AMERICAN SOCIETY of
GENE & CELL
THERAPY

Engineering Protein-Secreting Plasma Cells by Homology-Directed Repair in Primary Human B Cells

King L. Hung,[1] Iana Meitlis,[1,3] Malika Hale,[1,3] Chun-Yu Chen,[1] Swati Singh,[1] Shaun W. Jackson,[1] Carol H. Miao,[1] Iram F. Khan,[1] David J. Rawlings,[1,2] and Richard G. James[1,2]

[1]Center for Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, Washington, USA; [2]Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington, USA

The ability to engineer primary human B cells to differentiate into long-lived plasma cells and secrete a *de novo* protein may allow the creation of novel plasma cell therapies for protein deficiency diseases and other clinical applications. We initially developed methods for efficient genome editing of primary B cells isolated from peripheral blood. By delivering CRISPR/CRISPR-associated protein 9 (Cas9) ribonucleoprotein (RNP) complexes under conditions of rapid B cell expansion, we achieved site-specific gene disruption at multiple loci in primary human B cells (with editing rates of up to 94%). We used this method to alter *ex vivo* plasma cell differentiation by disrupting developmental regulatory genes. Next, we co-delivered RNPs with either a single-stranded DNA oligonucleotide or adeno-associated viruses containing homologous repair templates. Using either delivery method, we achieved targeted sequence integration at high efficiency (up to 40%) via homology-directed repair. This method enabled us to engineer plasma cells to secrete factor IX (FIX) or B cell activating factor (BAFF) at high levels. Finally, we show that introduction of BAFF into plasma cells promotes their engraftment into immunodeficient mice. Our results highlight the utility of genome editing in studying human B cell biology and demonstrate a novel strategy for modifying human plasma cells to secrete therapeutic proteins.

INTRODUCTION

Long-lived plasma cells stably reside in the bone marrow for decades and secrete large quantities of antibodies.[1,2] Consequently, plasma cells engineered to produce *de novo* proteins have the potential to be curative therapies for protein deficiency diseases, prophylaxis for infectious diseases, and many other applications. However, the development of plasma cell therapeutics has been limited by technical challenges in the *in vitro* modification, culture, expansion, and differentiation of primary human B cells. B cells can be transduced at high rates by recombinant adenovirus[3] or Epstein-Barr virus[4] (EBV) vectors, which deliver transgenes as episomes. However, episomal DNA expression is lost over time, limiting use of these vectors in applications that require long-term transgene expression. Unlike non-integrating vectors, gamma retrovirus (γRV) and lentivirus (LV) randomly integrate into the host genome and can be used to introduce stably expressing transgenes. However, these vectors are inefficient at transducing primary human B cells.[5,6] LVs that employ alternative envelopes, including that of baboon retrovirus,[7] measles virus,[5,8] or gibbon-ape leukemia virus,[9] exhibit higher B cell transduction rates (up to ~50%) but have low viral titers, which make large-scale production challenging. Because γRV and LV vectors do not efficiently transduce B cells, whereas transduction by non-integrating vectors results in only transient transgene expression, neither platform is currently effective for delivering long-term expression of exogenous genes to B cells on a therapeutic scale.

An alternative method for introducing stable protein expression is genome editing via homology-directed repair (HDR). Following cleavage by an engineered site-specific nuclease, DNA double-strand breaks are resolved through non-homologous end joining (NHEJ), an error-prone DNA repair pathway that typically leads to variable insertions or deletions (indels), or HDR, which repairs DNA by copying a homologous donor template. Delivery of exogenous DNA flanked by DNA homologous to the genomic sequence around the break site can lead to incorporation of the exogenous sequence in a site-specific manner. HDR-mediated genome editing in B cells may have several advantages over viral vector transduction for therapeutic applications, including decreased risk of insertional mutagenesis and sustained transgene expression. We and others have recently achieved high-efficiency HDR delivery of therapeutic transgenes to hematopoietic cells, including primary human T cells and hematopoietic stem cells,[10–12] but similar approaches are yet to be applied in the modification of primary human B cells.
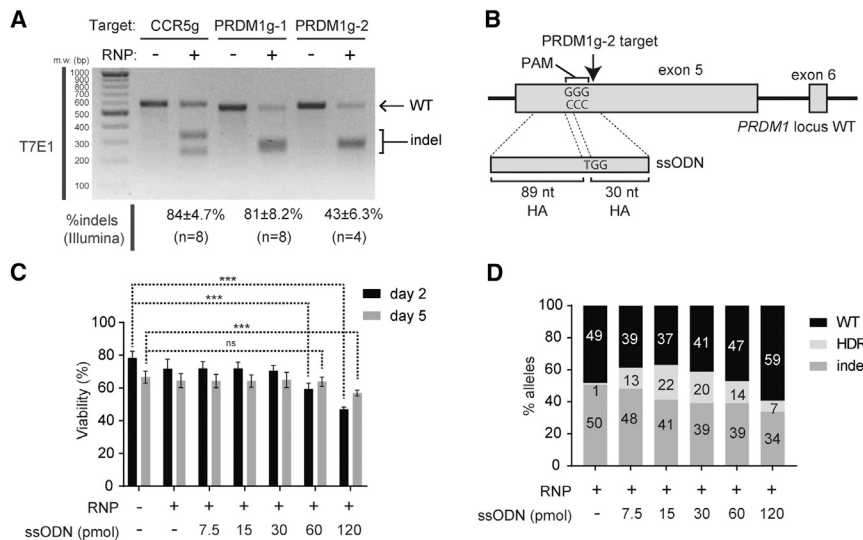
Figure 1. Cas9 RNP Induces Site-Specific Indels or a Precise Single Nucleotide Change in the Presence of an ssODN in Primary Human B Cells

(A) CD19+ B cells were isolated and activated in vitro for 2 days, mock treated or transfected with Cas9 RNPs targeting CCR5 or PRDM1 (CCR5g, PRDM1g-1, or PRDM1g-2), and cultured for 5 additional days. Total genomic DNA was isolated on day 5 and target regions were PCR amplified and analyzed using the T7 endonuclease 1 (T7E1) assay (one representative experiment shown) or Illumina sequencing for percentages of on-target indels (112,000 reads per experimental condition). (B) Diagram of wild-type PRDM1 locus, PRDM1g-2 target location, and the ssODN donor template containing a single nucleotide change. (C and D) B cells were activated for 2 days and mock treated and electroporated with Cas9 RNP-PRDM1g-2 alone or with the ssODN donor template at serially increasing doses from 7.5 pmol to 120 pmol. (C) Viabilities of B cells 2 days and 5 days after genome editing (n = 3, three donors). No significant difference in viability was observed between mock and up to 30 pmol ssODN plus RNP on either day 2 or day 5. Bar graph shows mean ± SEM. (D) Percentage of HDR, indel, and wild-type (WT) alleles in total genomic DNA extracted 5 days after genome editing as assessed by Illumina sequencing (greater than 1 million reads per experimental condition). n represents the number of independent experiments. We used one-way ANOVA with the Sidak correction for multiple comparisons; ***p < 0.001; ns, not significant.

