG 1:10000, Sigma-Aldrich).

Ag specific T cell response determination.

Enumeration of IFN releasing T cells by antigen-specific stimulation by IFN γ elispot. Multiscreen filter plates (Millipore-Merck) were coated overnight 4°C with capture Ab for IFN γ (clone R4-6A2 5 µg/mL 50 µL/well, BD Pharmingen), blocked with PBS 1% BSA for 2 hours at 37°C. Plates were washed twice with PBS and equilibrated with culture medium for 10 minutes at room temperature before seeding cells. Magnetically sorted splenic CD8 T cells (1x10⁵ cell/well, by mouse CD8 T cell isolation Kit, Miltenyi) or circulating T cells (1x10⁵ cells/well) were plated in complete RPMI (10% FBS, pen /strep, Na-pyruvate, non-essential amino acid and β -mercapto-ETOH) in triplicate with syngeneic *wt* EL4 cell line (1x10⁵ cells/well) or constitutively expressing the transgene (hFVIII, hIDUA) for 42h hours at 37C 5% CO₂ in the presence of IL-2 100u/mL (Proleukin). At the end of the culture plates were washed 7 times (PBS 0.05%Tween-20) and detection Ab for IFN γ (clone XMG1.2 0.5 µg/mL 50 µL/well, BD Pharmingen) was added for 2 hours at RT. After washing, Avidin-POD solution (Roche, 1:5,000, 50 µL/well) was then added and incubated for 1 hour at room temperature, and spots 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 from *wt* EL4 stimulated cells was subtracted to Ag stimulated condition and reported as IFN γ -producing cells in 1x10⁶ splenic CD8 or PBMC.

Antigen-specific T cell proliferation was determined by plating total splenocytes in 96-well plate (7x10⁵ cells/well) in complete RPMI. Cells were left unstimulated or stimulated with increasing concentration of recombinant hFVIII.BDD protein (ReFACTO, Pfizer) or recombinant hIDUA (Aldurazyme, Sanofi) in triplicate. After 5 days of culture, 1 µCi/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 a ratio between the mean counts *per* minute (cpm) in each stimulated condition and the mean counts of the unstimulated cell.

RNAseq and analysis

Cellular RNA was extracted using RNeasy+ mini kit (Qiagen), following the manufacturer's instructions. DNA was digested on columns using the RNase-Free DNase Set (Qiagen) and stored at -80°C.

Samples were sent to Genewiz for library preparation and sequencing (Illumina NovaSeq platform, paired-end reads, 2 × 150 bp). Pre-processing of raw RNA-seq reads was performed using FastQC (v0.11.6) to assess sequencing quality. Adapter sequences and low-quality bases were trimmed using Trim Galore (v0.6.6). Filtered reads were aligned to the mouse reference genome (GRCm39) using STAR (v2.7.11b) with default parameters. Only reads uniquely mapped to the genome were retained for downstream analyses. Gene-level read counts were quantified using featureCounts (v2.0.1), based on gene annotations from GENCODE (v36, primary assembly). Differential gene expression analysis was performed using the DESeq2 R/Bioconductor package (v1.30.0). Resulting P-values were adjusted for multiple testing using the Benjamini–Hochberg method, and genes with an adjusted P-value (FDR) < 0.05 were considered differentially expressed. Over-Representation Analysis (ORA) was performed using the Gene Ontology – Biological Process (GO-BP) database through the clusterProfiler package (v4.14.4). All graphical outputs was generated using the ggplot2 package in RStudio.

Statistical analysis

Statistical analyses were performed upon consulting with professional statisticians at the San Raffaele University Center for Statistics in the Biomedical Sciences (CUSBS). Non-parametric test, Mann-Whitney or Kruskal-Wallis tests, were performed when comparing two or more experimental groups.

List of Supplementary Materials

Fig. S1 to S14

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Author contributions: Conceptualization: AA, MM, AC; Methodology: AA, MM, CC, SI, FR, MF, MB; Investigation: AA, MM, CC, SI, FR, MF, MB; Visualization: AA, MM; Funding acquisition: AC, LN; Project administration: AC; Supervision: AA, MM, AC, LN; Writing – original draft: AA; Writing – review & editing: AA, MM, CC, LN, AC.

Competing interests: AC and LN are co-founders and quota holders of Genespire, a start-up aimed at developing liver-directed LV gene therapy for inherited diseases. MM, CC, AC, and AA are inventors on patents or patent applications owned by Genespire, Fondazione Telethon, or San Raffaele Scientific Institute that relate to the work presented in this manuscript. The remaining authors declare no competing interests.

Data and materials availability: All data are available in the main text or the supplementary materials. The LV and reagents described in this manuscript are available to interested scientists upon signing an 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 Genespire. All data are available in the main text or the supplementary materials.

Raw and processed RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE338074. During the review process, the dataset can be accessed using the private reviewer token sbyluqsgrbeddon. The data will be released publicly upon publication.

Figure 1

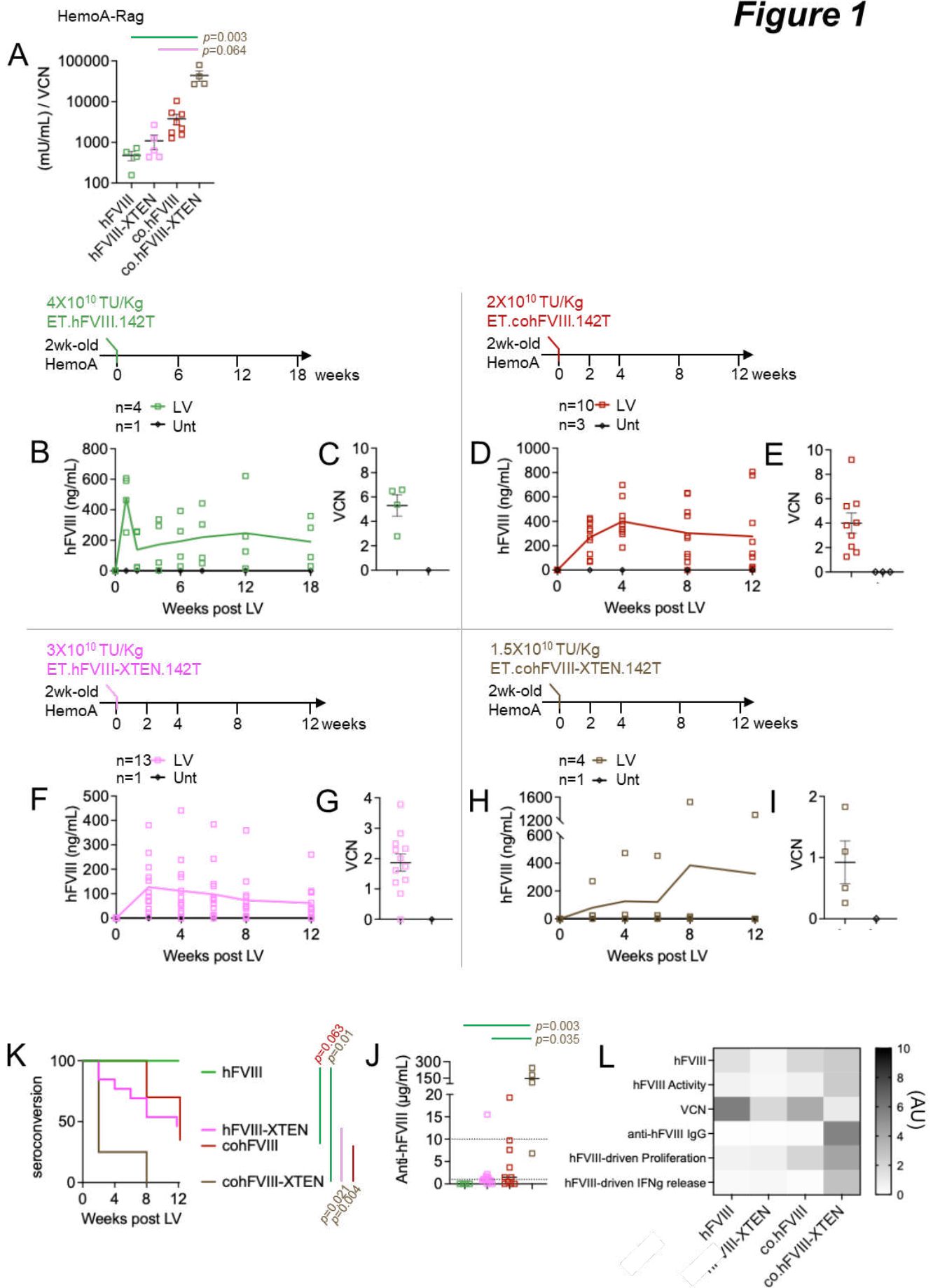


Figure 1. hFVIII sequence variants improving translation and increasing half-life impact on immunogenicity in *in vivo* gene therapy.

Single values and mean of the ratio between the plasma hFVIII activity, at the end of monitoring period, and the LV copies *per* diploid genome (VCN) detected in the liver of HemoA-Rag immune-deficient hemophilic mice, treated at two-week-old with LV.ET.hFVIII.142T (4×10^{10} TU/kg, n=4, green open squares), LV.ET.hFVIII-XTEN.142T (3×10^{10} TU/kg, n=5, orchid open squares), LV.ET.cohFVIII.142T (2×10^{10} TU/kg, n=8, red open squares), and LV.ET.cohFVIII-XTEN.142T (1.5×10^{10} TU/kg, n=4, brown open squares). This represents the potential therapeutic efficacy of each LV.FVIII variant, without the confounding factor of immune activation (**A**). Single values with connecting means of hFVIII concentration in the plasma of HemoA mice, i.v. treated at two-week-old with LV.ET.hFVIII.142T (4×10^{10} TU/kg, n=4, green open squares) (**B**), LV.ET.cohFVIII.142T (2×10^{10} TU/kg, n=10, red open squares) (**D**), LV.ET.hFVIII-XTEN.142T (3×10^{10} TU/kg, n=13, orchid open squares) (**F**), LV.ET.cohFVIII-XTEN.142T (1.5×10^{10} TU/kg, n=4, brown open squares) (**H**). Single values and mean with standard error of the mean (SEM) of VCN measured in the total liver of LV-treated mice and in untreated controls (black open diamonds) (**C**, **E**, **G**, **I**). Immunogenicity of each LV.FVIII variant was evaluated based on the appearance of anti-hFVIII IgG ($>1 \mu\text{g/mL}$, seroconversion) over time (**K**) and titer at the end of monitoring (**J**). A heatmap reporting normalized data on expression, copies, and anti-FVIII-specific cellular and humoral immune responses (see complete in Fig. S1) provides a direct comparison of *in vivo*-tested LV variants. The gray scale represents relative levels of each parameter, with darker shades indicating higher values (**L**). Kruskal-Wallis test was performed comparing mice with LV variants (*p* values are shown; **A**, **J**).

Figure 2

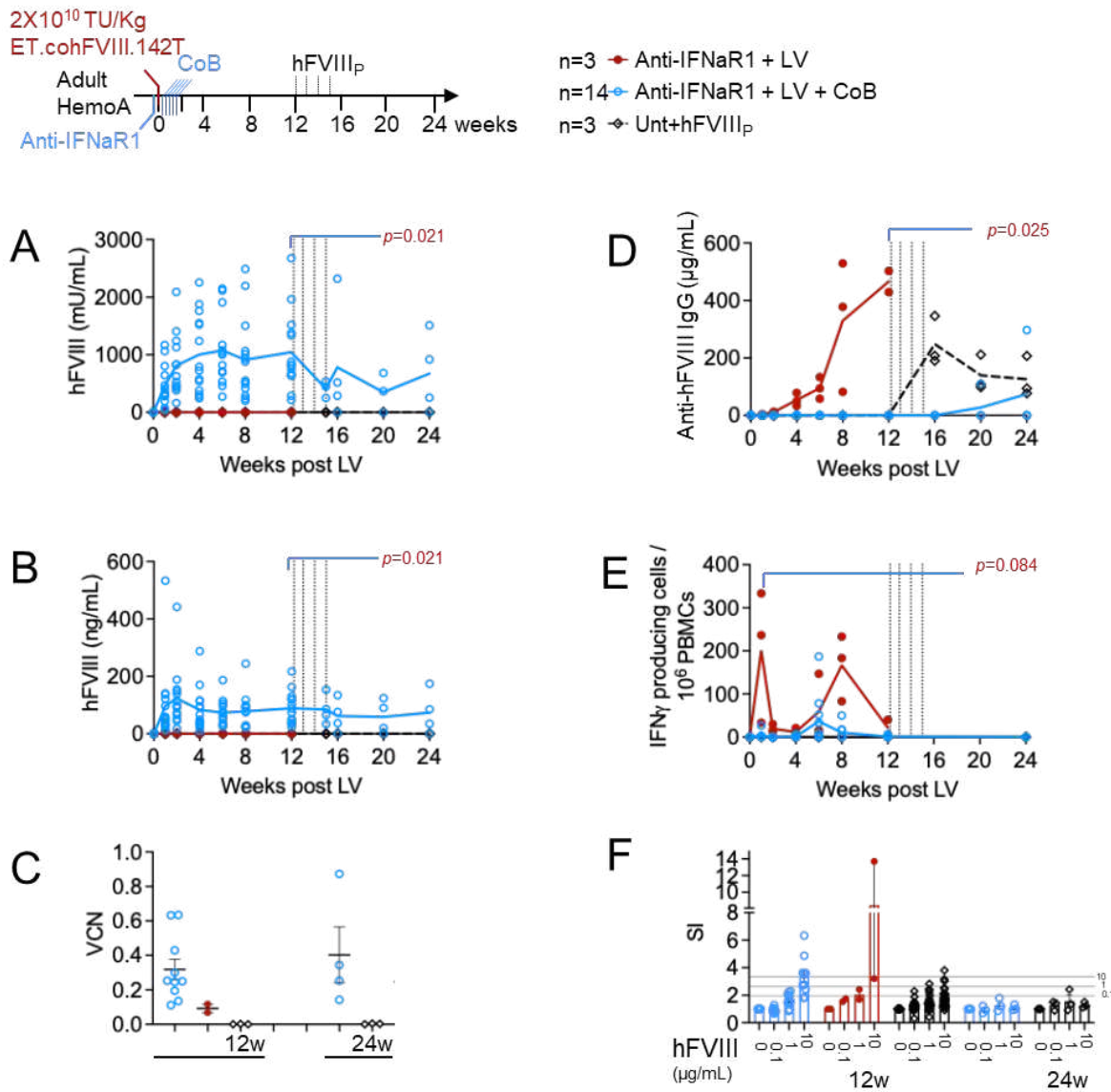


Figure 2. Co-stimulatory blockade (CoB) regimen impedes immune-mediated clearance of hFVIII-expressing hepatocytes after *in vivo* GT to resolve hemophilia A.

