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Production of therapeutic levels of human FIX-R338L by engineered B cells using GMP-compatible medium

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B cells can differentiate into plasmablast and plasma cells, capable of producing antibodies for decades. Gene editing using zinc-finger nucleases (ZFN) enables the engineering of B cells capable of secreting sustained and high levels of therapeutic proteins. In this study, we established an advanced *in vitro* good manufacturing practice-compatible culturing system characterized by robust and consistent expansion rate, high viability, and efficient B cell differentiation. Using this process, an optimized B cell editing protocol was developed by combining ZFN/adeno-associated virus 6 technology to achieve site-specific insertion of the human factor IX R338L Padua into the silent *TRAC* locus. *In vitro* analysis revealed high levels of secreted human immunoglobulins and human factor IX-Padua. Following intravenous infusion in a mouse model, human plasma cells were detected in spleen and bone marrow, indicating successful and potentially long-term engraftment *in vivo*. Moreover, high levels of human immunoglobin and therapeutic levels of human factor IX-Padua were detected in mouse plasma, correlating with 15% of normal human factor IX activity. These data suggest that the proposed process promotes the production of functional and differentiated engineered B cells. In conclusion, this study represents an important step toward the development of a manufacturing platform for potential B cell-derived therapeutic products.

INTRODUCTION

B cells are key effectors of the adaptative immune response. After antigen engagement, B cells differentiate into plasmablast and plasma cells.[1] In the bone marrow niche, the long-lived plasma cells can secrete large quantities of antibodies and survive for decades.[2] Recent developments with precise editing technologies open the possibility of engineering plasmablast and plasma cells to act as autologous cell factories, capable of delivering sustained and high doses of therapeutic proteins.[3–5] In this study, *ex vivo* B cell engineering was performed by combining electroporation of zinc-finger nuclease (ZFN) mRNA with adeno-associated virus 6 (AAV6) donor DNA template delivery.[6,7] In the literature, to date, most B cell engineering methods use fetal bovine serum (FBS) or human AB serum in combination with classical research media such as DMEM, RPMI, or Iscove's modified Dulbecco's medium.[8–18] However, animal-derived materials are strongly discouraged in good manufacturing practice (GMP) cell therapy manufacturing. Moreover, the use of FBS or human AB serum is known to decrease the efficiency of B cell engineering by inhibiting AAV6 transduction and consequently, targeted integration (TI) of the donor DNA sequence.[19] Therefore, we developed an *in vitro* culturing system optimized for B cell expansion, differentiation into plasmablast/plasma cells, and site-specific transgene insertion into target loci using a xeno-free medium compatible with GMP production. As a proof of concept, B cells were engineered for the expression of the human factor IX (FIX), more specifically the hyperactive FIX-R338L variant (also called Padua).[20] FIX deficiency in patients with hemophilia B leads to uncontrollable bleeding events, and the severity depends on the levels of this clotting factor. To improve current standard or extended half-life FIX replacement therapies,[21] alternative approaches are in clinical development like AAV gene therapies,[22] supported by the recent approval of etranacogene dezaparvovec, an AAV5-Padua human FIX gene therapy.[23] However, we believe that the development of an innovative *ex vivo* FIX-B cell therapy approach is of high interest, since it would not be limited by some downsides of current AAV-mediated *in vivo* gene therapy such as the requirement of high vector doses, the pre-existing immunity to the viral capsid in the human population, and the challenges of redosing with AAV vectors.[24–27]

RESULTS

Optimized conditions for expansion and differentiation of human primary B cells in a xeno-free culture medium

Various culture conditions were evaluated for optimal expansion, differentiation, and viability of human naive primary B cells. Using a

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Table 1. Composition of B cell culture media used for expansion and differentiation

Component	Expansion medium	Diff-1 medium	Diff-2 medium
CD40L multimer	0.5 μg/mL		0.25 μg/mL
Cross-linking antibody	1/1,000		1/2,000
ODN 2006	1 μg/mL		0.5 μg/mL
IL-2	50 ng/mL	50 ng/mL	50 ng/mL
IL-10	50 ng/mL	50 ng/mL	50 ng/mL
IL-15	10 ng/mL	10 ng/mL	10 ng/mL
IL-6	–	50 ng/mL	50 ng/mL
IFN type 1	–	500 U/mL	500 U/mL
APRIL and/or BAFF	–	200 ng/mL 200 ng/mL	200 ng/mL

APRIL, a proliferation inducing ligand; BAFF, B cell activation factor; CD40L, CD40 ligand.