The CRISPR/CRISPR-associated protein 9 (Cas9) system is an RNA-guided nuclease platform that is easily engineered to efficiently target specific sites in the genome for cleavage, generating double-strand DNA breaks.[13,14] The use of site-specific nucleases for gene disruption or HDR in B cells is currently limited to transformed or lymphoma-derived cell lines and murine models and has required plasmid- or LV-based CRISPR/Cas9 delivery.[15–17] Here, we describe high-efficiency genome editing in human peripheral blood B cells (75%–90% gene disruption or 10%–40% HDR) by delivering CRISPR/Cas9 ribonucleoprotein (RNP) complexes alone or in combination with single-stranded DNA oligonucleotide (ssODN) or adeno-associated virus (AAV) repair templates, respectively. We show that edited primary B cells can be subsequently differentiated in culture into plasma cells that produce physiological doses of therapeutic proteins, including human factor IX (FIX).

RESULTS
Optimized Conditions for Expansion of Primary Naive Human B Cells

Rapid cell cycling[18] and/or persistence in the S/G2 phases of the cell cycle[19,20] promote HDR in both cell lines and primary hematopoietic cells. Based on previous reports demonstrating rapid expansion of primary human B cells ex vivo, we initially used a combination of stimulants[21–23] (hereafter called "B cell activation cocktail") that included artificially oligomerized CD40 ligand (MEGACD40L; two linked CD40L trimers) in association with CpG, interleukin-2 (IL-2), IL-10, and IL-15. We isolated primary human CD19+ B cells from peripheral blood mononuclear cells (PBMCs) and cultured them for 13 days with this B cell activation cocktail. This treatment resulted in a ∼36-fold expansion of B cells (Figure S1A) while preserving viability at >60% (Figure S1B). Most cells maintained a naive

B cell phenotype (CD27⁻CD138⁻CD38^{low/−}CD19^{high}IgM⁺IgD⁺; Figure S1C) over the 13-day culture; however, immunoglobulin D (IgD) expression was gradually downregulated (Figure S1C), demonstrating some ex vivo skewing due to stimulation. Together, these data demonstrate that the B cell activation cocktail facilitates rapid cycling of naive and activated primary human B cells.

Cas9-Mediated Disruption of CCR5 and PRDM1 in Primary Human B Cells

To assess the efficiency of Cas9-induced indels in B cells, we designed CRISPR guide RNAs that target CCR5 (which is not expressed in human B cells[24] and has no known significance in plasma cell development) or PRDM1 (encoding BLIMP1, a protein required for B cell differentiation into plasma cells). After optimizing electroporation using mRNA (Figure S2), we transfected 30 pmol Cas9-guide RNP complexes into B cells, cultured them for 5 additional days under activating conditions, and extracted total genomic DNA to assess nuclease-induced indels using the T7 endonuclease 1 assay (Figure 1A). Illumina sequencing confirmed on-target indels and revealed that the RNPs induced high indel frequencies at each guide target site (43%–84%; Figure 1A). Western blot also verified concomitant PRDM1 protein reduction in the PRDM1-expressing TMD8 lymphoma cell line (Figure S3A; ∼65% knockdown). Finally, we found that B cells remained viable despite these genome modifications (Figure S3B). These data show that high-efficiency Cas9-mediated gene disruption is achievable in primary human B cells.

HDR-Mediated Single Nucleotide Substitution at the PRDM1 Locus Using an ssODN Donor Template

Cas9-induced DNA lesions can be seamlessly repaired via the HDR pathway in the presence of a donor template with homology

sequences flanking the lesion.[25] We initially tested an ssODN as a donor template based on promising results in other cell types.[26] We designed a 120-base ssODN containing asymmetric homology arms[26] (89 bases 5′ and 30 bases 3′) that flank the PRDM1g-2 target site, with a single nucleotide change at the 90th position that mutates the last nucleotide of the protospacer adjacent motif (PAM) (GGG to GGT; Figure 1B). This single nucleotide change was designed to both prevent Cas9-mediated cleavage of the repaired sequence and serve as a molecular marker for HDR. In this experiment, primary human B cells were activated for 2 days and then transfected with Cas9 RNP in conjunction with various doses of the ssODN. 2 and 5 days following transfection, cells transfected with ≤30 pmol ssODN had comparable viabilities to Cas9 RNP-transfected control cells (Figure 1C). In cells receiving 15 or 30 pmol ssODN, sequencing of the PRDM1 target region on day 5 post-transfection revealed 20%–22% of alleles had undergone HDR, whereas another 37%–41% had indels (Figure 1D), an overall editing rate that was marginally higher than that observed in the Cas9 RNP control (Figure 1D). Thus, ssODN donor templates can be used to achieve high rates of HDR in primary human B cells, with low cytotoxicity.

Cas9-Mediated Disruption of Genes That Regulate Plasma Cell Development

We next investigated whether Cas9-induced gene disruption can be used to study gene roles in human plasma cell development and antibody production. To do so, we developed an experimental workflow that includes inducing NHEJ-mediated gene disruption in activated naive human B cells using Cas9 RNPs and subsequently differentiating naive B cells into plasma cells (CD19^{low}CD38^{high}CD27^{+}CD138^{+}) using a three-step plasma cell culture system (see Figure 2A, the Materials and Methods section, and previous description[27] for detailed conditions). As a proof of concept, we studied four genes encoding transcription factors previously reported to regulate plasma cell development in murine studies: IRF4 and PRDM1/BLIMP1 are required for plasma cell differentiation[28,29] (Figure 2B), whereas PAX5 and BACH2 antagonize plasma cell differentiation in mice[30,31] (Figure 2B). We designed CRISPR guides targeting each of these four genes, transfected primary B cells with Cas9 RNP targeting each gene independently, and subsequently induced in vitro plasma cell differentiation (Figure 2A). As a control, we also transfected cells with Cas9 RNPs targeting CCR5. Following differentiation, we detected significantly lower percentages of CD19^{low}CD38^{high} and CD27^{+}CD138^{+} plasma cells in cultures transfected with PRDM1- or IRF4-targeting RNPs compared to cells transfected with CCR5-targeting RNP or mock transfected cells (mean indel percentages = 89%, 83%, and 92% at the PRDM1, IRF4, and CCR5 target sites, respectively; Figures 2C and 2D). Because antibody secretion is a major function of plasma cells, we measured IgM and IgG levels in cell culture supernatants by ELISA and found IgG to be significantly decreased in the PRDM1- and IRF4-targeted B cell cultures (Figure 2E). Together, as predicted, these data imply that both PRDM1 and IRF4 are required for human plasma cell differentiation and antibody production.[28,29] Conversely, we observed significant increases in the percentages of plasma cells in cultures transfected with either