Eight-week-old adult HemoA mice (n=17) were treated with an anti-IFN α R1 blocking monoclonal antibody (mAb) 3 hours before receiving a 2x10¹⁰ TU/kg dose of LV.ET.cohFVIII.142T. A co-stimulatory blockade (CoB) regimen, consisting of 5 doses of anti-CD154 and CTLA4-Ig, was then administered 2-3 days apart in most of LV treated mice (n=14, cerulean open dots), while the controls did not receive CoB (n=3, dark red open dots). Twelve weeks after LV injection n=10 mice were euthanized for endpoint analysis. In the other CoB treated mice (n=4) and in naïve HemoA (n=3) mice hFVIII protein was administered i.v. (Re-facto 4x10U, weekly) as a pro-immunogenic exposure (challenge) to hFVIII, and mice were monitored for an additional 12-week period. Single values with connecting means of hFVIII activity (**A**) and concentration (**B**) in the plasma of HemoA mice, at the indicated time points after LV administration. VCN was evaluated in genomic DNA isolated from liver biopsies of each mouse at the indicated time points; individual data and the average LV copies are shown (**C**). hFVIII-specific immune responses were monitored at the indicated time points by determining anti-FVIII IgG titer by ELISA (**D**), frequency of circulating IFN γ -releasing T cells by ELISPOT (**E**). hFVIII- driven proliferation of splenic T cells was measured by ³H-thymidine incorporation and reported as Stimulation Index (SI), SI =[(mean CPM

Ag stimulated triplicate) : [(mean CPM unstimulated triplicate)]. Ag-specific proliferation is considered significant if > than the mean SI of naïve HemoA mice (n=23) at the relevant Ag concentration. Individual measurements and the group mean $\pm$ SEM are reported (C, F). Kruskal-Wallis test was performed at the indicated time point comparing mice treated with or without CoB (*p* values are shown; A, B, D, E).

Figure 3

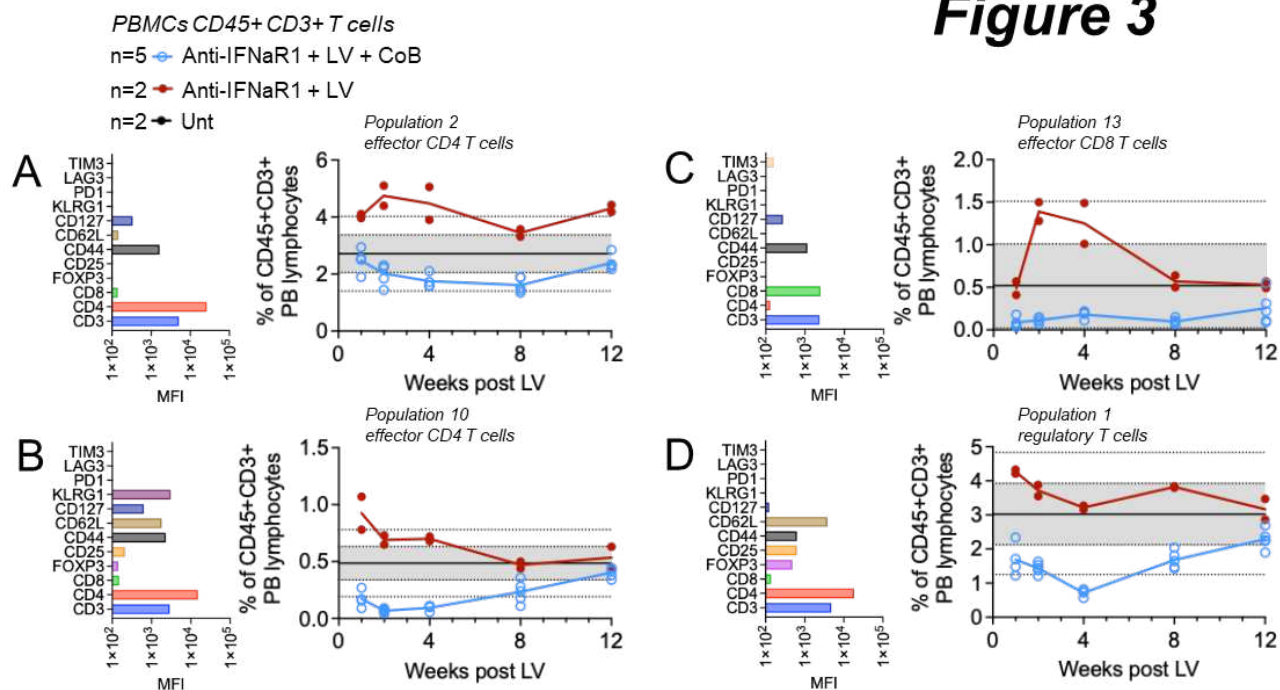


Figure 3. Co-stimulatory blockade (CoB) regimen transiently alters circulating T cell populations.

Anti-IFN α R1 mAb-pre-treated adult HemoA mice injected with LV.ET.cohFVIII-XTEN.142T and subsequently treated (n=5, cerulean open dots), or not (n=2, dark red dots) with the CoB regimen. Mice were bled and T cells were analyzed by flow cytometry at the indicated time points. Unsupervised multiparametric analysis was performed on single-cell immunophenotypic cytofluorimetric data from circulating T cells. Among the T cell subpopulations identified (Fig. S8), those annotated as effector CD4 (A, B) or CD8 (C) and regulatory (D) T cells, which showed altered frequencies compared to untreated HemoA controls (gray area = normal % $\pm$ 1SD), are reported. Geometric Mean Fluorescence Intensity (MFI) and the frequency of each population are reported at the indicated time point. Data for each lymphocyte population, single-mouse frequencies, and mean values are shown.

Figure 4

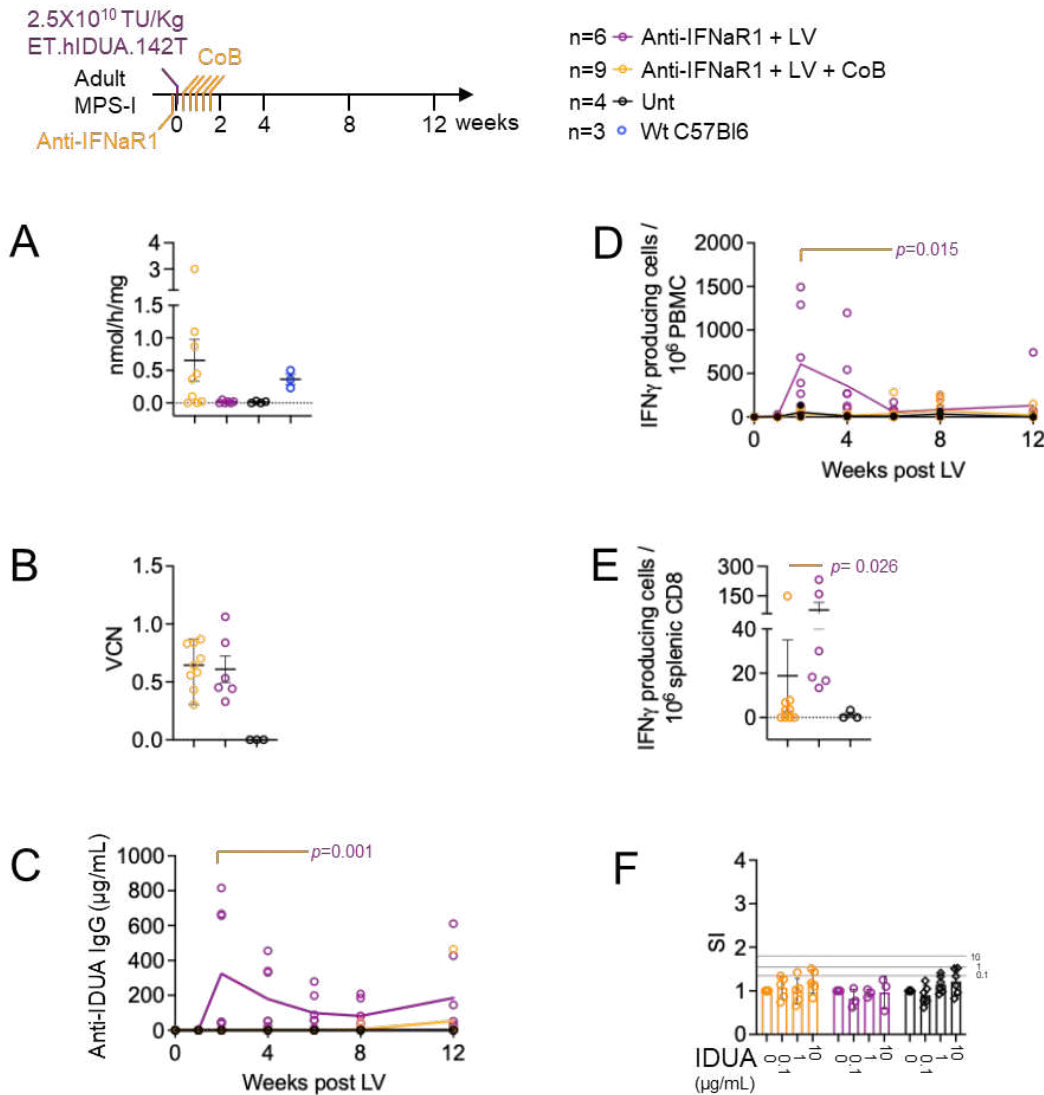


Figure 4. Co-stimulatory blockade (CoB) regimen improves efficacy of hepatocyte-directed gene therapy for mucopolysaccharidosis type I (MPS-I).

Eight-week-old adult *Idua*^{-/-} MPS-I mice (n=15) were treated with an anti-IFN α R1 mAb 3 hours before receiving a 2.5x10¹⁰ TU/kg dose of LV.ET.hIDUA.142T. A co-stimulatory blockade (CoB) regimen, consisting of 5 doses of anti-CD154 and CTLA4-Ig, was then administered 2-3 days apart (n=9, mustard open dots), while control mice (n=6, orchid open dots) did not receive CoB. All mice were monitored for 12 weeks. IDUA enzymatic activity (A) was evaluated in the liver; data are shown for each mouse and the group average. VCN was evaluated in genomic DNA isolated from liver biopsies of each mouse 12 weeks post LV injection; individual data and the mean LV copy number are shown (B). hIDUA-specific immune responses were monitored at the indicated time points by determining anti-IDUA IgG titer by ELISA (C), frequency of circulating (D) or splenic (E) IFN γ -releasing T cells by ELISPOT. hIDUA-driven proliferation of splenic T cells was measured by ³H-thymidine incorporation and reported as Stimulation Index (SI) (F). Ag-specific proliferation is considered significant if > than the mean SI of naïve MPS-I mice (n=6) at the relevant Ag concentration. Individual measurements and the group mean $\pm$ SEM are reported (A, B, E, F). Kruskal-Wallis test was performed at the indicated time point comparing mice treated with or without CoB (p values are shown; C, D, E).

Figure 5

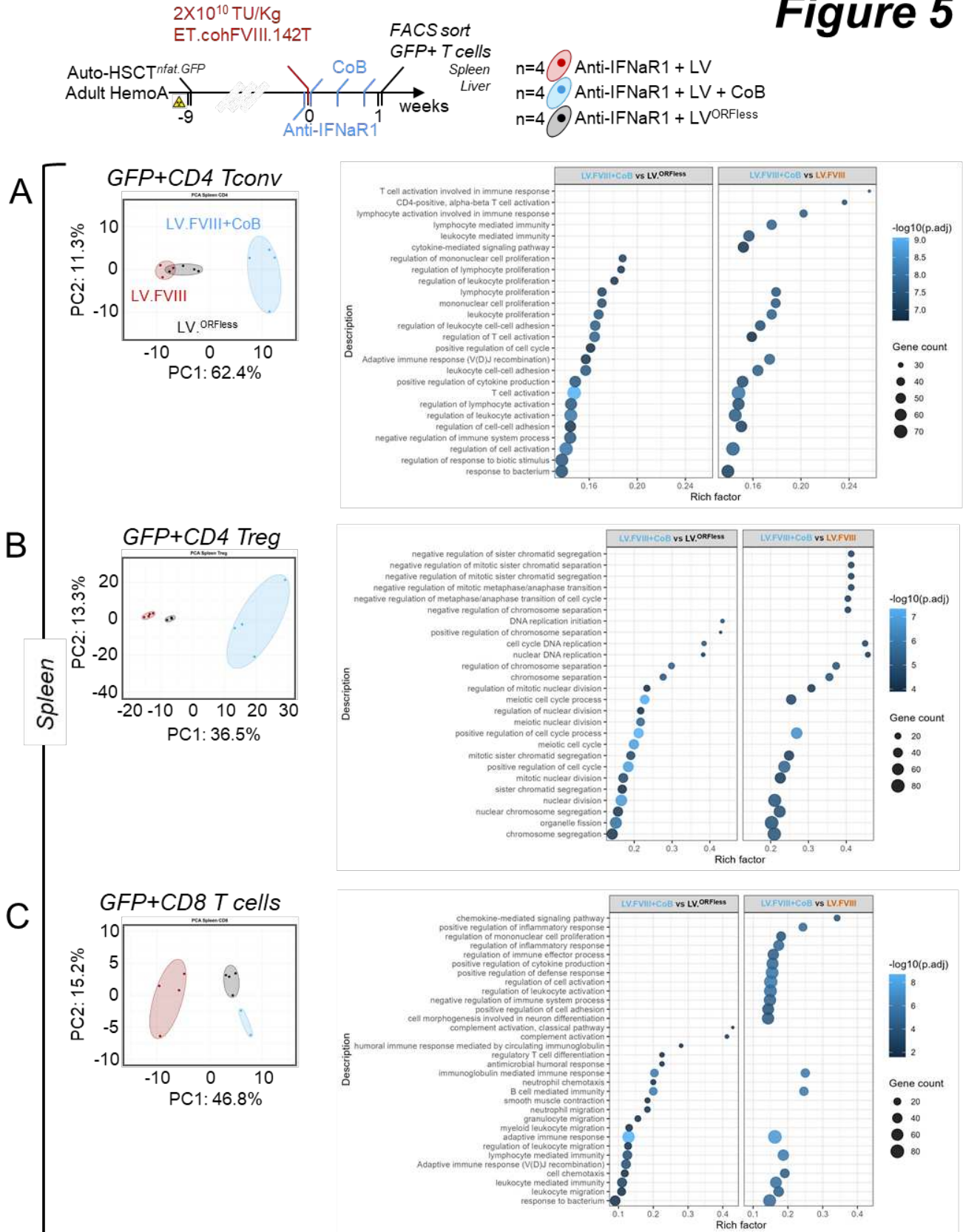


Figure 5. Co-stimulatory blockade (CoB) regimen alters the transcriptome of T cells activated by antigen recognition, delivered *in vivo* via LV-based gene therapy.