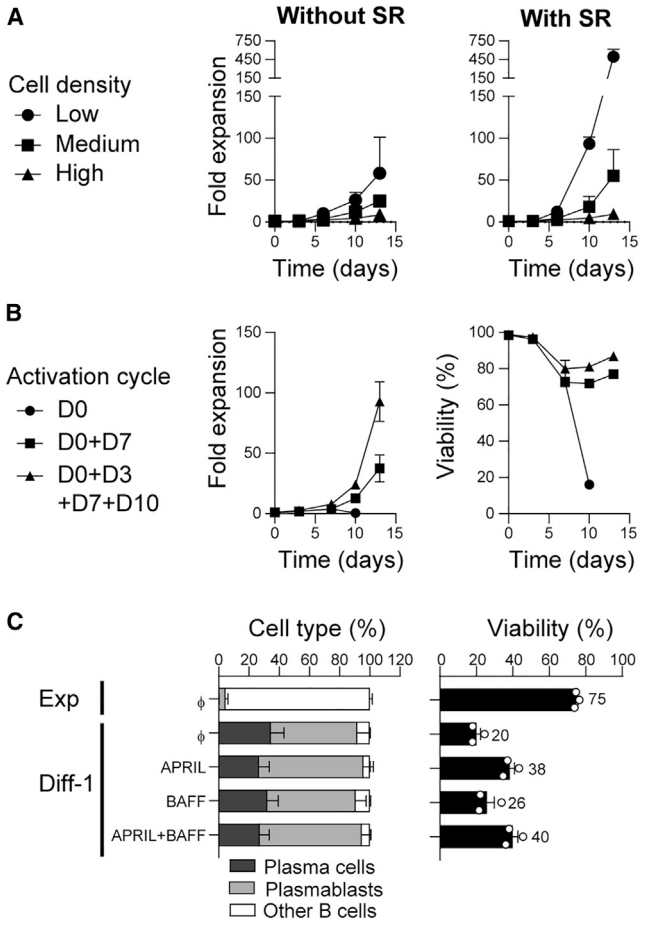

Figure 1. Critical parameters for optimal expansion and viability of differentiated B cells

(A) Evaluation of B cell concentration (1.5 [low], 5 [medium], or 10 [high] × 10^5 cells/mL) on B cell expansion capacity over 2 weeks in absence (left) or presence (right) of SR solution (n = 2 donors). (B) Expansion capacity and viability of B cell culture following 1, 2, or 4 activation cycles (n = 2–13 donors). (C) Impact of APRIL and/or BAFF proteins on the Diff-1 protocol at D14 (n = 3 donors). Data are represented as the mean ± SEM.

xeno-free GMP-compatible medium supplemented with human interleukin (IL)-2, IL-10, and IL-15 cytokines (Table 1), human B cell expansion was more efficient at low cell density and following the addition of a protein-enriched supplement called serum replacement (SR) (Figure 1A). The activation regimen was also found to be critical. Repeated activations every 3–4 days through CD40 and TLR9 stimulation, using cross-linked CD40L antibodies and CpG oligonucleotide (ODN), respectively, were necessary to maintain an optimal B cell viability over the 2 weeks of culture (Figure 1B). For the development of a differentiation protocol in plasmablast and plasma cells, freshly isolated B cells were first expanded for 1 week and then transferred in a first-generation differentiation (Diff-1) medium containing human IL-2, IL-10, IL-15, IL-6, and interferon (IFN) type 1 (Table 1) in the absence or presence of a proliferation-inducing ligand (APRIL), known to promote human B cell differentiation (see Table 1). Despite a very efficient differentiation of the culture in plasmablast ($CD38^+CD138^-$) and plasma cells ($CD38^+CD138^+$), the viability was poor, but the presence of APRIL was able to increase viability by at least 2-fold, from 20% to 40% (Figures 1C and S1). In parallel, the impact of the B cell-activating factor (BAFF), also known to be central for B cell expansion and survival *in vivo*, was evaluated alone or in combination with APRIL. In these culture conditions, BAFF had no impact, either on differentiation or overall B cell viability (Figure 1C). To further improve the viability and develop a second-generation differentiation (Diff-2) protocol, low doses of activation molecules and expansion cytokines were added to the culture (see last column, Table 1 and Figure 2A); the expansion protocol harbored optimal viability conditions. This Diff-2 protocol positively impacted the expansion capacity (>300-fold) (Figure 2B) and viability (>80%) (Figure 2C), comparable with the expansion control condition, while retaining a potent differentiation capacity into plasmablasts and, to a lesser extent, into plasma cells in only 14 days (Figure 2A). B cell subpopulations were analyzed over time based on IgD and CD27 expression profiles in the expansion and Diff-2 protocols (see gating strategy in Figure S2). As shown in Figures 2D and 2E, the naive phenotype was quickly lost in favor of a memory phenotype ($IgD^-/CD27^+$), but with a higher CD27 expression during differentiation (Figure 2F). Furthermore, the monitoring of the plasmablast and plasma cells phenotype using CD38 and CD138 markers (Figure 3A) showed differentiation in plasmablasts ($CD38^+CD138^-$) in only 3 days (D7–D10), and to a lesser extent in plasma cells (5%–10% of $CD38^+CD138^+$) after 7 days (D7–D14). As expected, B cell differentiation induced a decrease in CD19 and CD20 B cell marker expression (Figures 3B and S3) and an increase of human immunoglobulin (HuIg) secretion in the culture supernatants (IgA and IgM) (Figure 3C). Therefore, the Diff-2 protocol with optimal expansion, viability, and differentiation capacity was selected for further experiments.