PAX5- or BACH2-targeting RNPs (mean indel percentages = 80% and 86% at the PAX5 and BACH2 target sites, respectively; Figures 2C and 2D). We also observed concomitant increases in IgM and IgG secretion in PAX5-targeted cultures (Figure 2E). Again, as predicted, these data indicate that Cas9-mediated disruption of PAX5 or BACH2 enhances human plasma cell differentiation. Further, the IgG/IgM ratio in BACH2-targeted B cell cultures was markedly lower than in mock or CCR5-targeted cultures (Figures 2E and S4A) and plasmablasts in PAX5-targeted cultures exhibited decreased surface CD19 expression (Figure S4B), both consistent with previous studies in mice.[30,32,33] Finally, we confirmed that independent guide RNAs targeting the same genes lead to similar alterations in plasma cell differentiation (Figure S4C). Taken together, these results demonstrate that CRISPR/Cas9-induced gene disruption in primary B cells is useful for interrogating gene products that may modulate human plasma cell development and function.

Site-Specific HDR at the PRDM1 Locus Using Co-delivery of RNP and AAV Donor Template

HDR using ssODN donor templates is not suitable for delivering payloads larger than ~400 bases due to current limitations of the fidelity of ssODN synthesis. In contrast, AAV can package up to ~4.7 kb of the ssDNA donor template. We and others have used AAV to deliver candidate HDR templates, leading to high levels of HDR in multiple cell types and at a variety of loci.[34–36] Of note, there are no published data regarding the capacity of AAV to transduce primary human B cells. To investigate AAV transduction efficiency in human B cells, we designed a self-complementary AAV (scAAV) with a GFP coding sequence driven by MND,[37] a robust retroviral-derived ubiquitous promoter. We transduced activated B cells with this vector, which was packaged using various serotypes, and quantified GFP expression 2 days post transduction by flow cytometry. Regardless of serotype, we observed minimal loss of cell viability following viral exposure (Figure S5A). We observed the highest percentage of GFP^{+} cells (mean = 43%) and highest mean fluorescence intensity in B cells transduced with AAV serotype 6 (Figure 3A).

Because the packaging size of single-stranded AAV (ssAAV) is greater than that of scAAV, we next assessed the efficacy of HDR-mediated integration of larger payloads by delivering ssAAV and Cas9 into B cells. Initially, we designed an AAV6 donor template containing an MND-driven GFP cassette flanked by 400-bp homology arms at the PRDM1g-2 target site (PRDM1-GFP; Figure 3B). The PAM was not included in the repair template, thereby rendering the repaired sequence non-cleavable by Cas9 (Figure 3B). After activation and electroporation with or without Cas9 RNP, B cells were transduced with AAV6 PRDM1-GFP at various MOIs and cultured for 11 additional days with the same activating factors. Although we initially observed a 30% viability loss at the highest AAV dose, these cultures eventually recovered and exhibited comparable viabilities by day 11 (Figure S5B). At the highest AAV MOI, we observed persistent GFP expression in ~10% of cells that received both the PRDM1-targeting RNP and AAV repair template, whereas 2% of cells that were treated with AAV alone had persistent GFP expression
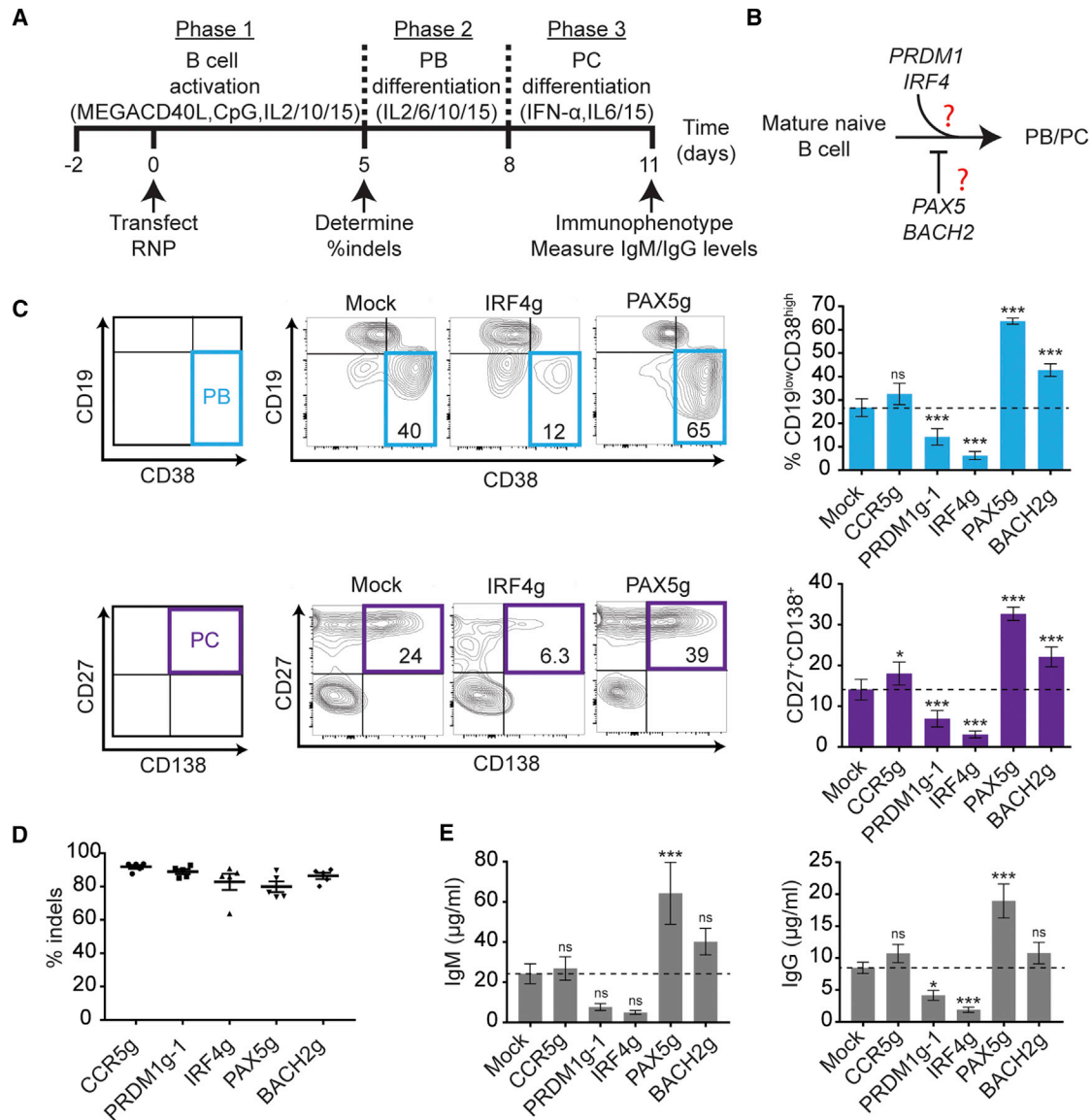
Figure 2. Cas9-Mediated Disruption of PRDM1, IRF4, PAX5, or BACH2 Alters B Cell Phenotype