HemoA^{nfat.GFP} chimeric mice (n=12) received 2×10^{10} TU/kg of LV encoding for co.hFVIII (n=8) with (n=4) or without CoB (n=4), and controls were treated with 2×10^{10} TU/kg of LV^{ORFless} (n=4) not encoding for any transgene. One week post-LV injection, splenic GFP-expressing conventional (A), regulatory CD4 (B),

and CD8 (C) T cells were FACS-sorted for RNA sequencing. Filtered reads were aligned to the mouse reference genome, read counts were quantified, and differential gene expression analysis was performed. Genes with an adjusted P value (FDR) < 0.05 were considered differentially expressed. Principal component analysis and dot-plot reporting the top-20 biological processes downregulated (A-C) by CoB regimen compared to LV.FVIII or LV.ORFless are shown.

Figure 6

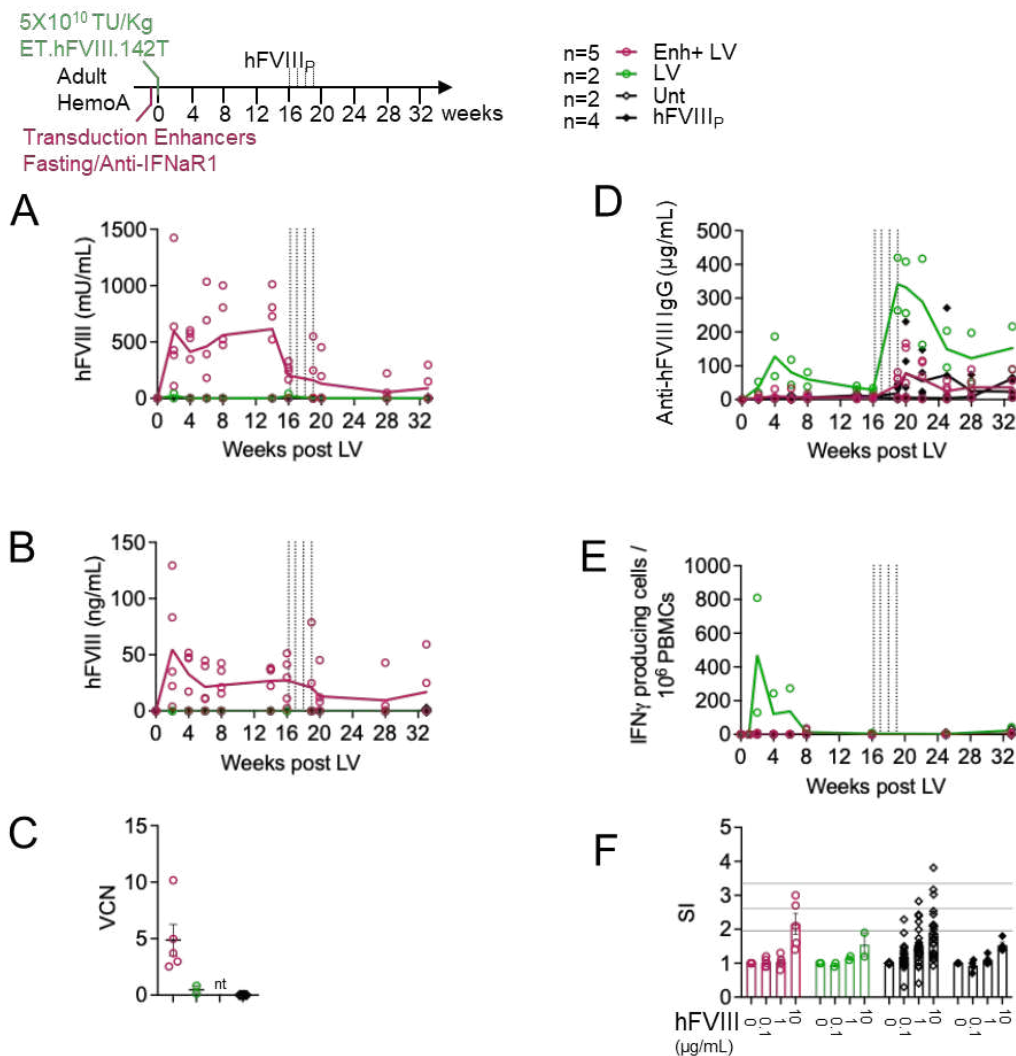


Figure 6. Application of sub-therapeutic FVIII-encoding sequences for *in vivo* gene therapy requires enhancers of transduction.

Eight-week-old adult HemoA mice were either fasted for 24 hours and treated with an anti-IFN α R1 mAb 3 hours before receiving LV.ET.hFVIII.142T (5×10^{10} TU/kg, n=5, dark pink open dots), or exclusively receiving LV.ET.hFVIII.142T (5×10^{10} TU/kg, n=2, dark green open dots). Control mice were left untreated (n=6, black open diamonds). Sixteen weeks after LV injection, hFVIII protein was administered i.v. (Re-facto 4×10^4 U, weekly) to LV-treated (n=7) and naïve HemoA (n=4, black diamonds) mice as a pro-immunogenic exposure to hFVIII, and mice were monitored for an additional 16-week period. Single values with connecting means of hFVIII activity (A) and concentration (B) in the plasma of HemoA mice, at the indicated time points after LV administration. VCN was evaluated in genomic DNA isolated from liver biopsies of each mouse at the indicated time points; individual data and the median LV copies are shown (C). hFVIII-specific immune responses were monitored at the indicated time points by determining anti-

FVIII IgG titer by ELISA (**D**), frequency of circulating IFN γ -releasing T cells by ELISPOT (**E**). hFVIII-driven proliferation of splenic T cells was measured by ^3H -thymidine incorporation (**F**) and reported as Stimulation Index (SI). Ag-specific proliferation is considered significant if $>$ than the mean SI of naïve HemoA mice (n=23) at the relevant Ag concentration. Individual measurements and the group mean $\pm$ SEM are reported (C, F).

Figure 7

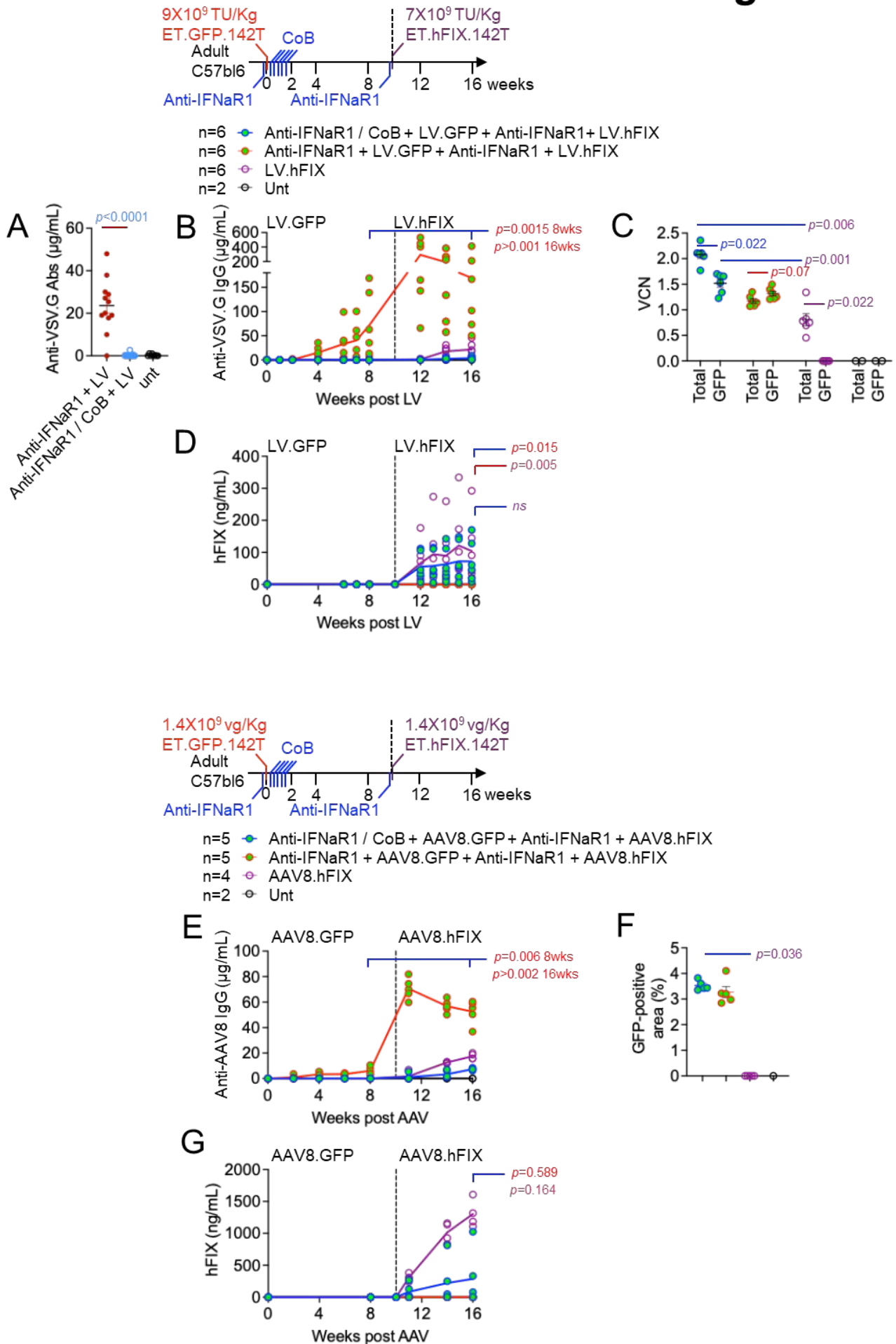


Figure 7. Co-stimulatory blockade (CoB) regimen allows viral vector re-dosing in immunocompetent mice.

Anti-VSV.G IgG titer was quantified by ELISA in plasma from HemoA mice receiving GT with or without CoB (A). Adult C57BL6 mice (n=12) received i.v. anti-IFN α R1- mAb 3 hours before injection of 9×10^9 TU/kg of LV.ET.GFP.142T. Half of them underwent the (CoB) regimen, consisting of 5 doses of anti-CD154 mAb and CTLA4-Ig, administered 2-3 days apart (n=6, blue open dots), while the other half remained untreated as controls (n=6, red open dots). Ten weeks after the first LV injection, all the previously treated mice, together with additional 5 naïve mice (n=6, dark pink open dots), received the anti-IFN α R1- mAb again and 7×10^9 TU/kg of LV.ET.hFIX.142T (n=18). Anti-VSV.G IgG titer was quantified by ELISA in plasma from at the indicated time points in mice from the above-described experimental groups (B). 16 weeks after the first LV dose, total liver VCN and VCN deriving specifically from GFP integrated copies, proxy of first transduction were evaluated (C). hFIX released in circulation was quantified by ELISA at the indicated time points before and after hFIX-encoding LV (D). An identical experimental scheme was repeated, providing AAV8.ET.GFP.142T first and AAV8.ET.hFIX.142T later. Anti-AAV8 capsid IgG titer was determined in plasma by ELISA (E). GFP expression was quantified as the area of the liver section analyzed by immunofluorescence (F). hFIX released in circulation was quantified by ELISA at the indicated time points before and after hFIX-encoding AAV8 (G). Individual measurements and the group mean $\pm$ SEM are reported (A, C, F). Kruskal-Wallis test was performed at the indicated time point comparing mice treated with or without CoB (*p* values are shown; A-G).

Figure 8

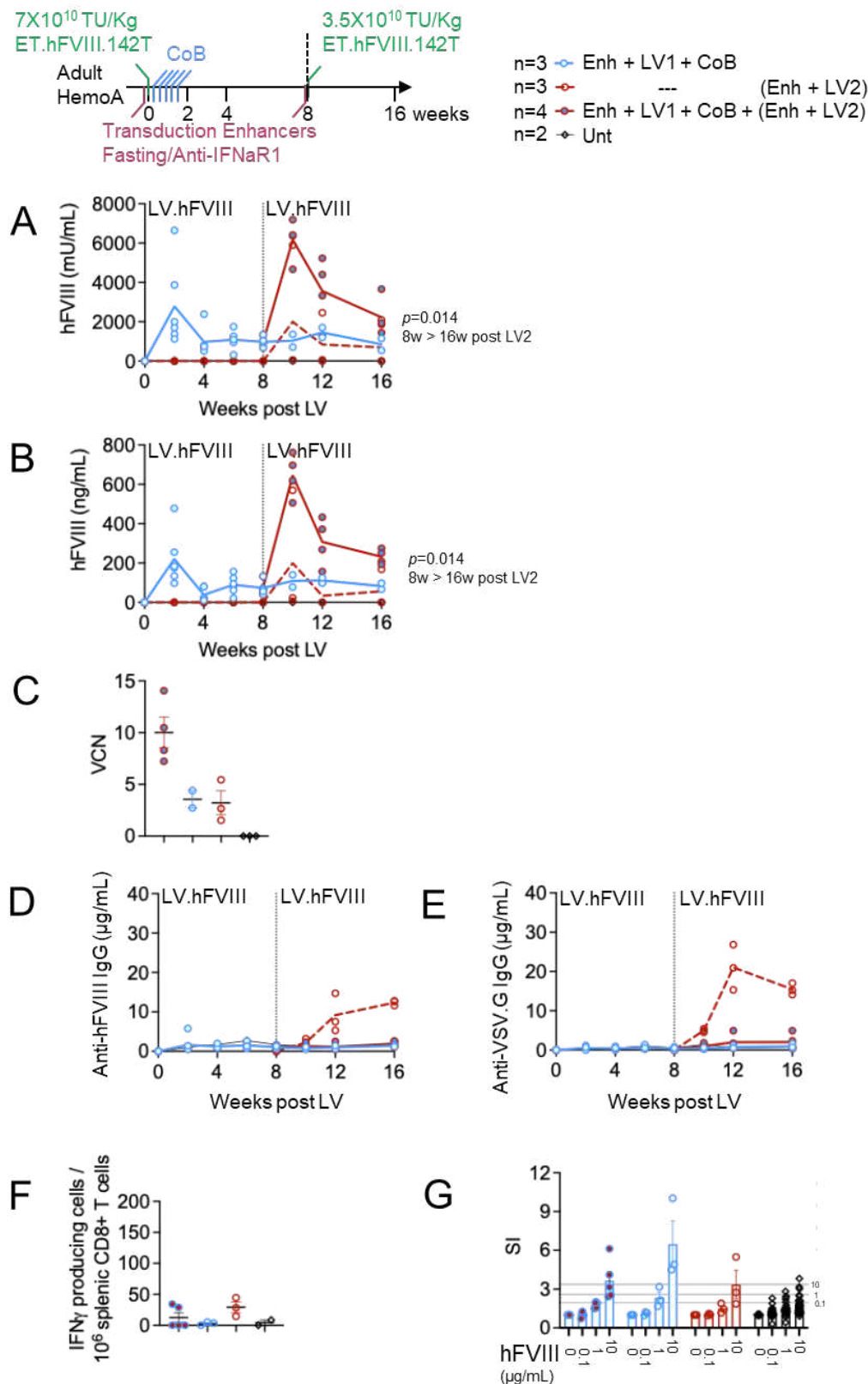


Figure 8. Co-stimulatory blockade (CoB) regimen allows viral vector re-dosing in immunocompetent mice.