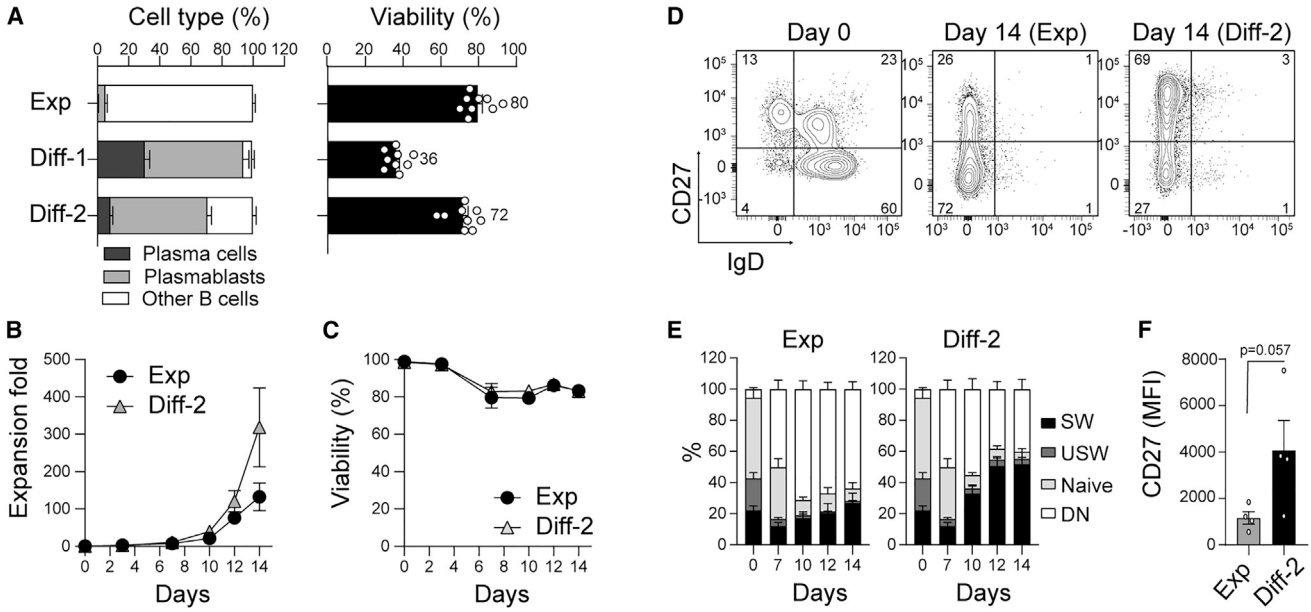

Figure 2. B cell culture monitoring in optimal expansion and differentiation protocol in GMP-compatible medium

(A) Development of a Diff-2 protocol (for details, see Table 1) to improve cell viability after two weeks of culture (n = 9 donors). B cell expansion (B) and viability (C) were monitored during 2 weeks of culture in expansion (Exp) or differentiation (Diff-2) medium (11–15 donors). (D) CD27 and IgD expression profile in B cells at day 0 or after 2 weeks in Exp or Diff-2 medium. (E) Two weeks monitoring of the ratio of double-negative, naive, switched, or unswitched B cell populations in Exp or Diff-2 culture medium (n = 8 donors). (F) CD27 expression (mean fluorescence intensity) enrichment in diff-2 medium at day 14 (n = 4 donors). Data are represented as the mean ± SEM and two-tailed Mann-Whitney test.

Production of FIX-engineered B cells by combining ZFN and AAV6-FIX donor DNA technologies for site-specific integration into *AAVS1* and *TRAC* loci

B cell engineering was performed during the expansion phase by combining electroporation of ZFN tandem mRNA with AAV6 donor template delivery to achieve site-specific insertion of a human FIX-Padua cassette by homologous recombination (HDR) into the safe harbor *AAVS1* locus or the silent *TRAC* locus (Figure 4A). The ZFN editing efficiency, monitored by following insertion or deletion (indel) frequency (Figure 4B), was comparable between *AAVS1* and *TRAC* loci. However, the FIX production in culture supernatants was higher upon integration into the *TRAC* locus compared with the *AAVS1* locus (Figure 4C). Interestingly, we observed a better correlation between TI efficiency monitored by next-generation sequencing analysis and the level of FIX secretion for the *TRAC* locus conditions (Figure 4D, left vs. right panel). This phenomenon was not linked to the FIX sequence, since the same was observed following site-specific HDR integration of a cassette expressing the green fluorescent protein (GFP) used as a control (Figure S4). B cell engineering into the *TRAC* locus is impacted less by donor-to-donor variability and is, therefore, more reliable for therapeutic transgene expression in B cells compared with the *AAVS1* locus.

Human FIX-engineered B cells injected in immunodeficient NOD SCID Gamma (NSG) mice secrete therapeutic levels of human FIX-Padua *in vivo*