(A) Experimental workflow of the plasma cell differentiation assay. This workflow includes a three-step differentiation culture that is divided by a B cell activation phase (phase 1), a plasmablast differentiation phase (phase 2), and a plasma cell differentiation phase (phase 3) using the different cocktails of soluble factors and cytokines specified. Cells were transfected with Cas9 RNP after 2 days of initial activation, indels were assessed in genomic DNA on day 5, and phenotypes and IgM/IgG secretion were quantified on day 11. (B) Schematic diagram of the roles of transcription factors PRDM1, IRF4, PAX5, and BACH2 as established by murine studies. (C–E) Primary B cells were activated and either mock treated or transfected with Cas9 RNP targeting CCR5, PRDM1, IRF4, PAX5, or BACH2 and were subsequently differentiated *in vitro*. (C) Top: representative flow plots showing CD19 and CD38 expression in live singlets and bar graph showing mean percentages of CD19lowCD38high plasmablasts 11 days post transfection. Bottom: representative flow plots showing CD27 and CD138 expression and a bar graph showing percentage of CD27+CD138+ plasma cells 11 days post transfection (n = 5, three donors). (D) Genomic DNA was isolated 5 days after RNP transfection for allelic indel analysis by Illumina sequencing (n = 5). (E) Amounts of IgM (left) and IgG (right) in each culture as measured by ELISA (n = 4, three donors). All bar graphs show mean ± SEM. n represents the number of independent experiments. Dotted lines mark mock levels. We used one-way ANOVA with the Sidak correction for multiple comparisons; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; ns, not significant. Statistical comparisons were made compared to mock. IFN-α, interferon-α; PB, plasmablast; PC, plasma cell.

(Figure 3C). In addition, we co-treated cells with the *PRDM1*-targeting RNP and a non-targeting AAV BFP control (MND-BFP without homology arms; BFP-noHA) and observed ∼1% BFP expression at the endpoint (Figure 3C), indicating that the majority of GFP expression resulting from co-delivery of *PRDM1*-targeting RNP and PRDM1-GFP was likely driven by HDR-mediated integration. We
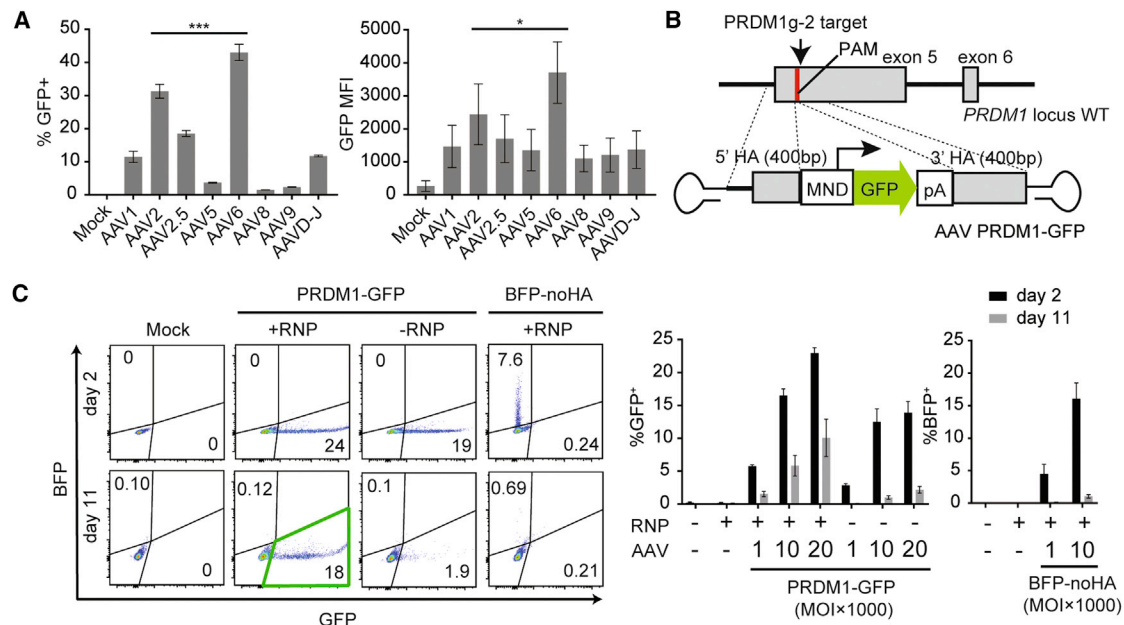
Figure 3. HDR-Mediated GFP Addition at the PRDM1 Locus Using Cas9 RNP and an AAV6 Donor Template Leads to Progressively Increased GFP Expression in a Dose-Dependent Manner

(A) B cells were activated for 2 days and were either mock treated or transduced with the GFP-expressing scAAV, which was packaged using a comprehensive panel of AAV serotypes (1, 2, 2.5, 5, 6, 8, 9, or D-J) at a MOI of 25,000. Shown are the percentages of GFP+ cells (left) and mean fluorescence intensities (right) (n = 3, three donors). (B) Schematic of wild-type PRDM1 locus, PRDM1g-2 target location, and an AAV GFP expression cassette with 400-bp flanking PRDM1 homology arms (AAV PRDM1-GFP). The 3-base PAM sequence is deleted from the AAV template homology sequence. (C) B cells were activated for 2 days and were either mock treated or transfected with Cas9 RNP-PRDM1g-2, with or without AAV transduction. Cells were subsequently cultured under the same activating condition for 11 days. Left: representative flow plots showing BFP and GFP expressions on day 2 and day 11 after genome editing. Green box highlights the GFP+ population in the gene-edited B cells. Right: bar graph showing percentages of GFP+ cells on day 2 and day 11 after gene editing (n = 4, four donors). All bar graphs show mean ± SEM. n represents the number of independent experiments. We used one-way ANOVA with the Sidak correction for multiple comparisons; *p < 0.05; ***p < 0.001; ns, not significant. HA, homology arm; MFI, mean fluorescence intensity; pA, SV40 poly-adenylation signal; PAM, protospacer adjacent motif; WT, wild-type.

also found that higher AAV doses correlated with increased percentages of GFP+ cells. Finally, we found that increasing homology arm lengths from 400 bp to 1.0 kb did not lead to higher levels of HDR (measured as persistent GFP expression; Figures S6A and S6B). Together, these data support the conclusion that co-delivery of Cas9 RNP and AAV can lead to efficient, targeted genomic integration of transgene, likely via the HDR pathway.