Twenty-four-hour-fasted adult HemoA mice (n=8, cerulean open dots) received anti-IFN α R1 mAb (transduction enhancers) 3 hours before 7×10^{10} TU/kg of hFVIII encoding LV (LV1). Eight weeks post the first LV1, LV1-treated HemoA mice (n=4, cerulean dots), and naïve age-matched (n=3, brown open dots) were systemically injected 3.5×10^{10} TU/kg. Animals were monitored for 16 weeks to determine hFVIII

activity by chromogenic assay (**A**), and plasmatic concentration (**B**), vector copies in the liver (**C**), titer of anti-hFVIII (**D**) and anti-VSV.G IgG by ELISA (**E**). Frequency of circulating IFN γ -releasing T cells by ELISPOT (**F**), and proliferation of splenic T cells by ^3H -thymidine incorporation (**G**) were determined by hFVIII *in vitro* restimulation and reported as Stimulation Index (SI). Ag-specific proliferation is considered significant if $>$ than the mean SI of naïve HemoA mice (n=23) at the relevant Ag concentration. Individual measurements and the group mean $\pm$ SEM are reported (C, F, G). The Mann-Whitney paired test was performed to compare mice pre- and post-second LV infusion (*p*-values are shown; A, B).

SUPPLEMENTARY MATERIALS

Transient costimulatory blockade overcomes immune barriers to durable and redosable liver-directed gene therapy

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Supplementary Figure 1. hFVIII sequence variants to improve translation and half-life impact on immunogenicity of in vivo Gene Therapy.

Supplementary Figure 2. Interferon alpha-mediated innate signaling blockade is not effective in controlling anti-hFVIII immunity in 2-week-old HemoA mice.

Supplementary Figure 3. Anti-CD3-mediated T cell inactivation temporarily protects hFVIII-expressing hepatocytes from immune-mediated clearance in 2-week-old HemoA mice.

Supplementary Figure 4. Transient B cell depletion modulates anti-hFVIII immunity, allowing GT-driven hFVIII expression to persist in 2-week-old HemoA mice.

Supplementary Figure 5. Transient B cell depletion failed in modulating anti-hFVIII immunity driven by in vivo GT in adult HemoA mice.

Supplementary Figure 6. Co-stimulatory blockade (CoB) regimen impedes immune-mediated clearance of co.hFVIII-expressing hepatocytes after in vivo GT in 2-week-old HemoA mice.

Supplementary figure 7. Co-stimulatory blockade (CoB) regimen impedes immune-mediated clearance of co.hFVIII.XTEN-expressing hepatocytes after in vivo GT into adult HemoA mice.

Supplementary figure 8. Immunophenotype of circulating T cells in the circulation after hemophilia A gene therapy in mice receiving CoB immunomodulatory regimen.

Supplementary figure 9. Strategy for the isolation of T cells activated in vivo by LV gene therapy.

Supplementary figure 10. Unsupervised immunophenotypic analysis of splenic activated CD4 T cells after LV gene therapy.

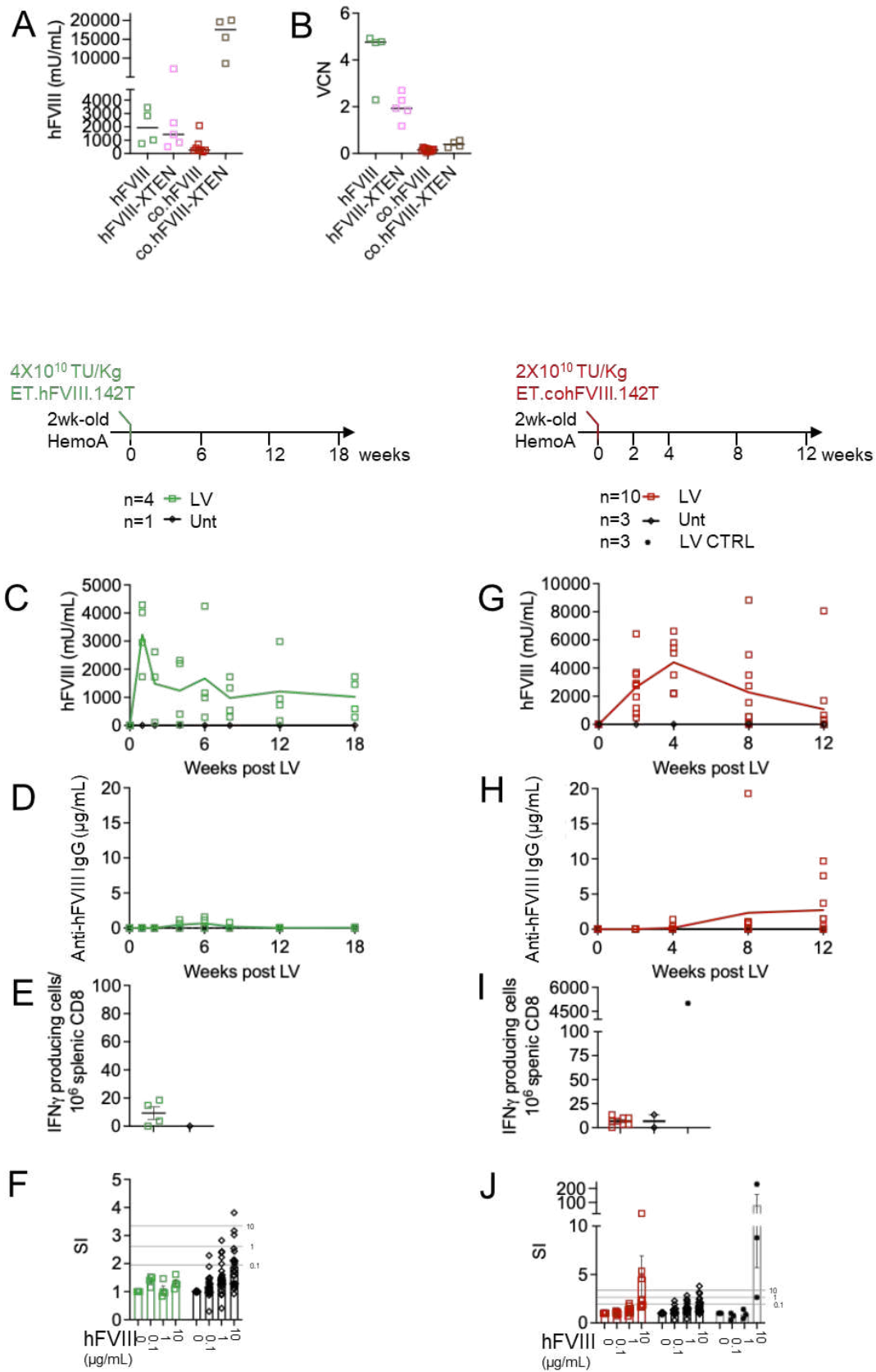
Supplementary figure 11. Unsupervised immunophenotypic analysis of splenic activated CD8 T cells after LV gene therapy.

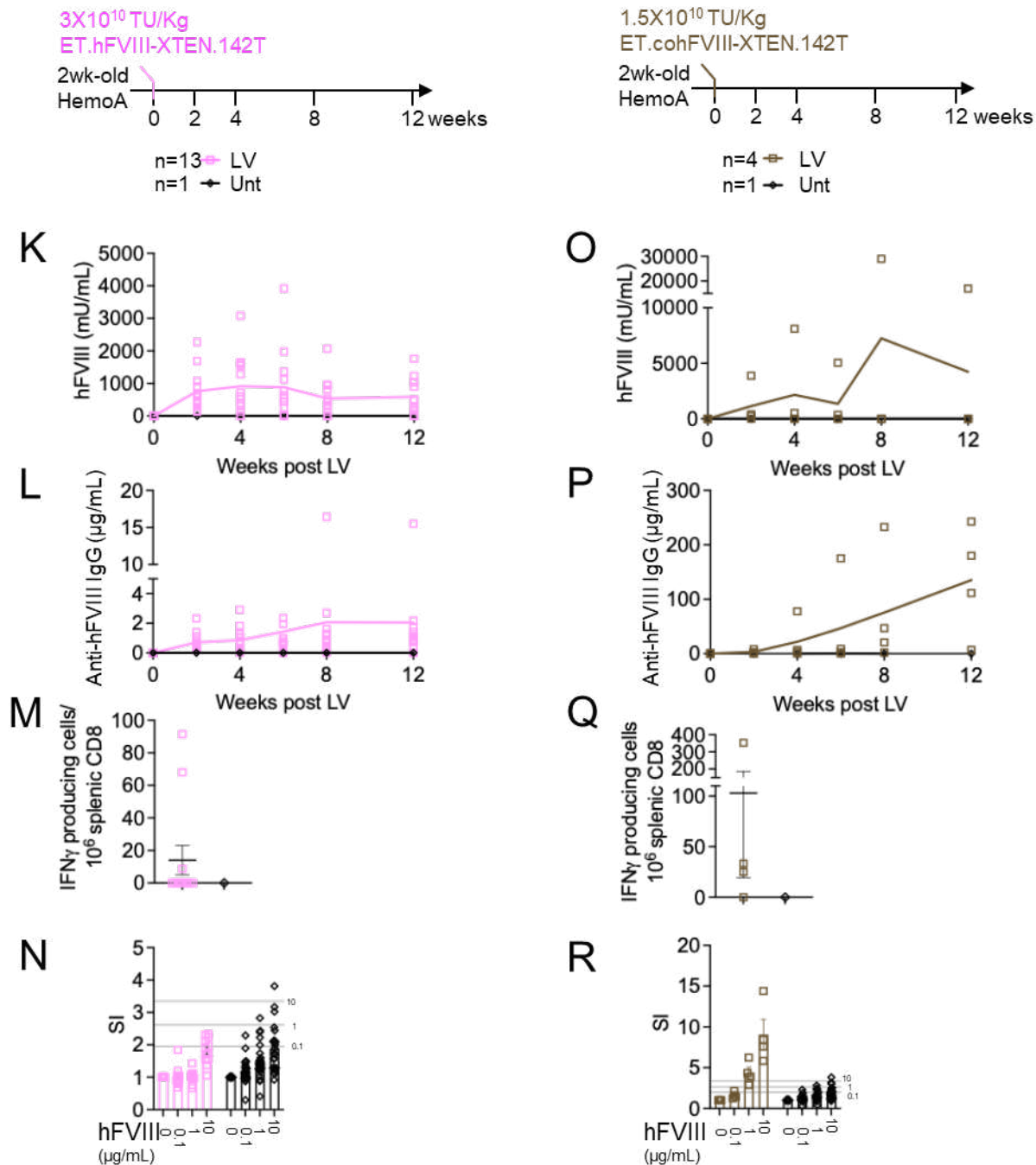
Supplementary figure 12. Unsupervised immunophenotypic analysis of liver-infiltrating activated CD4 T cells after LV gene therapy.

Supplementary figure 13. Unsupervised immunophenotypic analysis of liver-infiltrating activated CD8 T cells after LV gene therapy.

Supplementary figure 14. Co-stimulatory blockade (CoB) regimen alters the transcriptome of T cells activated by antigen recognition, delivered in vivo via LV-based gene therapy.

Supplementary Figure 1

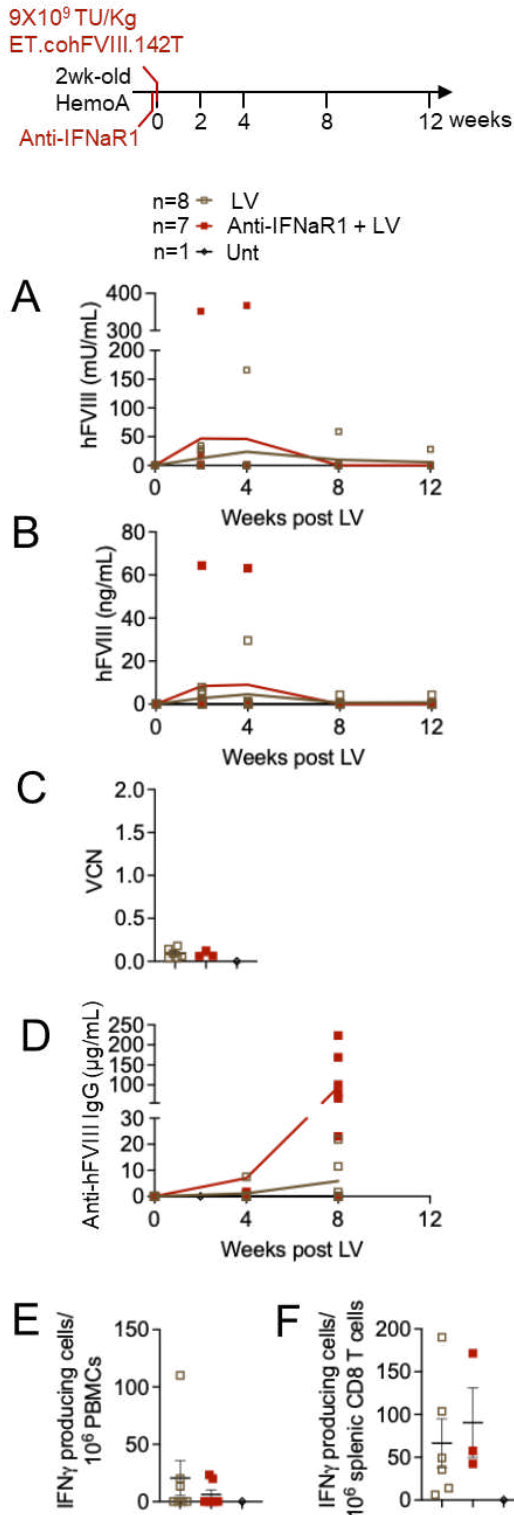




Supplementary Figure 1. hFVIII sequence variants to improve translation and half-life impact on immunogenicity of *in vivo* Gene Therapy.