Since the *TRAC* locus was most effective at delivering a sustained and high-level expression of FIX-Padua *in vitro*, it was selected for the *in vivo* proof of concept. For that, differentiated B cells engineered at the *TRAC* locus for FIX-Padua were injected at day 14 into NSG mouse model pre-engrafted with human memory T cells (Figures 5A and S5) to sustain human B cell engraftment as described previously.[28] Before *in vivo* injection, to ensure that the FIX-Padua produced by FIX-engineered (FIX-Eng) B cell is active, the FIX activity secreted by the latter has been determined using a commercial chromogenic assay (Figure S6 and supplemental methods). As expected, the FIX-Padua is highly active, approximately 5% of normal pooled plasma. Next, engraftment efficiency of FIX-eng B cells, and levels of HuIg and FIX-Padua secretion were monitored for 4 weeks. At approximately 3 weeks post-injection, a peak of HuIg production was observed (Figure 5B), indicating an efficient engraftment of human plasmacytes. Concerning the FIX-Padua secretion, we observed a dose-response curve with an optimal level of secretion at approximately 25 ng/mL and 100 ng/mL for FIX-eng B cells with a TI of 5% and 20%, respectively (Figure 5C). In the blood, FIX-eng B cells were not detectable at any time point, probably the consequence of their *in vitro* differentiation in plasmablasts and plasma cells, migrating preferentially into secondary lymphoid organs. Indeed, plasma cells were readily detected in the mouse spleen and bone marrow (Figure S7) at the time of sacrifice (day 41), indicating successful and potential long-term engraftment (Figures 5D and 5E, and gating strategy in Figure S8). The phenotypic characterization of the sub-populations using human CD38 and CD138 showed a strong differentiation into plasma cells and plasmablasts, at approximately 40%–50% and 30% respectively. Since the phenotype of engineered B cells at the time of injection (Diff-2 vitro day 14) showed mainly plasmablasts

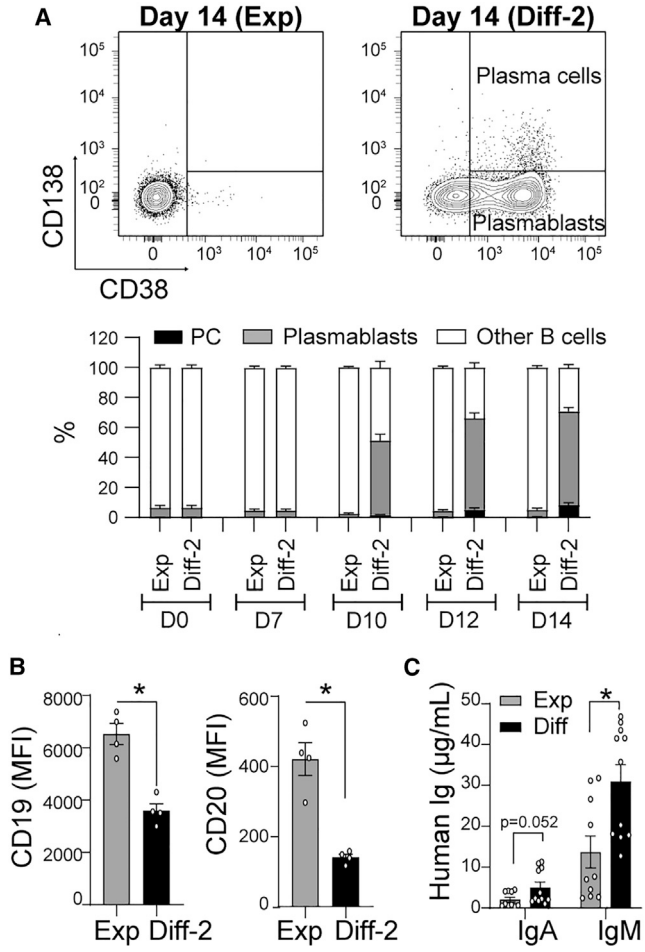

Figure 3. B cell differentiation in plasmablasts and plasma cells

B cell differentiation profiles were monitored during 2 weeks of culture in expansion medium (Exp) or 1 week of culture in expansion followed by 1 week of culture in differentiation medium (Diff-2). (A) Representative $CD38^{+}CD138^{+}$ cells in Exp or Diff-2 conditions (top) and ratio of plasmablasts, plasma cells, and other B cells in expansion (Exp) or differentiation (Diff-2) culture protocol over time (bottom; n = 8–15 donors). (B) CD19 and CD20 expression (mean fluorescence intensity) decrease in Diff-2 medium at day 14 (n = 4 donors). (C) Monitoring of IgA and IgM secretion in Exp or Diff-2 culture medium at day 14 (n = 10–11 donors). Data are represented as the mean ± SEM. Two-tailed Mann-Whitney test. *p < 0.05.