Generation of Active FIX-Secreting Human Plasma Cells via HDR-Mediated Integration at the CCR5 Locus

Engineering exogenous protein production in plasma cells may have therapeutic applications in protein deficiency diseases such as hemophilia B, which is caused by a deficiency of FIX. To assess whether primary B cells could be engineered to secrete active proteins with predicted functionality, we used an HDR-mediated knockin strategy to engineer *de novo* expression of FIX. Of note, our HDR studies targeting the *PRDM1* locus (described above) were anticipated to underrepresent HDR efficiencies due to the negative impact of *PRDM1* disruption on plasma B cell differentiation and the relative inefficiency of the guide used in the studies (PRDM1g-2; Figure 1A). Therefore, we targeted the *CCR5* locus as a "safe harbor" because (1) it is not transcriptionally active in human B cells;[24] (2) it is not

required for plasma cell differentiation (Figures 2A–2D); and (3) heterozygous and homozygous null mutations in *CCR5* are innocuous to human carriers.[38] To generate plasma B cells capable of secreting exogenous human FIX, we co-delivered an AAV vector containing an MND-driven FIX (codon-optimized FIX-R338L Padua variant[39]) expression cassette, with the same flanking CCR5 homology arms as described above (CCR5-FIX), into primary human B cells with or without *CCR5*-targeting RNP (Figure 4A). To boost plasma cell differentiation in these cultures, we also co-delivered *PAX5*-targeting RNPs in a subset of CCR5-targeted cells. Although we observed similar HDR rates of ~15%–20% at the *CCR5* locus across conditions (Figure 4B), following plasma cell differentiation, we observed increases in CD19^{low}CD38^{high} plasmablast percentages in the cultures that received both *CCR5*- and *PAX5*-targeting RNPs (Figure 4C). With or without delivery of *PAX5*-targeting RNPs, we detected high levels of *de novo* FIX secretion in the cultures (Figure 4D). In addition, dual-edited HDR cultures co-treated with both *CCR5*- and *PAX5*-targeting RNPs exhibited similar levels of FIX production (Figure 4D).

To assess whether the FIX produced by gene-edited plasma cells is active, we supplemented cultures with vitamin K1 to enable
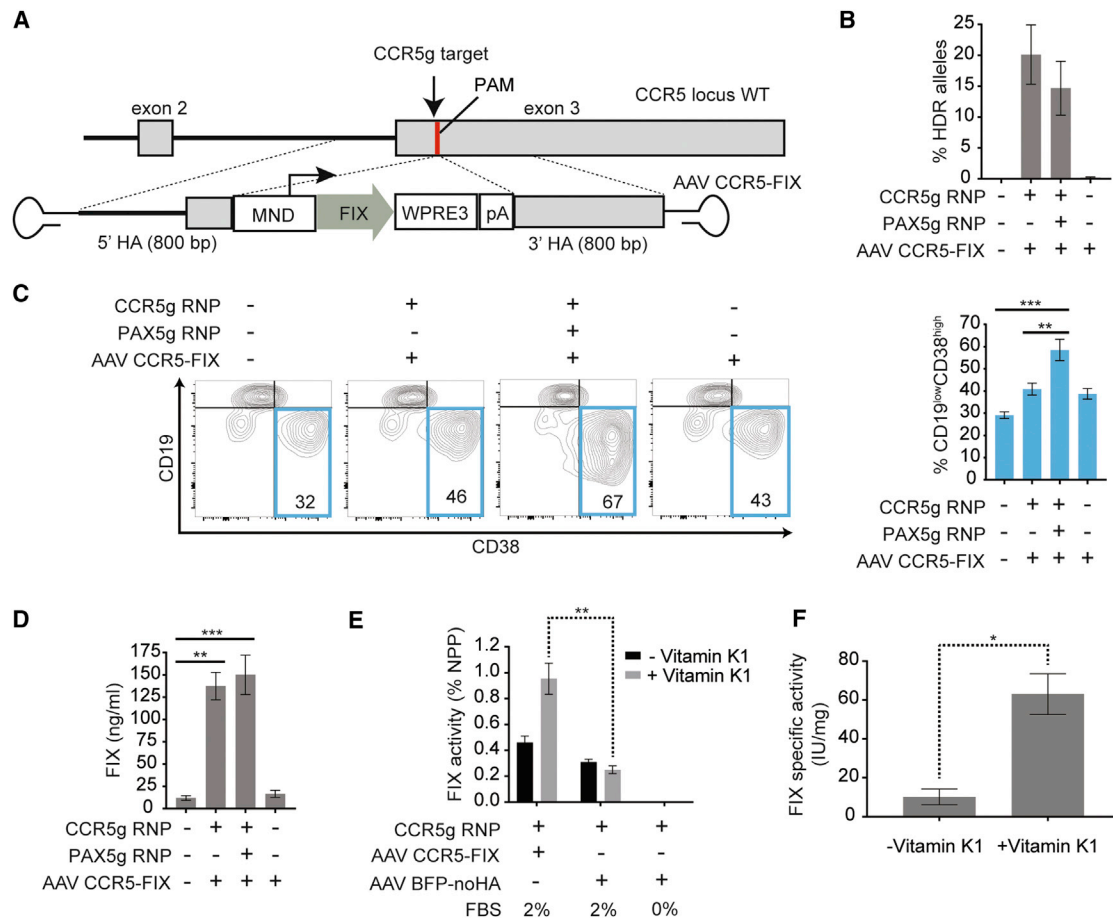
Figure 4. HDR-Mediated Integration of FIX Coding Sequence at the CCR5 Locus Leads to High Levels of FIX Secretion by Gene-Edited Plasma Cells *Ex Vivo*

(A) Schematic of wild-type CCR5 locus, CCR5g target location, and a FIX-expressing AAV construct with 800-bp flanking CCR5 homology arms (AAV CCR5-FIX). (B–D) Primary B cells were gene edited using the AAV CCR5-FIX donor template and CCR5-targeting RNP (CCR5g RNP), with or without PAX5-targeting RNP (PAX5g RNP). B cells were subsequently differentiated *in vitro*. (B) Frequency of on-target FIX integration in total alleles on day 11 after genome editing as assessed by digital droplet PCR (n = 2, two donors). (C) CD19 and CD38 expression on day 11. Left: representative flow plots; right: mean percentages of CD19lowCD38high plasmablasts (n = 3, two donors). (D) FIX production at day 11 after genome editing as measured by ELISA (n = 3, two donors). (E and F) Gene-edited B cells were differentiated *in vitro* as before; at day 8 post genome editing, FBS content was reduced to 2% (unless otherwise specified) and cells were cultured with or without vitamin K1. At day 11, supernatants were collected for FIX chromogenic activity assay and ELISA (n = 3, three donors). Shown are the total FIX activity (E) and FIX-specific activity (F) (specific activity in IU/mg was obtained by dividing net activity values by antigen concentrations). AAV was added at 20,000 MOI. Bar graphs show mean ± SEM. n represents the number of independent experiments. We used one-way ANOVA with the Sidak correction for multiple comparisons (C–E) or paired two-tailed t test for comparison of two groups (F); **p < 0.01; ***p < 0.001. HA, homology arm; pA, SV40 poly-adenylation signal; PAM, protospacer adjacent motif; WPRE3, a shortened woodchuck hepatitis virus posttranscriptional regulatory element 55; WT, wild-type.