Activity of hFVIII in plasma and VCN, determined at the end of follow-up of immunodeficient HemoA.Rag mice treated with all vector variants at the same dose as the immunocompetent counterpart are reported (A, B) as source data of Figure 1A. Two-week-old HemoA mice were intravenously (i.v.) injected with LV.ET.hFVIII.142T (4×10^{10} TU/Kg, n=4) (C-F), LV.ET.cohFVIII.142T (2×10^{10} TU/kg, n = 8, LV.PGK.hFVIII immunogenic control, LV CTRL n=3) (G-J), LV.ET.hFVIII-XTEN.142T (3×10^{10} TU/kg, n = 13) (K-N), or LV.ET.cohFVIII-XTEN.142T (1.5×10^{10} TU/kg, n = 4) (O-R) or left untreated as control. To complete the data shown in Figure 1, plasmatic hFVIII activity (C, G, K, O), titer of anti-hFVIII IgG (D, H, L, P), frequency of FVIII-specific IFN γ -releasing CD8 T cells by ELISPOT (E, I, M, Q), and FVIII-driven cell proliferation by ³H-Thymidine incorporation are reported for each LV.FVIII variant (F, J, N, R). T cell proliferation is expressed as Stimulation Index, $SI = [(\text{mean CPM Ag stimulated triplicate}) : (\text{mean CPM unstimulated triplicate})]$. Ag-specific proliferation is considered significant if > than the mean SI of naïve HemoA mice (n=23) at the relevant Ag concentration.

Supplementary Figure 2

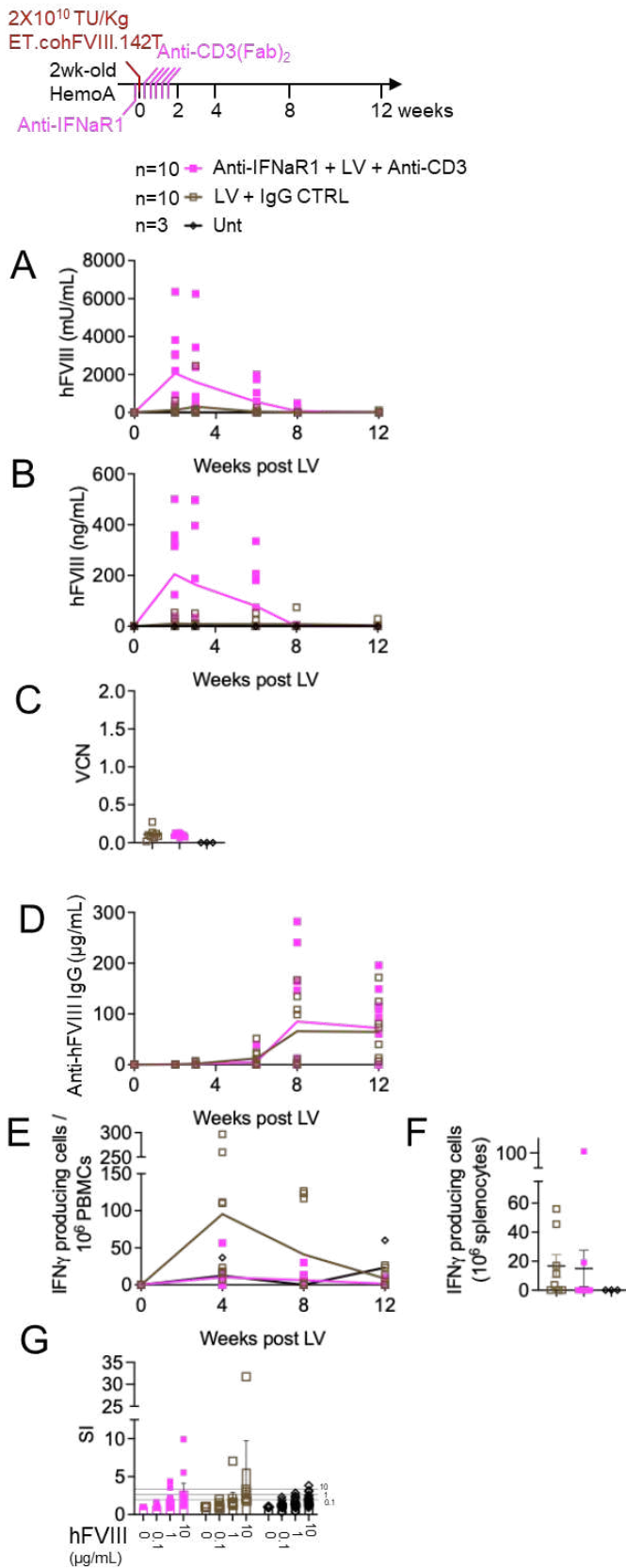


Supplementary Figure 2. Interferon alpha-mediated innate signaling blockade is not effective in controlling anti-hFVIII immunity in 2-week-old HemoA mice.

A cohort of 2-week-old HemoA mice was i.v. injected with 9x10⁹ TU/kg of LV.ET.cohFVIII.142T (n=15), and a subset (n=7) was pre-treated with an anti-IFNaR1-blocking monoclonal antibody (mAb). Therapeutic efficacy of LV.cohFVIII was evaluated by measuring plasma FVIII activity and concentration (**A**, **B**). Integrated VCN was assessed in genomic DNA isolated from liver biopsies of each mouse 12 weeks after LV injection; individual data and the median LV copy number are shown (**C**). hFVIII-specific immune responses were monitored at the indicated time points by determining anti-hFVIII IgG titer by ELISA (**D**),

and by measuring the frequency of circulating (E) and splenic (F) IFN γ -releasing T cells upon in vitro hFVIII restimulation by ELISPOT. Individual values and the group mean $\pm$ SEM are reported.

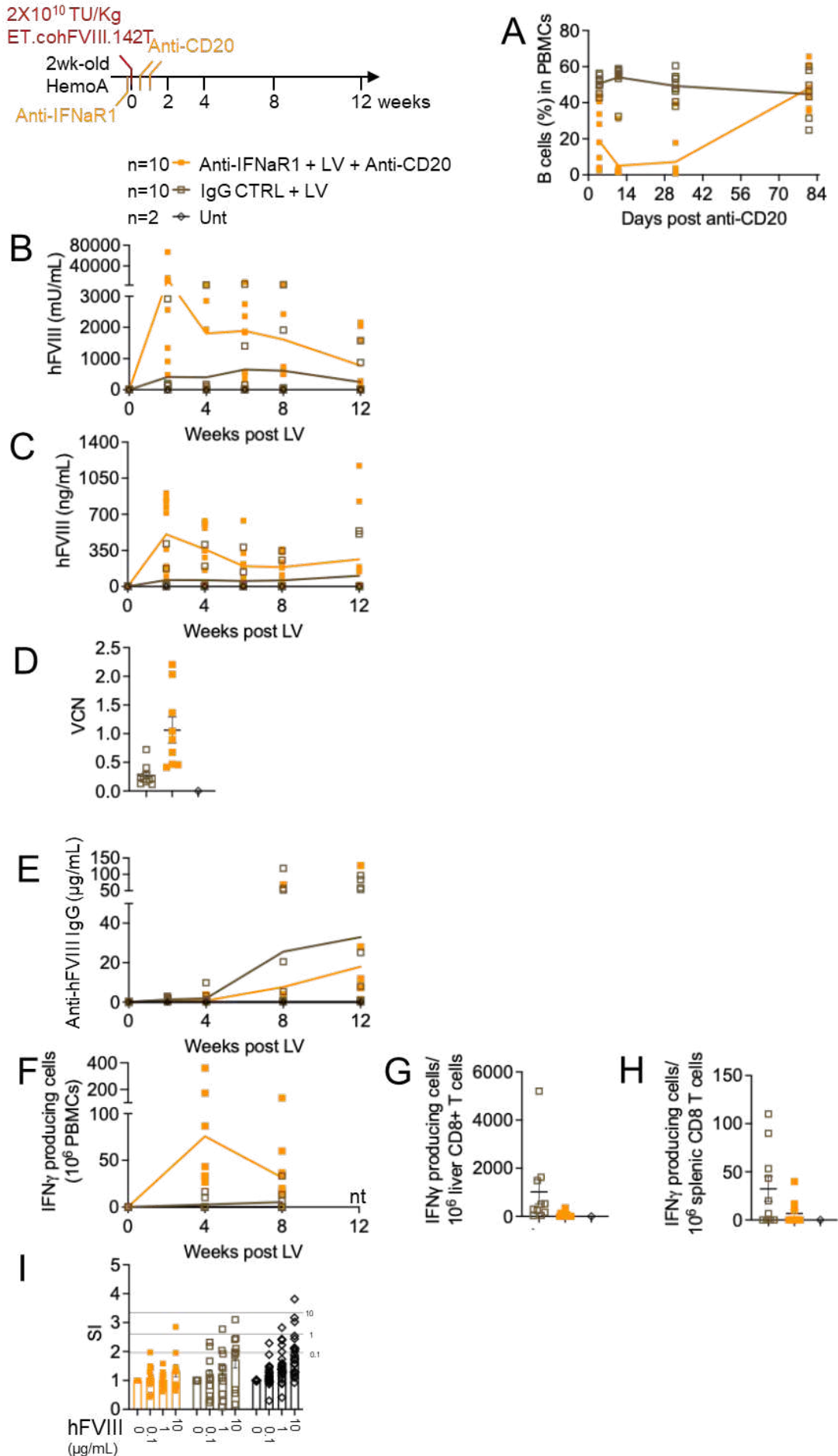
Supplementary Figure 3



Supplementary Figure 3. Anti-CD3-mediated T cell inactivation temporarily protects hFVIII-expressing hepatocytes from immune-mediated clearance in 2-week-old HemoA mice.

Two-week-old HemoA mice were pretreated i.v. with anti-IFN α 1 mAb or IgG control and *iv* injected with 2×10^{10} TU/kg of LV.ET.cohFVIII.142T (n=20). Mice receiving IFN α 1 mAb, starting day 3 post-LV injection, received 5 doses of anti-CD3F(ab)₂ depleting/inactivating mAb, 2-3 days apart. Therapeutic efficacy of LV.cohFVIII was evaluated in anti-CD3F(ab)₂-treated and control mice by measuring plasma FVIII activity and concentration (**A, B**). Integrated VCN was assessed in genomic DNA isolated from liver biopsies of each mouse 12 weeks post-LV injection; individual data and the median LV copy number are shown (**C**). hFVIII-specific immune responses were monitored at the indicated time points by determining anti-hFVIII IgG titer by ELISA (**D**), frequency of circulating (**E**) and splenic CD8 (**F**) IFN γ -releasing T cells by ELISPOT. hFVIII-driven proliferation of splenic T cells was measured by ³H-thymidine incorporation and reported as Stimulation Index (SI) (**G**). Ag-specific proliferation is considered significant if > than the mean SI of naïve HemoA mice (n=23) at the relevant Ag concentration. Individual values and the group mean $\pm$ SEM are reported.

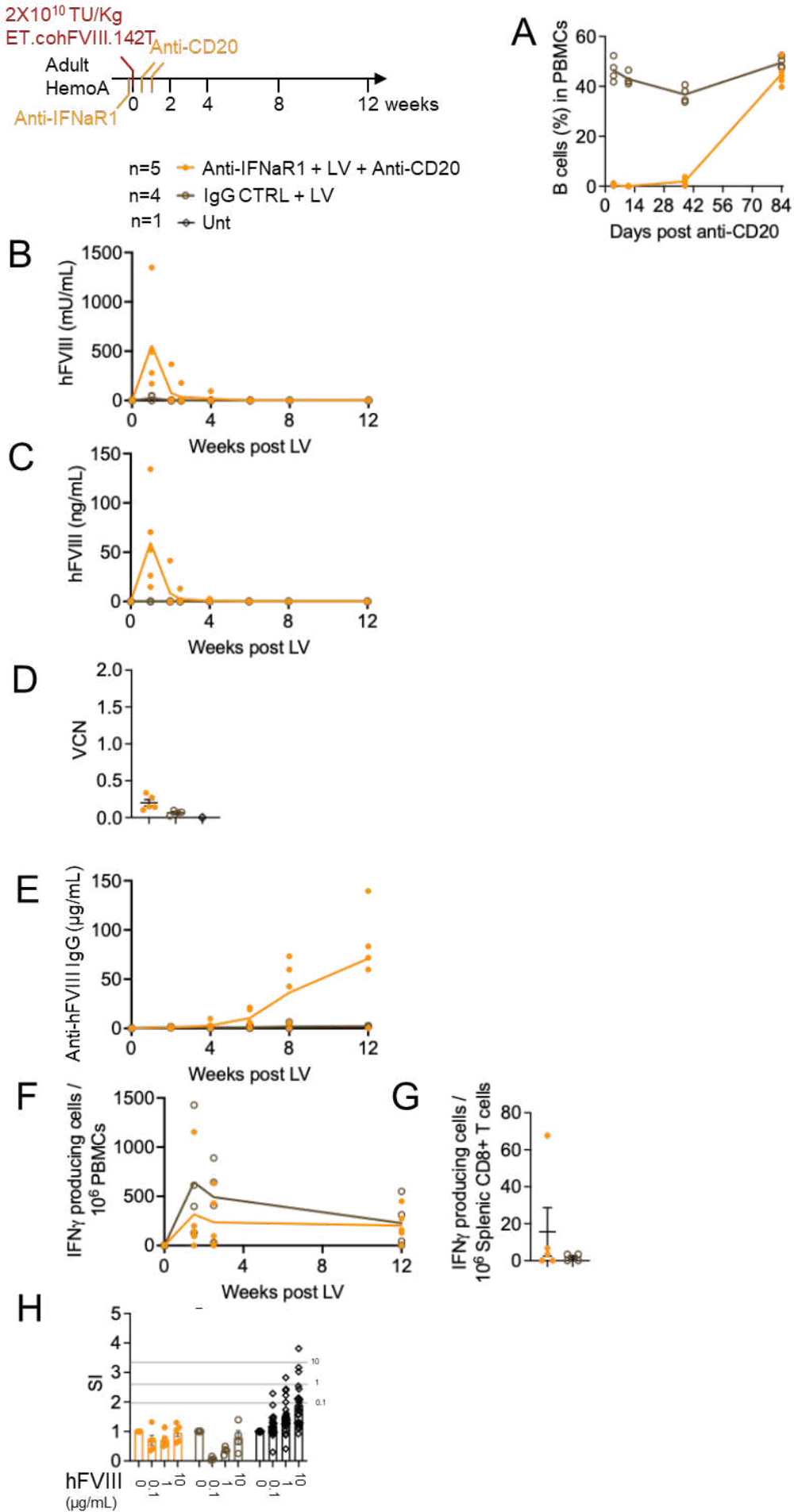
Supplementary Figure 4



Supplementary Figure 4. Transient B cell depletion modulates anti-hFVIII immunity, allowing GT-driven hFVIII expression to persist in 2-week-old HemoA mice.