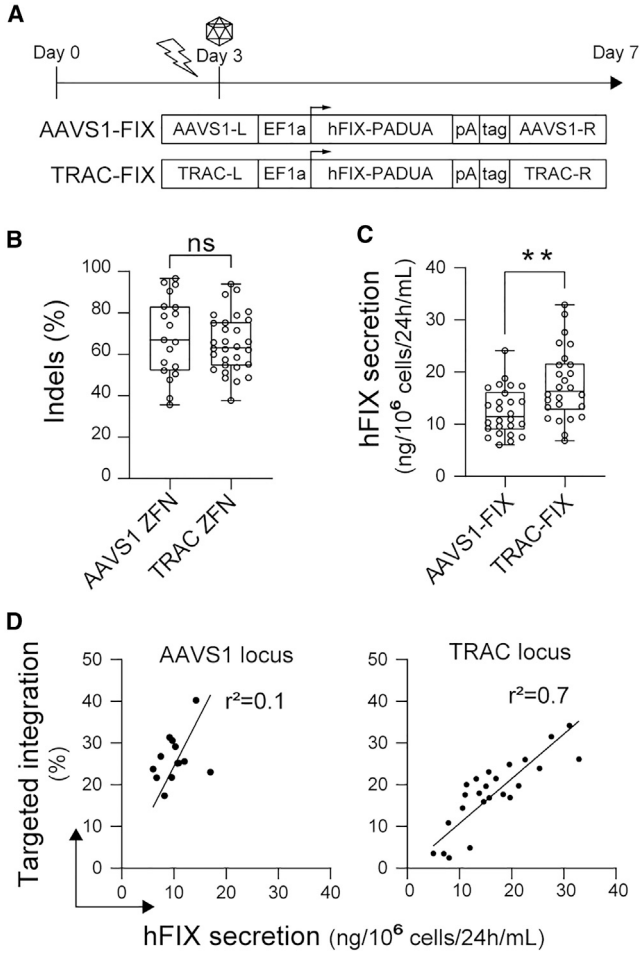

Figure 4. Editing and TI efficiency of human FIX sequence in human B cells

(A) Schematic of the B cell editing protocol and AAV6 constructs used. The human FIX (hFIX) donor cassette is targeted by homology-directed repair to the *AAVS1* or the *TRAC* locus using the flanking left and right homology arms (respectively 600 and 400 bp for AAVS1 and 423 bp and 393 bp for TRAC). EF1a, elongation factor 1a promoter; pA, bovine growth hormone polyA; Tag, randomized sequence for next-generation sequencing integration analysis. (B) Indels (%) obtained after editing with ZFN pairs directed against the *AAVS1* or the *TRAC* locus (n = 19–30 donors). Data are represented as mean ± SEM. (C) Level of hFIX expression (ng/10^6 cells/24 h/mL) after TI of AAV6 donor DNA in either *AAVS1* or *TRAC* locus. Data are represented as box and whiskers showing all points (n = 26 donors) two-tailed Mann-Whitney test. **p < 0.01, ns, not significant). (D) Correlation between TI efficiency in the *AAVS1* or the *TRAC* locus and hFIX expression was analyzed using a linear regression and r2 calculation.

(approximately 60%) and low amounts of plasma cells (<10%), this highlighted the capacity of engineered plasmablasts to differentiate very efficiently *in vivo* into plasma cells, especially in the bone marrow. Consistent with B cell differentiation, engrafted plasmablast and plasma cells from spleen and bone marrow showed a loss in cell surface expression of the CD20 marker, and high levels of CD27, BLIMP-1, and XBP-1 (Figure 6 and gating strategy in Figure S9).

DISCUSSION

Patient-derived B cells can be engineered to express a therapeutic protein, expanded, and differentiated into plasmablasts and plasma cells *ex vivo*. Upon re-infusion, cells are expected to engraft and serve as an *in vivo* source for sustained circulation of the therapeutic factor. However, *in vitro* B cell culture without animal-derived materials like bovine serum or human AB serum is very challenging. In this study, we optimized human engineered B cell culture using a xeno-free GMP-compatible medium. We showed that B cell expansion is more efficient at low cell density and can benefit greatly from the addition of the SR protein-enriched supplement. Continuous expansion and stable cell viability over time required repeated activations

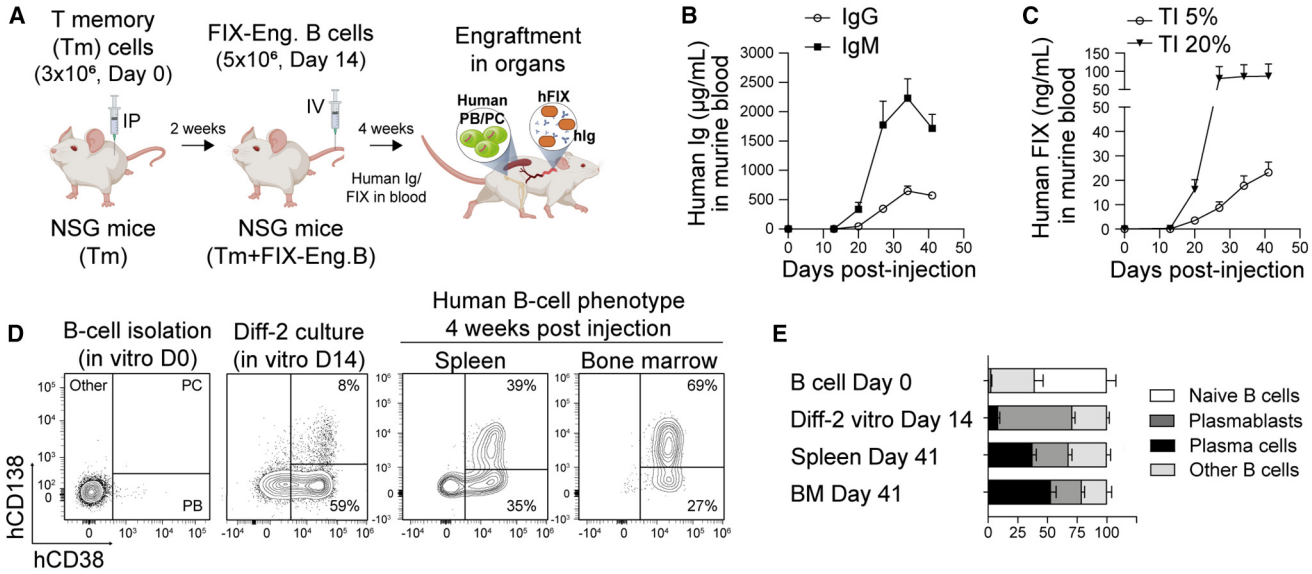

Figure 5. *In vivo* engraftment of control B cells and engineered B cells expressing human FIX