vitamin-K-dependent post-translational carboxylation of FIX and subsequently measured FIX activity levels using a chromogenic assay. We found that FIX activity was significantly higher in the vitamin-K1-supplemented, gene-edited plasma cell cultures (Figure 4E). The observed low level of activity in the BFP-noHA negative controls was absent when fetal bovine serum (FBS) was removed from cultures, suggesting that this non-specific assay background originates from FIX present in FBS. Importantly, FIX produced by gene-edited plasma cells exhibited high specific activity (∼63 IU/mg protein) in a vitamin-K-dependent manner (Figure 4F). These data demonstrate production of functional FIX via targeted HDR in gene-edited human

B cells using Cas9 RNP and AAV and illustrate the possibility of multi-locus modifications to simultaneously drive plasma cell differentiation and *de novo* protein secretion.

Engineered BAFF Secretion via HDR Improves Plasma Cell Survival *In Vitro*

In order to use gene-edited plasma cells in a therapeutic setting, their long-term survival will be required. One strategy to improve plasma cell survival is to engineer cells that secrete survival factors in an autocrine manner. As a first test of this approach, we used our HDR-mediated knockin strategy to engineer *de novo* expression of the

B cell activating factor (BAFF).[40] We designed two AAV6 repair templates: one contained MND-driven GFP and BAFF linked with a T2A self-cleaving peptide (CCR5-GFP-BAFF) and the other contained only an MND-BAFF expression cassette (CCR5-BAFF). Both donor constructs contained 800-bp homology arms around the *CCR5* guide target site (Figure 5A). Following RNP transfection and/or AAV transduction, we cultured cells using the three-step plasma cell culture system (Figure 2A). As in earlier *PRDM1*-targeting experiments, we observed persistent GFP expression (mean = 33% GFP$^+$) only in the condition containing both *CCR5*-targeting RNP and AAV (Figures 5B and S7). Regardless of the template used, we observed similar rates of HDR as quantified by digital PCR (~28%–31% HDR; Figure 5C). Although use of both donor templates led to BAFF secretion, B cells targeted by CCR5-BAFF (repair template solely containing BAFF) exhibited much higher levels of BAFF secretion (4.1-fold increase) relative to those targeted by CCR5-GFP-BAFF (template containing BAFF downstream of the T2A linkage) (Figure 5D). To determine if this exogenous BAFF is functional, we assessed cell expansion and plasmablast formation following genome editing and *in vitro* differentiation. In the CCR5-BAFF edited culture that contained the highest concentration of exogenous BAFF, we observed increases in cell number, viability, and percentage of CD19^{low}CD38^{high} plasmablasts relative to controls that lacked Cas9 RNP (Figures 5E and 5F). This is consistent with previous studies showing that BAFF promotes both human and murine B cell survival and differentiation.[40] Taken together, these results show that Cas9 RNP- and AAV-mediated genome editing can be used to generate functional, BAFF-secreting human plasma cells with improved *in vitro* survival.

BAFF-Expression Human Plasma Cells Exhibit Sustained Secretory Capabilities in NSG Mice

We next tested whether autocrine BAFF secretion might promote human plasma cell survival *in vivo* in immunodeficient NOD/SCID/gamma-c null (NSG) mice. We first generated gene-edited B cells using co-delivery of *CCR5*-targeting RNP and the CCR5-GFP-BAFF AAV donor template. Edited cells were differentiated into plasma cells *in vitro* using the three-step culture system and adoptively transferred into NSG mice via intravenous infusion (Figure 6A). In parallel, we transferred equal numbers of unedited plasma cells into a separate cohort of NSG recipients and also evaluated control unmanipulated animals. Serum samples were collected at days 10 and 21, and human protein was quantified by ELISA (Figure 6A). As expected, human BAFF was detected only in mice that received BAFF-expressing cells (Figure 6B). Human IgM and IgG were detected only in mice that received edited or unedited human plasma cells and not in control mice (Figure 6C). Notably, serum BAFF and IgM levels remained stable from day 10 to day 21 and human IgG levels progressively increased during this period only in recipients of BAFF-expressing edited plasma cells (Figures 6B and 6C). Most strikingly, we observed significantly higher levels of IgM and IgG in mice that received BAFF-expressing plasma cells (Figure 6C; difference in IgG levels at day 10 was not significant). These findings demonstrate that gene-modified plasma cells can maintain stable secretory capacities *in vivo* for at least 3 weeks and support our

hypothesis that expression of a human-relevant survival factor via genome editing can promote plasma cell secretory function and/or survival *in vivo*.

DISCUSSION

Here, we report high rates of gene disruption in primary human B cells at multiple loci using CRISPR/Cas9 RNPs. We apply this method to modeling the impact of genetic changes on human plasma B cell differentiation. We also demonstrate the use of Cas9 RNP in combination with ssODN or AAV6 repair templates to achieve high-efficiency HDR in B cells and engineer secretion of functional and therapeutically relevant proteins. Importantly, gene-modified cells obtained a plasma cell phenotype following 13 days of culture (including 2 days pre-editing) *in vitro* and are detectable in humanized mice for at least 3 weeks, suggesting the potential for the future development of adoptive cell therapies using engineered human plasma cells.

Cas9-mediated disruption of *PRDM1*, *IRF4*, *PAX5*, or *BACH2*, which either promote (*PRDM1* and IRF4) or inhibit (*PAX5* and *BACH2*) plasma cell differentiation in mice,[28–31] led to human B cell phenotypes consistent with these regulatory roles. The ability to easily disrupt genes or induce single-base changes using Cas9 RNP with or without ssODN-mediated HDR will likely facilitate further study of the impact of genetic changes on human B cell development. When combined with differentiation *in vitro*, this system could be used as a high-throughput model for studying genetic mutations that alter human B cell development, including somatic and/or germ-line mutations associated with a range of human B cell disorders (in B cell lymphoma and in autoimmune disorders, including systemic lupus erythematosus). It will enable a range of mechanistic studies designed to assess candidate genes identified by genome-wide association studies.