Anti-CD20 mAb-pre-treated 2-week-old HemoA mice were systemically injected with 2×10^{10} TU/kg of LV.ET.cohFVIII.142T (n=20), and half of them, starting from day 3 post-LV injection, received twice anti-CD20 depleting mAb, 7 days apart. B cell depletion was monitored in peripheral blood by FACS, and the frequency of CD19+CD45R.B220+ B cells was determined at the indicated time points (**A**). Therapeutic efficacy of LV.cohFVIII was evaluated in anti-CD20-treated and control mice by measuring plasma hFVIII activity and concentration (**B**, **C**). Integrated VCN was assessed in genomic DNA isolated from liver biopsies of each mouse 12 weeks post-LV injection (**D**). hFVIII-specific immune responses were monitored at the indicated time points by determining anti-hFVIII IgG titer by ELISA (**E**), frequency of circulating (**F**), hepatic (**G**), and splenic (**H**), CD8 IFN γ -releasing T cells by ELISPOT. IFN γ -releasing T cells by ELISPOT. hFVIII-driven proliferation of splenic T cells was measured by ^3H -thymidine incorporation and reported as Stimulation Index (SI) (**I**). Ag-specific proliferation is considered significant if $>$ than the mean SI of naïve HemoA mice (n=23) at the relevant Ag concentration. Individual values and the group mean $\pm$ SEM are reported.

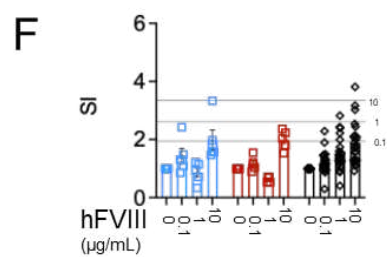
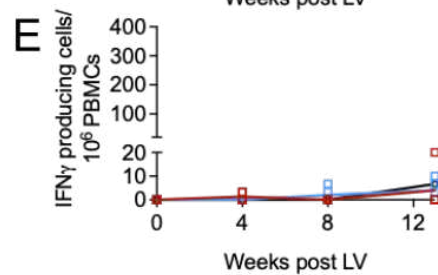
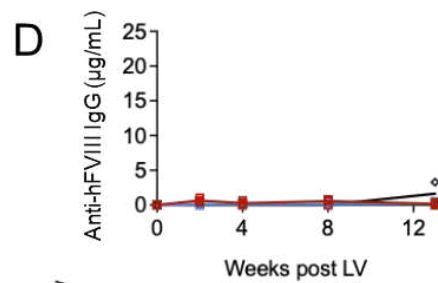
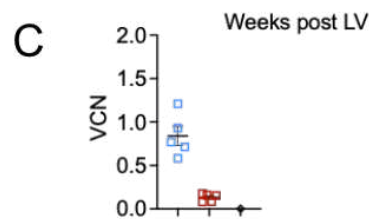
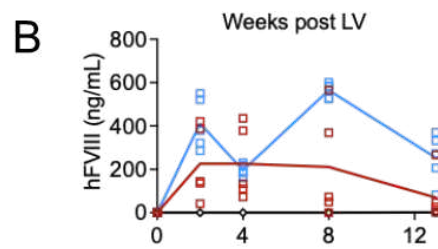
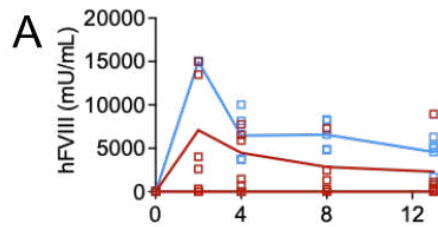
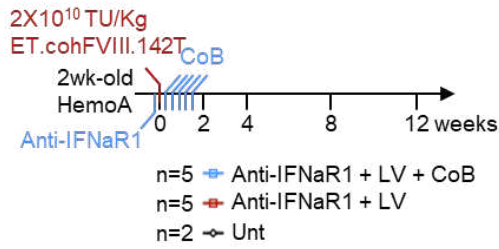
Supplementary Figure 5



Supplementary Figure 5. Transient B cell depletion failed in modulating anti-hFVIII immunity driven by *in vivo* GT in adult HemoA mice.

Anti-CD20 mAb-pre-treated adult HemoA mice were i.v. injected with 2×10^{10} TU/kg of LV.ET.cohFVIII.142T (n=9). On day 3 post-LV injection, anti-CD20 depleting mAb was injected twice, 7 days apart (n=5), or isotype IgG as control (n=4). B cell depletion was monitored in peripheral blood, and the frequency of CD19+CD45R.B220+ B cells was determined at the indicated time points (**A**). Therapeutic efficacy of LV.cohFVIII was evaluated in anti-CD20-treated and control mice by measuring plasma hFVIII activity and concentration (**B, C**). Integrated VCN was assessed in genomic DNA isolated from liver biopsies of each mouse 12 weeks post-LV injection (**D**). hFVIII-specific immune responses were monitored at the indicated time points by determining anti-hFVIII IgG titer by ELISA (**E**), frequency of circulating (**F**), and splenic (**G**), CD8 IFN γ -releasing T cells by ELISPOT. IFN γ -releasing T cells by ELISPOT. hFVIII-driven proliferation of splenic T cells was measured by ^3H -thymidine incorporation and reported as Stimulation Index (SI) (**H**). Ag-specific proliferation is considered significant if $>$ than the mean SI of naïve HemoA mice (n=23) at the relevant Ag concentration. Individual values and the group mean $\pm$ SEM are reported.

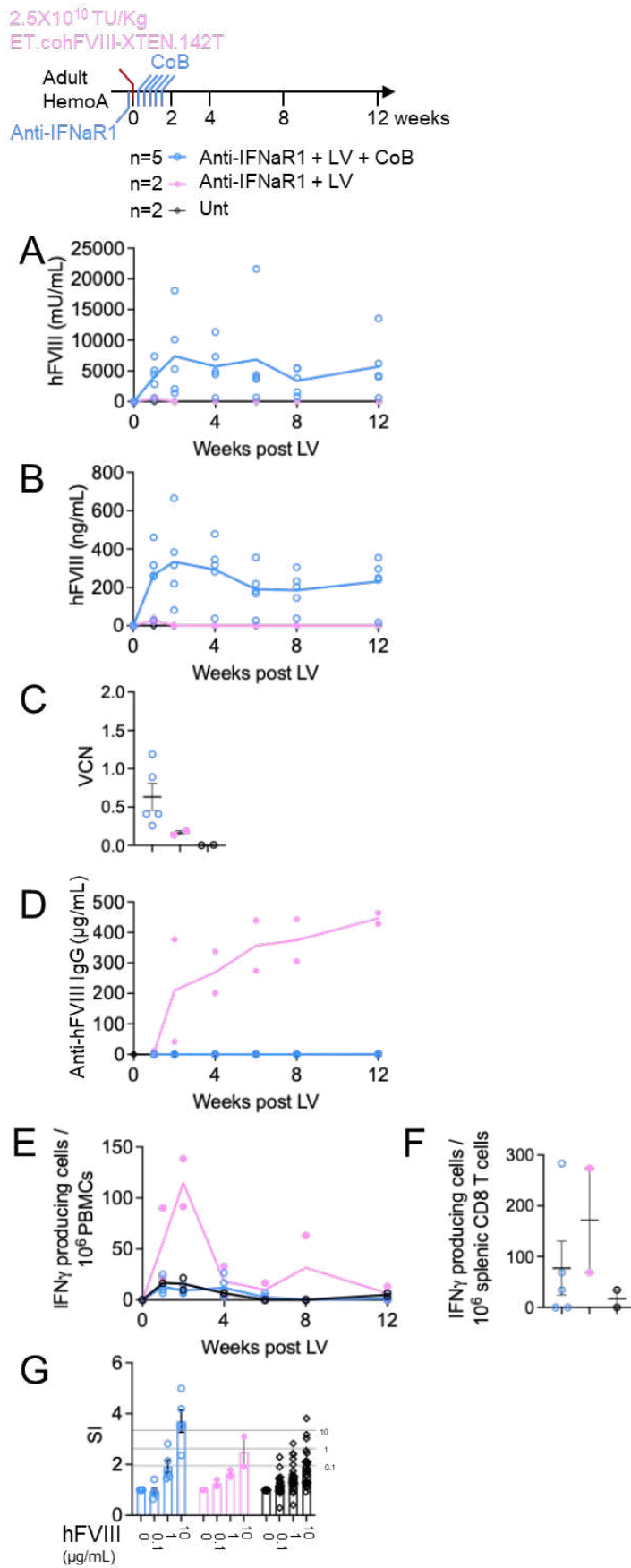
Supplementary Figure 6



Supplementary Figure 6. Co-stimulatory blockade (CoB) regimen impedes immune-mediated clearance of co.hFVIII-expressing hepatocytes after *in vivo* GT in 2-week-old HemoA mice.

Two-week-old HemoA mice (n=10) were treated with an anti-IFN α R1 mAb blocking mAb 3 hours before receiving i.v. a 2×10^{10} TU/kg dose of LV.ET.cohFVIII.142T. A co-stimulatory blockade (CoB) regimen, consisting of 5 doses of anti-CD40L and CTLA4-Ig, was then administered 2-3 days apart (n=14). Activity (A) and concentration (B) of hFVIII released by hepatocytes were evaluated in plasma at the indicated time points; VCN was evaluated in genomic DNA isolated from liver biopsies of each mouse at 12 weeks post LV injection (C). hFVIII-specific immune responses were monitored at the indicated time points by determining anti-FVIII IgG titer by ELISA (D), frequency of circulating (E) and splenic CD8 (F) IFN γ -releasing T cells by ELISPOT. IFN γ -releasing T cells by ELISPOT. hFVIII-driven proliferation of splenic T cells was measured by ^3H -thymidine incorporation and reported as Stimulation Index (SI) (G). Ag-specific proliferation is considered significant if > than the mean SI of naïve HemoA mice (n=23) at the relevant Ag concentration. Individual values and the group mean $\pm$ SEM are reported.

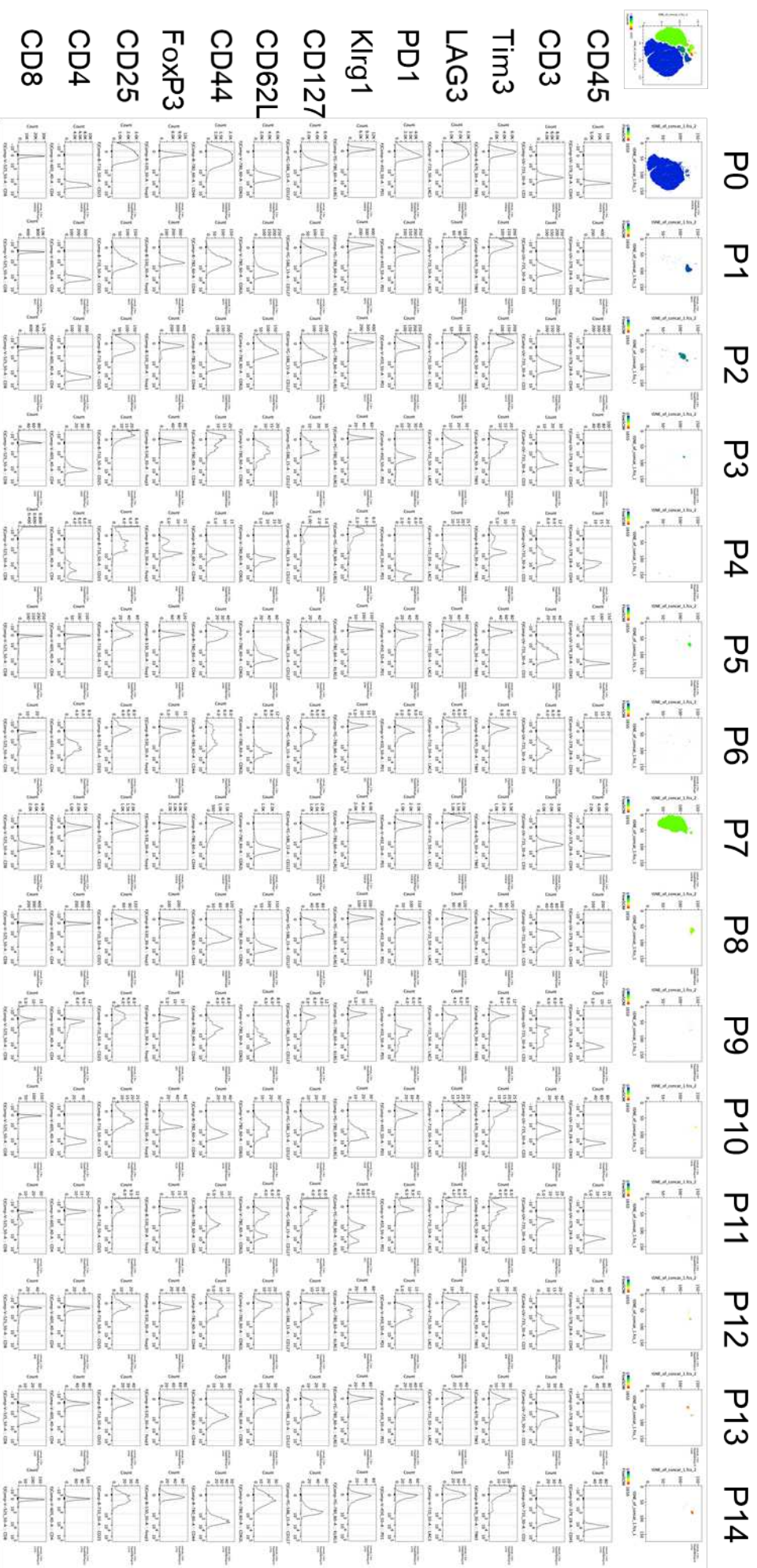
Supplementary Figure 7



Supplementary figure 7. Co-stimulatory blockade (CoB) regimen impedes immune-mediated clearance of co.hFVIII.XTEN-expressing hepatocytes after *in vivo* GT into adult HemoA mice.