(A) Schematic of the *in vivo* experimental protocol (created with Biorender). Four weeks after B cells engraftment, monitoring of (B) HuIgs (n = 41 mice, 12 donors) and (C) human FIX secretion in murine blood (TI 5% [n = 14 mice, 4 donors]; and TI 20% [n = 4 mice, 3 donors]). (D) Expression profile of hCD38 and hCD138 (D). (E) cell population ratio of naive B cells, plasma cells and plasmablasts just after B cell isolation (B cell day 0), after 2 weeks of culture *in vitro* (Diff vitro day 14) or in the mouse spleen (9 donors) and bone marrow (BM) (8 donors) after 41 days (n = 8–28 mice). Data are represented as mean ± SEM.

through CD40 and TLR9 stimulation. Our Diff-1 protocol resulted in the highly efficient production of plasmablasts and plasma cells. However, it showed a strong negative impact on overall viability. The addition of BAFF, an important factor *in vivo* for B cell expansion and survival,[29] did not have any positive effect on differentiated B cells viability in our culture conditions *in vitro*. In contrast, the addition of APRIL,[30] known to promote B cell differentiation, led to a 2-fold increase in viability. The largest improvement implemented in the Diff-2 protocol was the addition of low doses of activation molecules and expansion cytokines, leading to a strong improvement in the overall expansion (>300-fold) and viability (>80%), comparable with the expansion control condition, while retaining a potent differentiation capacity into plasmablasts and, to a lesser extent, into plasma cells. Nevertheless, the strong improvement in overall production capacity of differentiated B cells between Diff-1 and Diff-2 protocols largely compensates for this decrease in plasma cell percentage. Under these optimized culture conditions, B cells were engineered using the ZFN editing technology in combination with an AAV6 donor DNA harboring an expression cassette for the FIX-Padua variant. Specific ZFNs were able to disrupt both the *AAVS1* locus and the *TRAC* locus with a similar indels profile, around 70%. Despite showing similar editing efficiency, the correlation between the TI efficiency and the transgene expression (either GFP or human FIX) was more robust for the *TRAC* target locus than the *AAVS1* locus. The transgene expression capacity into the *AAVS1* locus in B cells seemed to be influenced by the B cell donor-to-donor variability, a phenomenon already described in human induced pluripotent stem cells.[31,32] On the contrary to the *TRAC* locus, *AAVS1* is not a silent locus. *AAVS1* is located within the *protein phosphatase 1, regulatory (inhibitor) subunit 12C* (*PPP1R12C*) gene, expressed in the hematopoietic tissue, a potential reason for the variability in transgene expression from donor to donor after integration in this active locus. In a previous study, Hung et al.[16] produced FIX-Padua from human B cells edited in the *CCR5* locus using the CRISPR-Cas9 system and an FIX-expressing AAV6 as a donor DNA. The optimal TI activity achieved in their study in the *CCR5* locus was approximately 20% (±5%), while we reached up to 35% in the *TRAC* locus (Figure 4D), probably the consequence of the use of animal serum (10% FBS) known to inhibit AAV6 transduction[19] or eventually a more potent HDR activity on the *TRAC* locus versus *CCR5* in B cells. Next, to evaluate the *in vivo* functionality of human FIX-eng B cells, differentiated B cells engineered in the *TRAC* locus for FIX-Padua expression were injected into the NSG immunodeficient mouse model pre-injected with autologous memory T cells, known to promote very efficiently the B cell engraftment.[28] Four weeks after injection, engineered B cells were able to secrete large amounts of HuIgs and up to 100 ng/mL of FIX-Padua in murine blood, a circulating concentration corresponding with 15% of wild-type FIX (i.e., ≥15 times the FIX level observed in patients with severe hemophilia B [<1%] and ≥3 times the FIX level in moderate hemophilia B [1%–5%]). Interestingly, an extensive differentiation of engineered B cells directly into plasma cells *in vitro* is not strictly necessary, since plasmablasts produced *in vitro* can engraft and differentiate into plasma cells *in vivo*. Indeed, while plasma cells represented only 10% of the total population at the time of injection, the human B cell biodistribution showed enrichment in plasma cells after 4 weeks *in vivo*, with up to 40% in spleen and more than 50% in the bone marrow. This represents a manufacturing process advantage, since the differentiation into