Our development of a method for the efficient, site-specific introduction of transgenes via HDR opens the possibility of engineering plasma cells to act as autologous "cell factories" capable of delivering sustained, high doses of therapeutic proteins to patients. As a proof of concept, we engineered plasma cells to secrete FIX. Deficiency of FIX is the cause of hemophilia B, a genetic disease characterized by blood clotting defects. Current treatment for hemophilia B is limited to protein replacement therapy, which is costly and non-curative. Recent gene therapy trials reported long-term FIX expression in patients after intravenous injection of a liver-tropic AAV8-FIX vector;[41,42] however, pre-existing AAV-neutralizing antibodies to all known AAV serotypes are prevalent in humans[43–45] and greatly limit *in vivo* AAV transduction, making a significant group of patients with anti-AAV antibodies ineligible for treatment.[41] In addition, systemic AAV delivery induces humoral immunity against AAV that prevents subsequent delivery, thus limiting AAV therapy to a single dose and/or necessitating use of alternative serotypes. Delivery of *ex vivo* gene-edited plasma cells producing FIX would remove the requirement for systemic AAV delivery and likely avoid limitations posed by AAV-neutralizing antibodies.
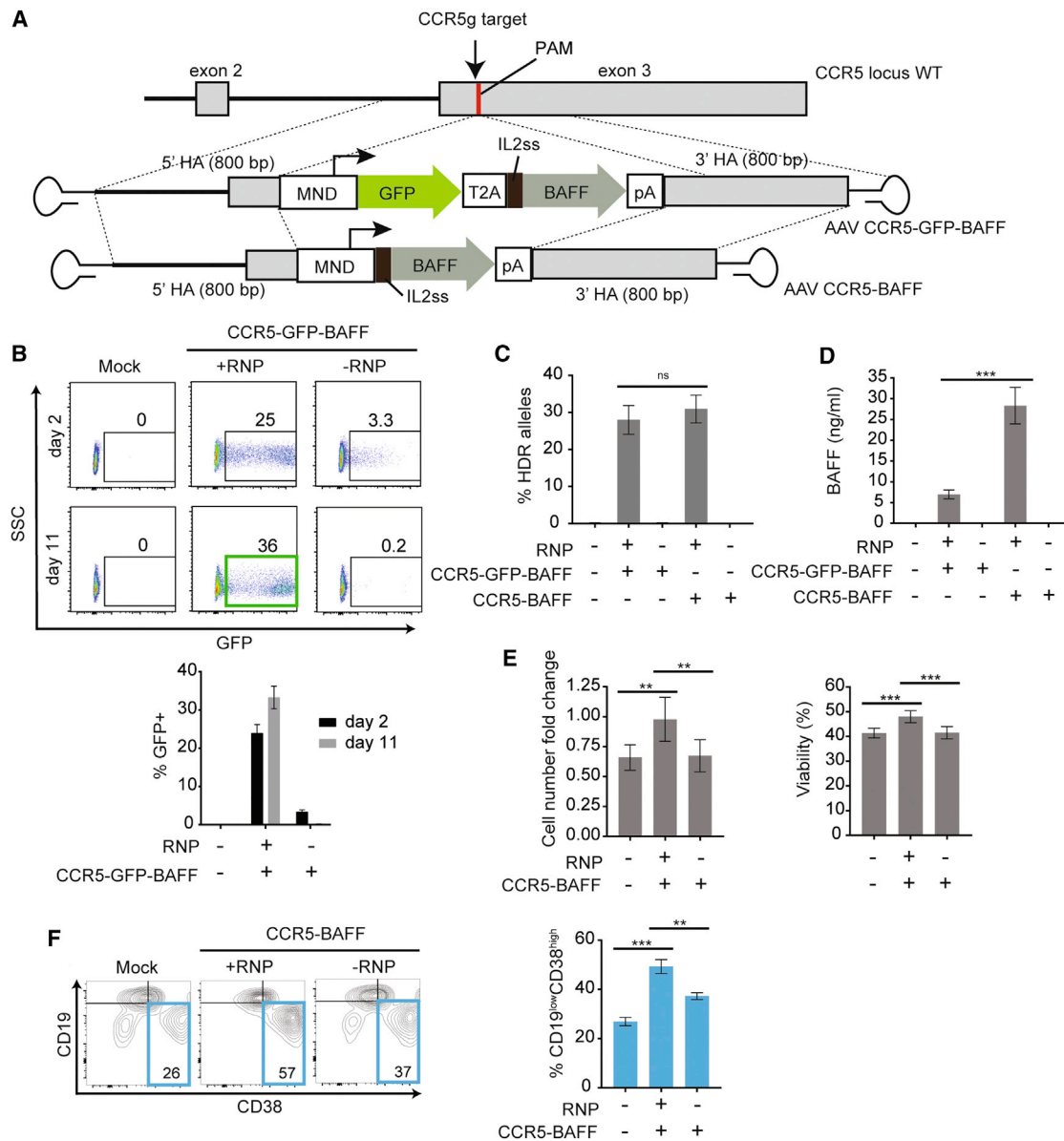
Figure 5. HDR-Mediated Integration of BAFF Coding Sequence at the CCR5 Locus Results in Persistent BAFF Secretion by Gene-Edited Plasma Cells and Increases Plasma Cell Differentiation and Viability

(A) Schematic of wild-type CCR5 locus, CCR5g target location, an AAV construct that co-expresses GFP and BAFF via T2A linkage (AAV CCR5-GFP-BAFF), and a BAFF-expressing AAV construct (AAV CCR5-BAFF) with identical 800-bp flanking CCR5 homology arms. (B–F) B cells were gene edited after 2 days of *in vitro* activation, and were subsequently differentiated into plasma cells using the three-step culture system. (B) Upper: representative flow plots showing GFP expression on day 2 and day 11 post gene editing in mock, CCR5-GFP-BAFF transduced cells, with or without Cas9 RNP. Lower: bar graph summarizing percentages of GFP+ cells on day 2 and day 11 post gene editing (n = 4, three donors). (C) Frequency of on-target donor template integration in total alleles on day 11 after genome editing as assessed by digital droplet PCR (n = 3, two donors). (D) Bar graph shows BAFF production as measured by ELISA at day 11 (n = 4, three donors). (E) Left: cells were counted at day 5 and day 11 post genome editing. Bar graph shows fold changes in cell numbers. Right: viabilities at day 11 by flow cytometry (n = 5, four donors). (F) Left: representative flow plots showing CD19 and CD38 expression at day 11 after genome editing using the AAV CCR5-BAFF vector. Right: bar graph summarizing percentages of CD19lowCD38high plasmablasts/plasma cells at day 11 (n = 5, four donors). Both AAV CCR5-GFP-BAFF and CCR5-BAFF were added at 20,000 MOI. All bar graphs show mean ± SEM. n represents the number of independent experiments. We used one-way ANOVA with the Sidak correction for multiple comparisons; **p < 0.01; ***p < 0.001. HA, homology arm; IL-2ss, IL-2 signal sequence; pA, SV40 poly-adenylation signal; PAM, protospacer adjacent motif; WT, wild-type.
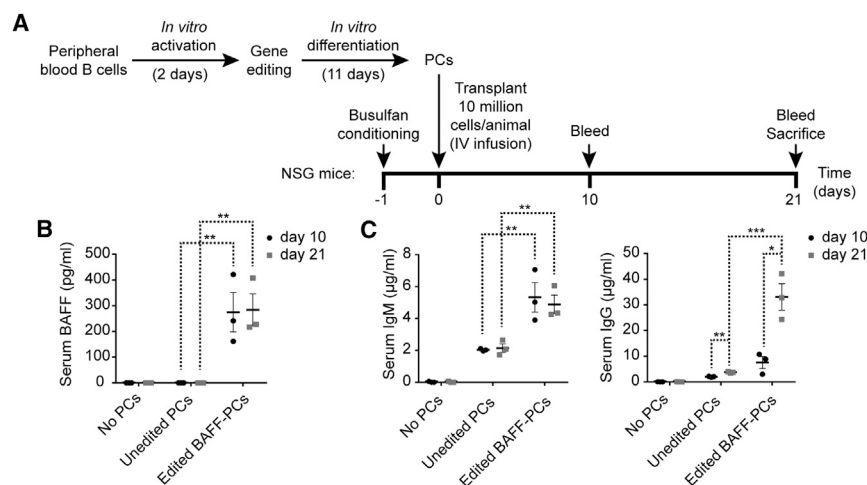
Figure 6. Gene-Edited, BAFF-Expressing Human Plasma Cells Stably Secrete BAFF and Immunoglobulins in NSG Mice