Eight-week-old adult HemoA mice (n=7) were treated with an anti-IFN α R1 mAb blocking mAb 3 hours before receiving i.v. a 2.5×10^{10} TU/kg dose of LV.ET.cohFVIII.XTEN.142T. A co-stimulatory blockade (CoB) regimen, consisting of 5 doses of anti-CD40L and CTLA4-Ig, was then administered 2-3 days apart (n=5). Activity (**A**) and concentration (**B**) of hFVIII released by hepatocytes were evaluated in plasma at the indicated time points; VCN was evaluated in genomic DNA isolated from liver biopsies of each mouse at 12 weeks post LV injection (**C**). hFVIII-specific immune responses were monitored at the indicated time points by determining anti-FVIII IgG titer by ELISA (**D**), frequency of circulating (**E**) and splenic CD8 (**F**) IFN γ -releasing T cells by ELISPOT. IFN γ -releasing T cells by ELISPOT. hFVIII-driven proliferation of splenic T cells was measured by ^3H -thymidine incorporation and reported as Stimulation Index (SI) (**G**). Ag-specific proliferation is considered significant if > than the mean SI of naïve HemoA mice (n=23) at the relevant Ag concentration. Individual values and the group mean $\pm$ SEM are reported.

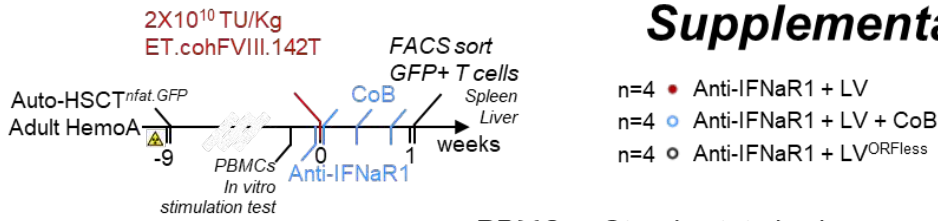
Supplementary Figure 8



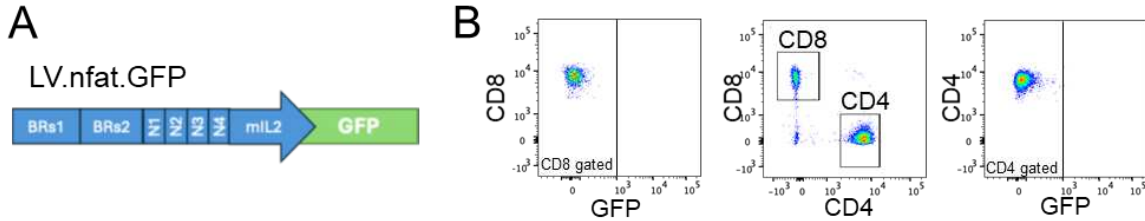
Supplementary figure 8. Immunophenotype of circulating T cells in the circulation after hemophilia A gene therapy in mice receiving CoB immunomodulatory regimen.

Multiparametric analysis was performed on single-cell immunophenotypic data from circulating T cells using FACS. Unsupervised identification of T cell subpopulations was performed in silico. The single-parameter fluorescence table for each population is reported.

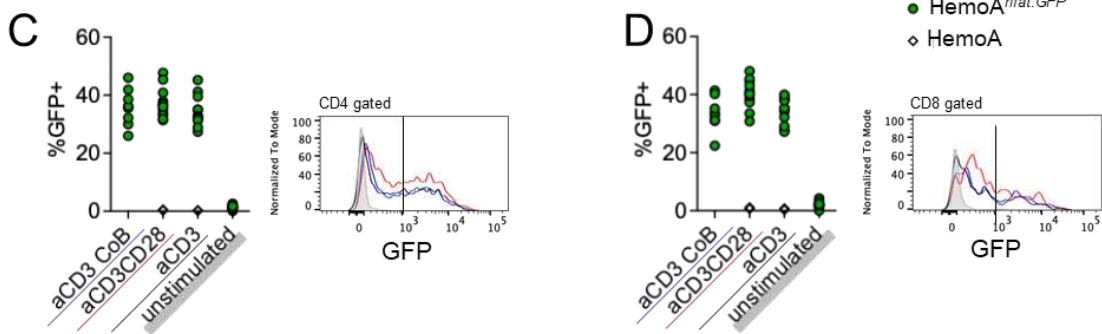
Supplementary Figure 9



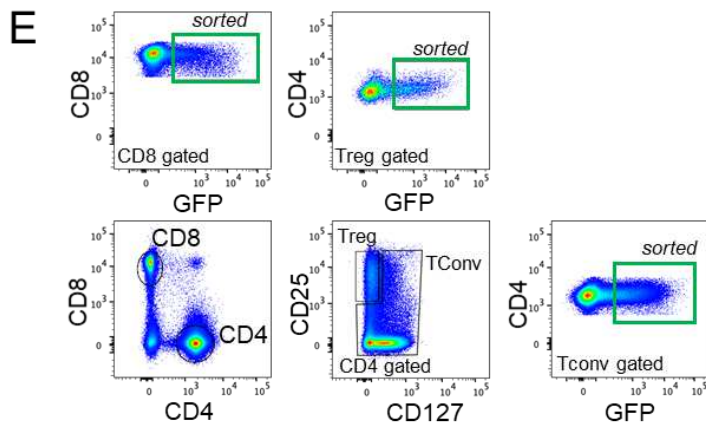
PBMCs - Steady state in vivo



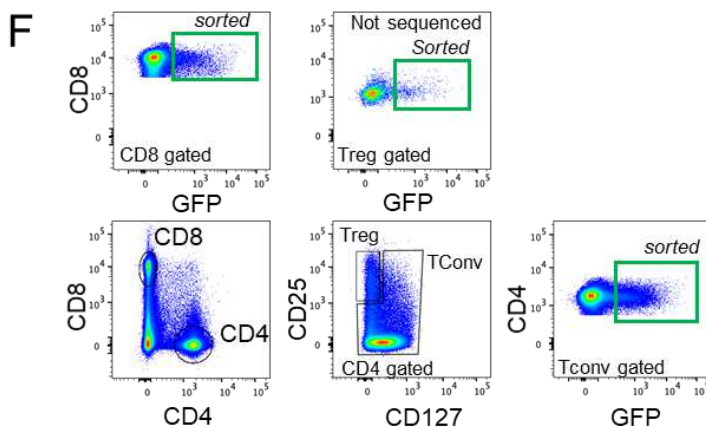
PBMCs - Activated in vitro



Splenic T cells - 7 days post LV - Activated in vivo

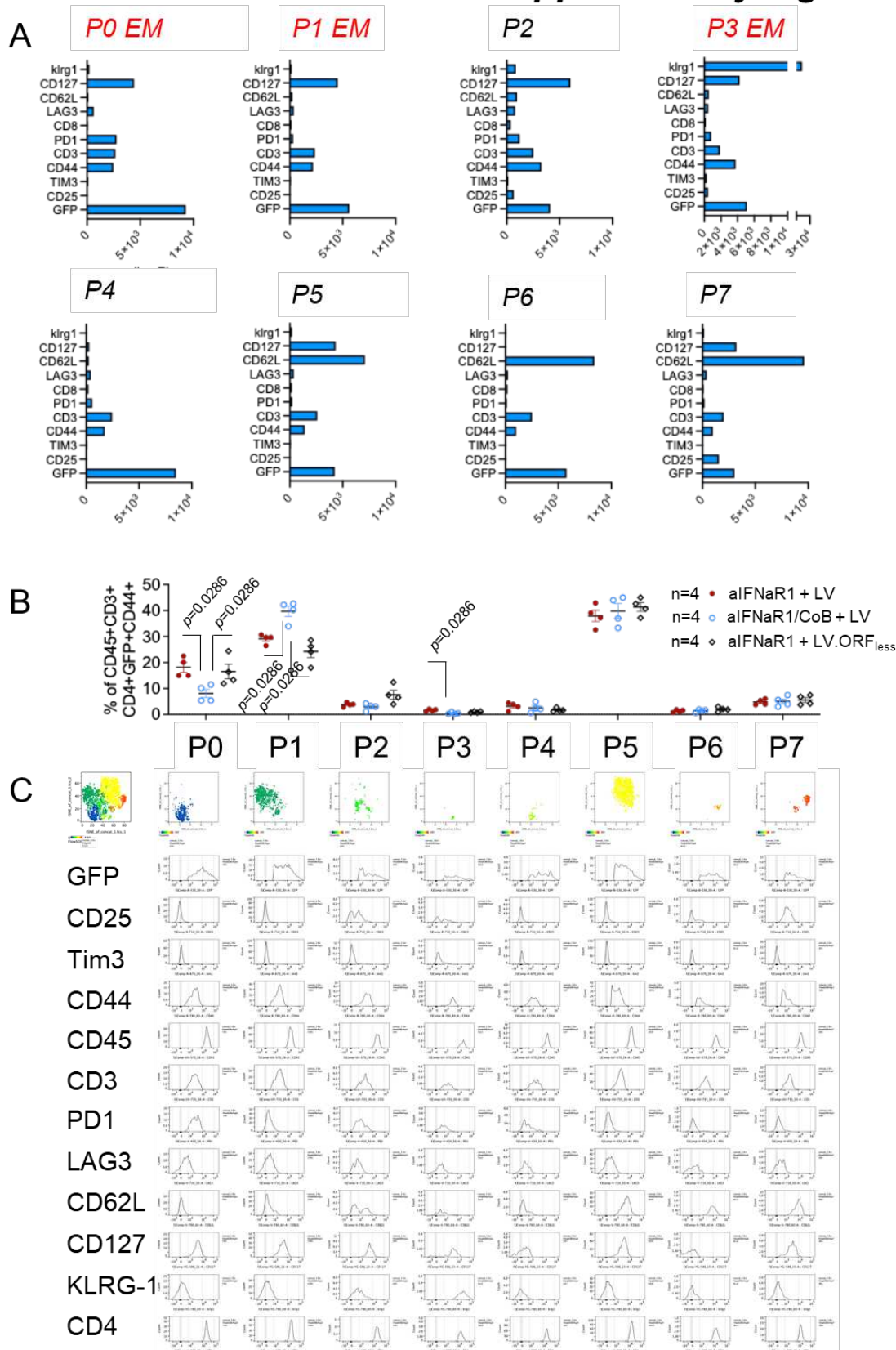


Liver infiltrating T cells - 7 days post LV - Activated in vivo



Supplementary figure 9. Strategy for the isolation of T cells activated *in vivo* by LV gene therapy.

Hemo A mice (n=12) were lethally irradiated and transplanted with autologous bone marrow-derived lineage-negative cells (Lin-) *in vitro* transduced with LV encoding for GFP under the control of a minimal murine interleukin-2 (mIL2) promoter induced by nuclear factor of activated T cell (NFAT) transcription factor following antigen recognition via T cell receptor (TCR). The synthetic NFAT-responsive promoter includes two Background Reduction signals (BRs), and 4 repeated NFAT binding sequences (N1, N2, N3, N4) upstream of the mIL2 promoter (A)³⁰. At complete immune reconstitution, 9 weeks post Lin^{-nfatGFP} transplantation, mice were bled to isolate circulating T cells. Minimal basal GFP expression by circulating T cells was evaluated by FACS at steady-state conditions (B). GFP expression driven by TCR signaling in *ex vivo* isolated T cells was tested *in vitro* by stimulating circulating T cells with anti-CD3 alone, or in the presence of anti-CD28 or CoB, or maintained in culture without stimulation as a control (C, D). GFP expression by CD4 and CD8 T cells in each culture condition of one representative HemoA^{nfat.GFP} chimeric mouse (C, D) and frequency of GFP-expressing T cells for all chimeric mice are reported (D, F). Circulating T cells from HemoA were stimulated with anti-CD3 ± anti-CD28 as a negative control.

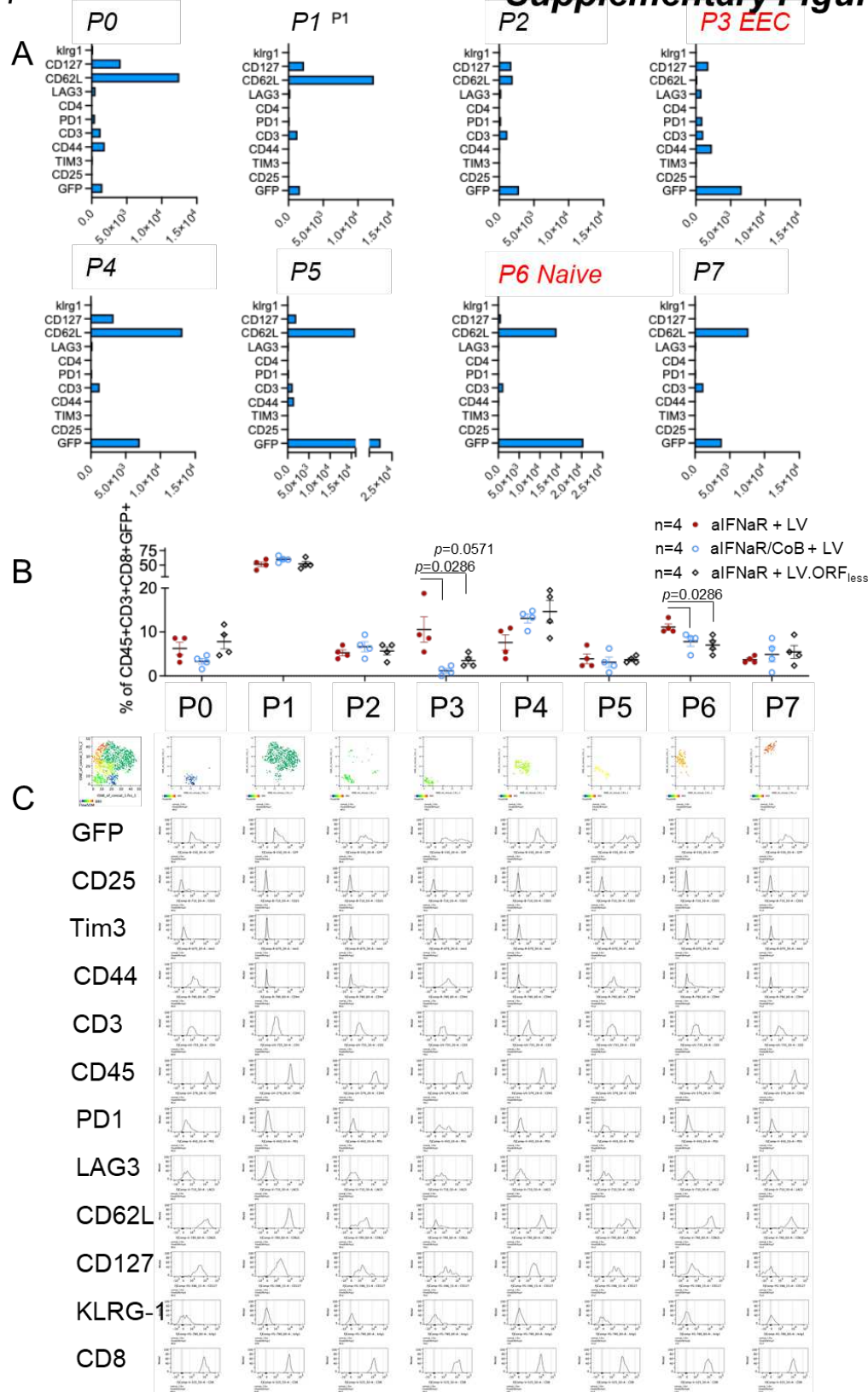


Supplementary figure 10. Unsupervised immunophenotypic analysis of splenic activated CD4 T cells after LV gene therapy.