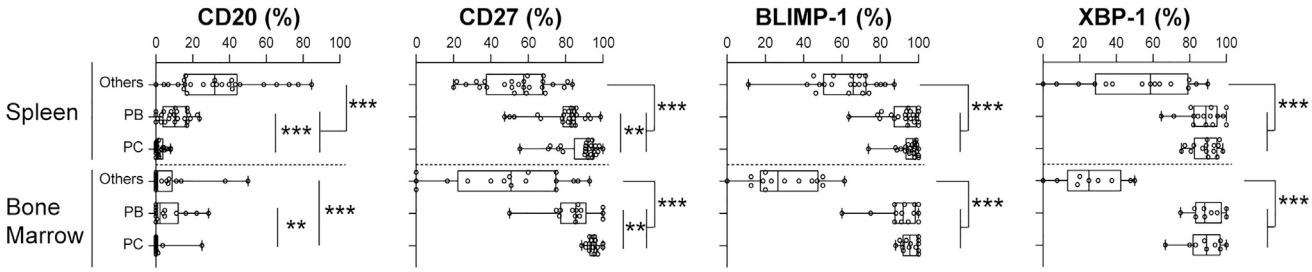

Figure 6. Immunophenotyping of control and FIX-eng B cells after *in vivo* engraftment

At day 41, isolation of human B cells (8 donors) engrafted in murine spleen (n = 25 mice) and bone marrow (n = 17 mice) and monitoring of cell surface expression of human CD20, CD27, BLIMP-1, and XBP-1 in the different sub-populations consisting of human plasma cells (PC) ($CD38^+CD138^+$), plasmablasts (PB) ($CD38^+CD138^-$) and other B cells (Others) ($CD38^-CD138^-$). Two-tailed Mann-Whitney test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

plasma cells *in vitro* is associated with extremely poor viability, as described in our results and in the literature.[33] In summary, we demonstrated that ZFN mRNA combined with rAAV6 donor DNA allows for efficient site-specific insertion of the FIX-Padua transgene into the human B cell *TRAC* locus, resulting in therapeutic levels of FIX-Padua expression in a B cell-engraftment mouse model. This study represents an essential step in developing a B cell therapy manufacturing platform for the potential treatment of patients with hemophilia B and a variety of other protein deficiencies.

MATERIALS AND METHODS

Human primary cell isolation and culture

Buffy coats were obtained from healthy volunteers (EFS, Marseille, France). For *in vitro* data, B cells were isolated directly from blood (EasySep direct human B isolation kit; StemCell Technologies, Saint-Egrève, France). For *in vivo* experiments, peripheral blood mononuclear cells were isolated by Ficoll gradient centrifugation. B cells and Tm cells were isolated by immunomagnetic CD19 positive selection (EasySep human CD19 positive selection kit II) and $CD45RO^+CD4^+$ negative selection (EasySep human memory $CD4^+$ T cell enrichment kit), respectively, following manufacturer instructions (StemCell Technologies). To limit the risk of GvHD in NSG mice, the post-enrichment purity for $CD45RO^+CD4^+$ Tm cells was higher than 90% (94% ± 3%) and $CD8^+$ T cell impurities of less than 0.3%.

B cells were cultured in CTS OpTmizer expansion medium supplemented with 2.6% expansion supplement, 10% immune cell SR (Thermo Fisher Scientific, Illkirch-Graffenstaden, France), 20 mM L-glutamin, 55 μM 2-mercaptoethanol, and 75 μg/mL Gentamicin (Sigma-Aldrich, Saint-Quentin-Fallavier, France). B cells were activated with 0.5 μg/mL hCD40L multimer, 1 μg/mL CpG-ODN 2006 (Miltenyi Biotec, Paris, France), 50 ng/mL Proleukin S (Euromedex, Souffelweyersheim, France), 50 ng/mL hIL-10, and 10 ng/mL hIL-15 (PeproTech, Neuilly-Sur-Seine, France).[16] For *in vitro* B cell differentiation, different protocols were tested (Table 1). Briefly, B cells were cultured in OpTmizer medium supplemented with 50 ng/mL Proleukin S, 50 ng/mL hIL-10, 10 ng/mL hIL-15, 50 ng/mL hIL-6, 200 ng/mL human APRIL (PeproTech),[34] 500 U/mL type I IFN (Bio-Techne, Noyal Châtillon sur Seiche, France) with or without 0.25 μg/mL hCD40L multimer, 0.5 μg/mL CpG-ODN 2006, or 200 ng/mL BAFF (Peprotech).

ZFN mRNA and AAV6 vector production

Heterodimeric ZFNs targeting the human *AAVS1* and *TRAC* loci were cloned into the pVAX-GEM2UX plasmid. Following plasmid linearization, mRNAs were produced using the mMessage mMachine T7 ultra-Kit (Thermo Fisher Scientific) and subsequently purified by lithium chloride precipitation. The quality and quantity of ZFN mRNA transcripts were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Les Ulis, France). AAV6-GFP and AAV6-FIX-Padua vectors were produced using a triple transfection method as previously described.[7] Briefly, HEK293 cells were transfected using the calcium phosphate method with an AAV Rep2/Cap6 helper plasmid, the pXX6 adenovirus helper plasmid, and an ITR-containing donor vector plasmid. After 3 days, cells were lysed by three rounds of a freeze/thaw cycle, and cell debris was removed by centrifugation. AAV6 vectors were then precipitated from the lysates using polyethylene glycol and purified by ultra-centrifugation overnight on a cesium chloride gradient. Vectors were formulated by dialysis and filter sterilized. AAV6 vector titration was performed by qPCR, as previously described.[35]

ZFN-mediated editing and AAV6 TI in human B cells

After expansion, B cells were suspended at 25×10^6 cells/mL in Maxcyte electroporation buffer (Maxcyte, Gaithersburg, MD). ZFN mRNA transcripts were then mixed with B cells at 90 μg/mL and electroporated with ExPERT GTx electroporator according to manufacturer's recommendations (Maxcyte). Next, cells were recovered, suspended in fresh medium, incubated 6 h at 37°C/5% CO_2 with AAV6 vectors (multiplicity of infection 10^5) and finally diluted at 5×10^5 cells/mL in B cell activation medium. After 4 days, editing and transduction efficiency were evaluated by monitoring indels, targeting integration, and transgene expression.

Indels and TI assessment

Indel analysis has been performed as described previously.[36] In short, targeted regions were amplified by nested PCR. PCR libraries were

barcoded, and the levels of modification were determined by paired-end deep sequencing on a MiSeq (Illumina, Evry, France) according to the manufacturer's instructions. To distinguish the TI sequence, a Miseq Tag sequence was incorporated into the AAV6 donor DNA, consisting of a DNA segment of human *TRAC* or *AAVS1*, randomly scrambled, to produce a unique amplicon for the quantification of TI events in the respective target site (see primers in Table S1).[37]

Cellular immunophenotyping

Cells were stained with specific conjugated monoclonal antibodies targeting CD3, CD4, CD8, CD19, IgD, CD20, CD27, CD38, CD138, CD45, CD45RA, CD45RO, and MHC-I (Miltenyi Biotec and Becton Dickinson, Le Pont de Claix, France). If required, cells were fixed and permeabilized with a staining buffer set (Thermo Fisher Scientific) and stained for XBP1 and BLIMP-1 intracellular markers (Becton Dickinson). For *in vivo* experiments, mouse spleen, lung, and bone marrow samples were passed through a 70-μm cell strainer to obtain a single cell suspension. Red blood cells were lysed with red blood cell lysis buffer for 5 min at room temperature (RT) (Merck, Fontenay Sous Bois, France). Next, cells were incubated with mouse Fc block (BD Biosciences, Franklin Lakes, NJ) and stained 10 min at RT with antibodies diluted 100-fold in fluorescence-activated cell sorting buffer (PBS-2% FBS). Marker expressions were measured by flow cytometry and analyzed using FlowJo software (BD Biosciences).

Quantification of HuIgs and human FIX-Padua

To assess HuIgs and FIX production by edited B cells, the latter were edited and expanded or differentiated *in vitro*. On days 4 and 11 post-editing, cells were collected and resuspended in fresh culture medium at 10^6 cells/mL. After 24 h, culture supernatants were collected. FIX secretion levels in cell culture supernatants or in mouse plasmas were determined by ELISA according to manufacturer's instructions (Human Factor IX ELISA Kit, Abcam, Paris, France). Human IgA, IgM, and IgG were measured using an MSD Multiplex assay according to manufacturer's instructions (Meso Scale Diagnostics, Rockville, MD).

Mouse model for human B cell engraftment

NSG mice were obtained from Charles River (Lyon, France). The 8-week-old NSG mice were housed in containment isolators and habituated for 1 week before experimental use. The animal protocol was approved by the French animal ethics committee. Mice were randomly assigned to groups. For *in vivo* experiments, human Tm and B cells were obtained and purified from the same donors. Freshly isolated Tm cells were injected intraperitoneally at day 0 (3×10^6 per mouse) and expanded/differentiated B cells were injected intravenously 2 weeks later at day 14 (5×10^6 per mouse). Throughout experiments, the body weight and graft-versus-host disease (GvHD) scores were monitored twice a week by operators blinded to treatment as described previously.[38] To limit the risk of GvHD, only memory T cells with a purity above 90% and containing less than 0.3%, CD8 T cells were selected. Overall, approximately15% of mice developed GvHD after 2–3 weeks leading to a failure in B cell engraftment. These mice were excluded from the study. Once a week, blood samples were collected in coated heparin LH microtainer (Becton Dickinson) by retro-orbital sampling under local anesthesia. At the end of the experiment (day 41), mice were euthanized, tissue and blood samples were collected and processed for flow cytometry immunophenotyping, and human Ig and FIX-Padua in mouse plasma were measured as described above.

Statistics

All statistical analyses were performed using GraphPad Prism v.6 (GraphPad Software Inc., San Diego, CA). Statistical significances were determined using non-parametric two-tailed Mann-Whitney tests.

DATA AND CODE AVAILABILITY

All requests for data will be reviewed by Sangamo Therapeutics, Inc., to verify whether the request is subject to any intellectual property or confidentiality obligations. If deemed necessary, a material transfer agreement between the requestor and Sangamo Therapeutics, Inc., may be required for sharing of some data. Any data that can be freely shared will be released.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2023.101111.

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AUTHOR CONTRIBUTIONS

M.D., D.M., G.S., C.J., I.M., and C.F.D. performed experiments; M.D., D.M., and D.F. designed experiments and analyzed and interpreted data; C.D. reviewed and submitted animal protocols to French authorities; C.D., J.F., and M.d.l.R. contributed to the discussions; M.D., D.M., M.d.l.R., and D.F. wrote the manuscript; D.F. supervised the study.

DECLARATION OF INTERESTS

All authors are Sangamo Therapeutics employees. Sangamo Therapeutics has filed a patent application covering the technology described in this paper.

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