Multiparametric analysis was performed on single-cell immunophenotypic data from splenic GFP-labeled CD4 T cells using FACS. Unsupervised identification of T cell subpopulations was performed in silico. Geometric mean fluorescence for each parameter across the population is shown in histograms (A), and

frequencies for each mouse and the group mean are shown in (B). The single-parameter fluorescence table (C) for each population is also reported.

Splenic *nfat.GFP+CD8+ T cells* **Supplementary Figure 11**



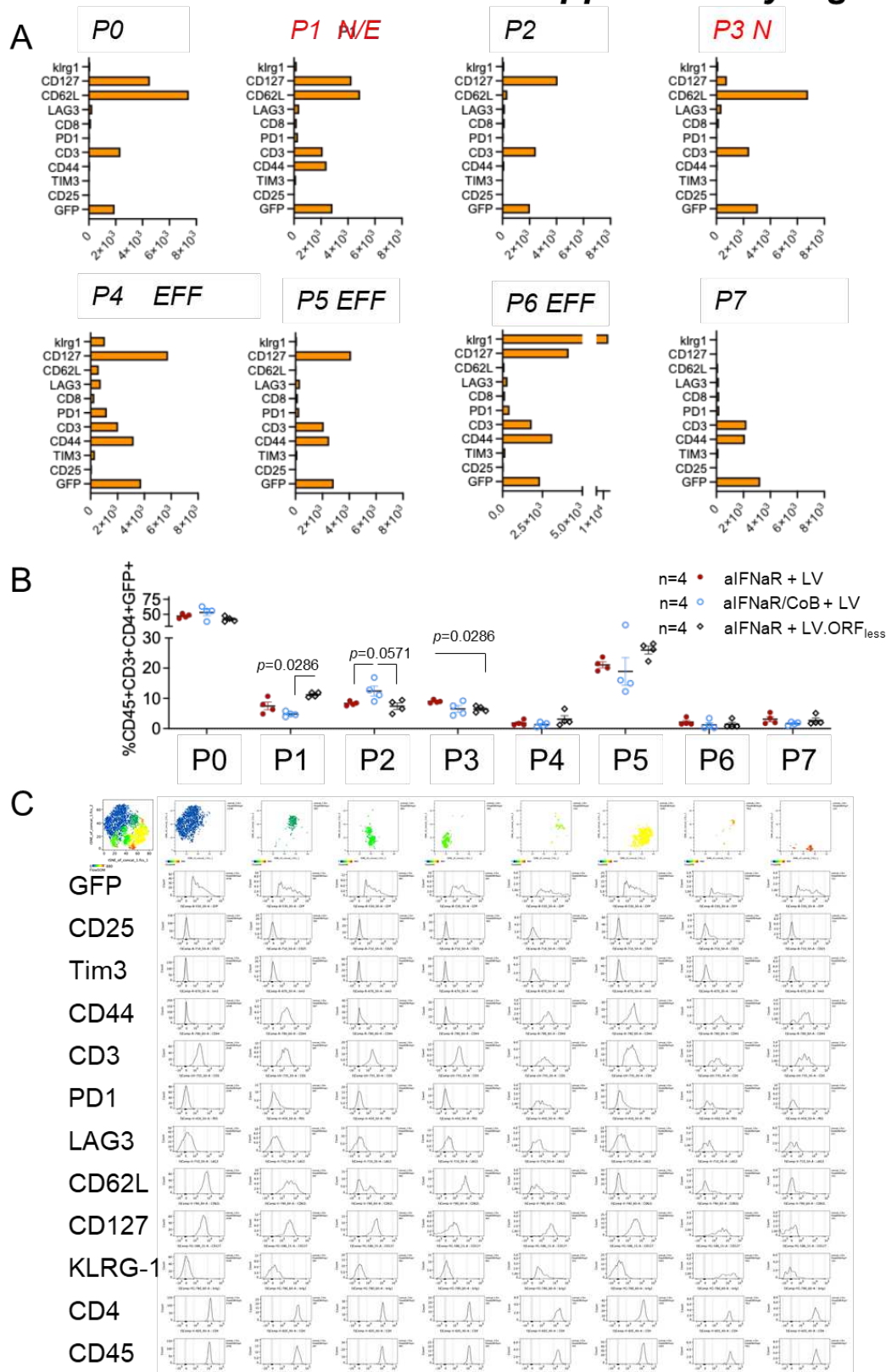
Supplementary figure 11. Unsupervised immunophenotypic analysis of splenic activated CD8 T cells after LV gene therapy.

Multiparametric analysis was performed on single-cell immunophenotypic data from splenic GFP-labeled CD8 T cells using FACS. Unsupervised identification of T cell subpopulations was performed in silico.

Geometric mean fluorescence for each parameter across the population is shown in histograms (A), and frequencies for each mouse and the group mean are shown in (B). The single-parameter fluorescence table (C) for each population is also reported.

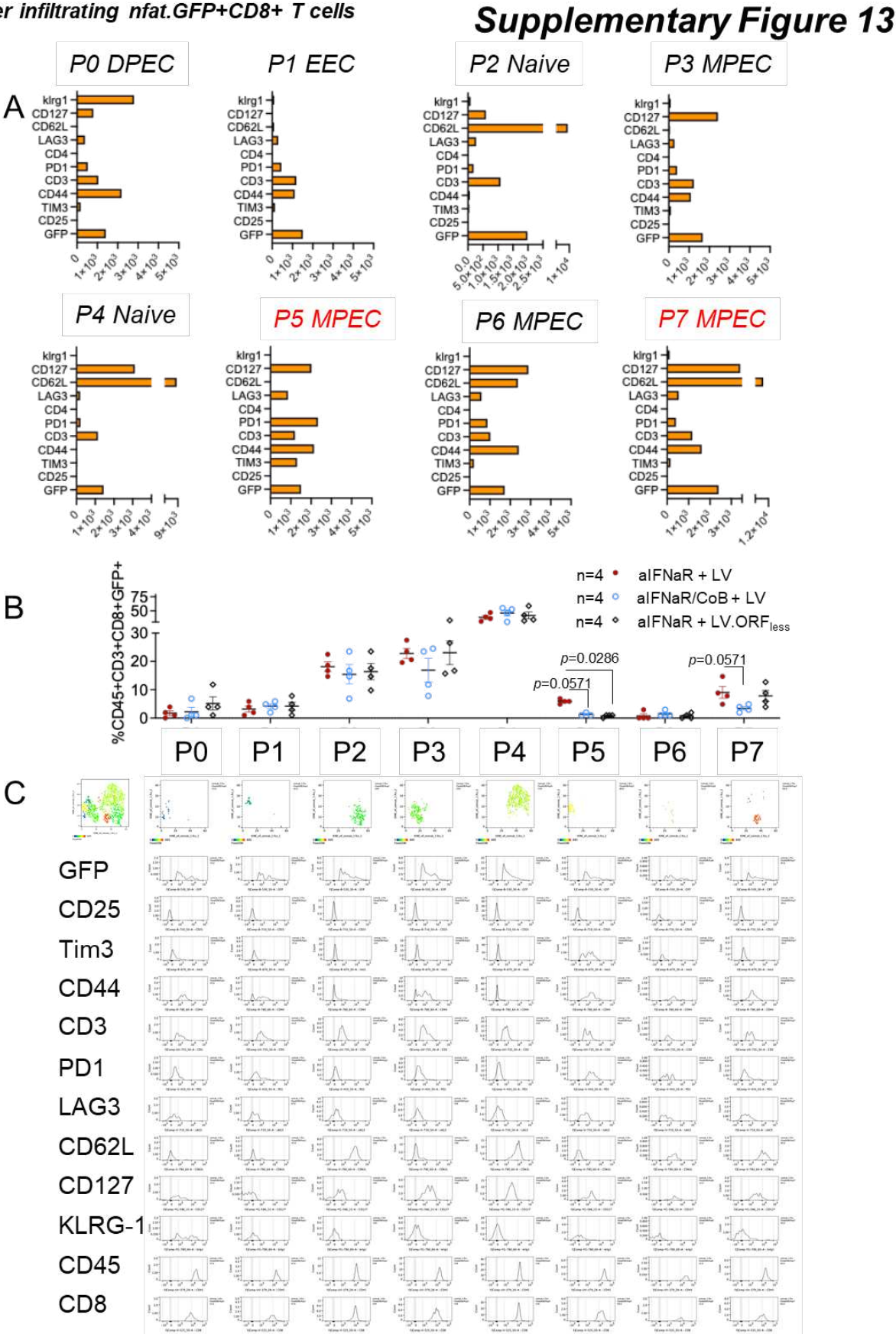
Liver infiltrating nfat.GFP+CD4+ T cells

Supplementary Figure 12



Supplementary figure 12. Unsupervised immunophenotypic analysis of liver-infiltrating activated CD4 T cells after LV gene therapy.

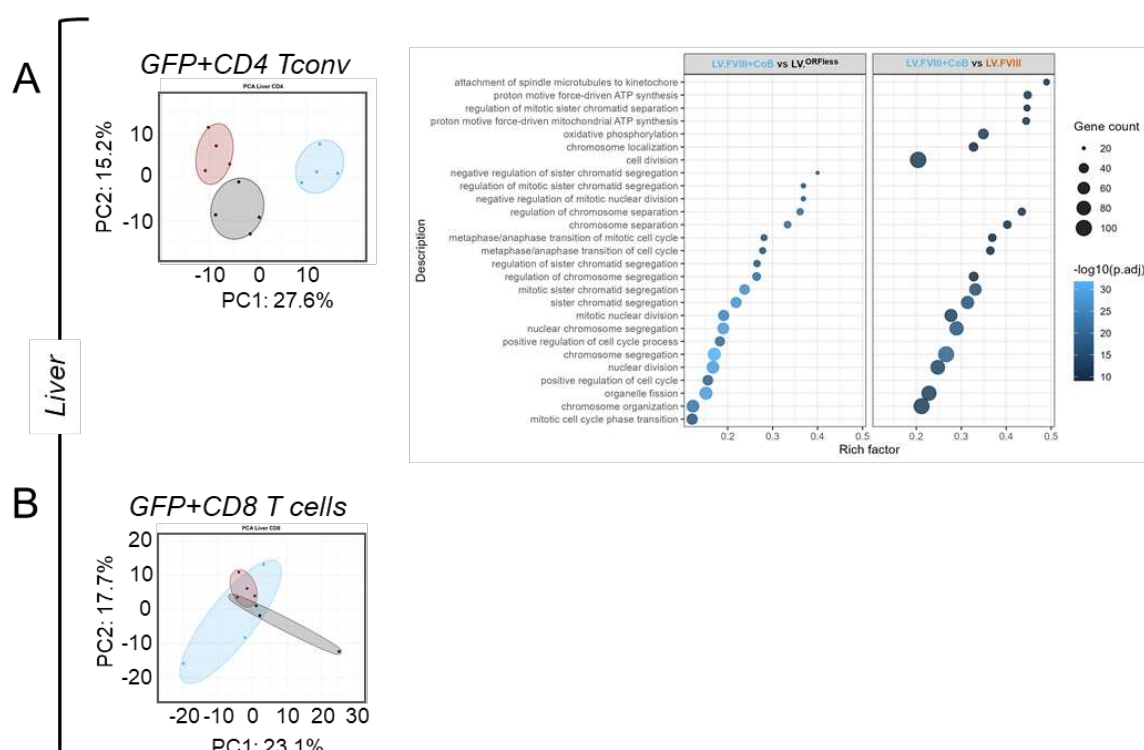
Multiparametric analysis was performed on single-cell immunophenotypic data from hepatic GFP-labeled CD4 T cells using FACS. Unsupervised identification of T cell subpopulations was performed in silico. Geometric mean fluorescence for each parameter across the population is shown in histograms (A), and frequencies for each mouse and the group mean are shown in (B). The single-parameter fluorescence table (C) for each population is also reported.



Supplementary figure 13. Unsupervised immunophenotypic analysis of liver-infiltrating activated CD8 T cells after LV gene therapy.

Multiparametric analysis was performed on single-cell immunophenotypic data from hepatic GFP-labeled CD8 T cells using FACS. Unsupervised identification of T cell subpopulations was performed in silico. Geometric mean fluorescence for each parameter across the population is shown in histograms (A), and frequencies for each mouse and the group mean are shown in (B). The single-parameter fluorescence table (C) for each population is also reported.

Supplementary Figure 14



Supplementary figure 14. Co-stimulatory blockade (CoB) regimen alters the transcriptome of T cells activated by antigen recognition, delivered *in vivo* via LV-based gene therapy.

HemoA^{nfat.GFP} mice chimeric (n=12) received i.v. 2×10^{10} TU/kg of LV encoding for co.hFVIII (n=8) with (n=4) or without CoB (n=4), and controls were i.v. treated with 2×10^{10} TU/kg of LV.ORFless (n=4) not encoding for any transgene. One-week post-LV injection, liver-infiltrating GFP-expressing CD4 conventional, CD4 regulatory, and CD8 T cells were FACS-sorted for RNA sequencing. Note that GFP+ Tregs from the liver were sorted, but the isolation yield of RNA was insufficient for sequencing. Genes with an adjusted P value (FDR) < 0.05 were considered differentially expressed. Principal component analysis and dot plot reporting the top 20 biological processes downregulated by the CoB regimen indicate T cell subsets relative to LV.FVIII or LV.ORFless were found and shown (A, B).