(A) Experimental layout of NSG mouse transplant. Gene-edited B cells expressing human BAFF were generated as before after 2 days of *in vitro* B cell activation and were subsequently differentiated into plasma cells using the three-step culture system. 11 days post genome editing, edited or control cells (10 million cells/animal) were delivered intravenously into busulfan-conditioned, NSG mice. A control animal cohort (no PCs) was also evaluated as noted. Blood samples were collected at day 10 and at day 21 when mice were sacrificed. (B and C) Serum proteins were quantified by ELISA (n = 3/cohort). Shown are serum BAFF levels (B) and serum human IgM and IgG levels (C). All graphs represent mean ± SEM. We used one-way ANOVA with the Sidak correction for multiple comparisons between groups and paired two-tailed t test for comparisons between two time points (day 10 versus day 21); *p < 0.05; **p < 0.01; ***p < 0.001. IV, intravenous; NSG, NOD/SCID/gamma-c null; PCs, plasma cells.

In addition, immune responses to therapeutic proteins comprise a key unaddressed challenge. Based on evidence that B cell delivery can induce tolerance,[46–48] an additional application of B cell editing technology would be to deliver smaller numbers of short-lived B cells producing FIX, or other relevant immunogenic proteins, with the goal of inducing tolerance to the secreted product. This approach would be useful alone as well as in a setting in which it might be paired with co-delivery of gene-edited plasma cells, producing a therapeutic protein anticipated to provoke an unwanted immune response.

In this proof of principle study, we have only begun to assess the *in vivo* stability, homing, and safety of engineered plasma cells. Our engraftment studies contrast with the majority of those previously reported using human cells in NSG and other immunodeficient murine models in that we have attempted to transplant mature cells into adult mice. In most other successful studies, human stem cells have been engrafted into humanized murine models, at times including co-engraftment with human fetal liver or thymus into neonatal animals. In these cases, CD34+ stem cells can differentiate into B cells and even mature B cells.[49] However, the majority of the engrafted cells remain in the transitional and/or immature developmental stages. In particular, development of PCs post-immune challenge has met with very limited success and can be increased by the provision of additional human cytokines.[49–51] Consistent with these ideas, our demonstration that engineering mature B cells to express human BAFF can confer engraftment advantage parallels studies showing that access to human cytokines, including IL-6,[49] SIRPA,[49] or T cell factors,[52] promote B cell maturation and the ability to respond to immunization in mice. Radbruch and colleagues have shown that it is possible to transfer mature murine plasma B cells into immune-deficient mice and that these cells can home to the spleen and bone marrow and stably produce functional antibody for at least 21 weeks.[53] For us to perform similar studies using human mature plasma cells, we will need to determine the key human growth or survival factors that promote and maintain a human plasma cell niche in murine models.

One important question is how many engrafted engineered plasma cells will be required to deliver therapeutic levels of an exogenous protein like FIX. Based on a study that quantified serum antibody levels and the number of antigen-specific plasma cells in immunized mice, a single antibody-secreting plasma cell can stably maintain ~50 ng serum IgG.[1,54] In patients with severe hemophilia B, 3% (or 30 IU/L) of persistent FIX is considered therapeutic,[42] which roughly translates into ~2.5 mg FIX per adult subject (based on 5 L blood). Consequently, if we were able to deliver a plasma cell that produced FIX at the same rate as an anti-tetanus secreting plasma cell, we would require ~50,000 engineered cells to deliver a therapeutic dose. The present rate of production in our plasma cell cultures is considerably lower than that of an anti-tetanus antibody-secreting cell: ~1–10 pg/edited cell per day. Therefore, given our current protein production levels, we would require engraftment of a much larger number of edited cells to reach therapeutic levels of FIX. However, we anticipate that simple changes to our methods, including elimination of competing secreted antibody, use of a plasma cell locus, and enrichment for edited plasma cells will substantially increase protein production per gene-edited cell and, therefore, the translational potential for this approach.

In summary, our study demonstrates high rates of locus-specific genome editing in primary human B cells. These engineered B cells retain the ability to differentiate into plasma cells *ex vivo* and secrete physiological doses of therapeutic proteins such as FIX. The ability to efficiently target specific loci enables us to drive B cell differentiation and secretory programs. Thus, creative multiplexing of gene disruption and HDR will likely provide ever more powerful tools to coordinately optimize protein secretion, cellular phenotype, and long-term survival.

MATERIALS AND METHODS
CRISPR/Cas9 Reagents and ssODNs
CRISPR RNAs (crRNAs) targeting the CCR5, PRDM1, IRF4, PAX5, and BACH2 loci (sequences in Table S1) were identified using the MIT CRISPR design tool (http://crispr.mit.edu/) and the Broad Institute single guide RNA (sgRNA) design tool (http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) and synthesized (IDT) containing phosphorothioate linkages and 2′O-methyl modifications.[26] ssODNs were commercially synthesized by IDT (Ultramer DNA Oligonucleotides) with phosphorothioate linkages. crRNA and trans-activating crRNA (tracrRNA; IDT) hybrids were mixed with Cas9 nuclease (IDT) at a 1.2:1 ratio and delivered with or without ssODNs to cells by Neon electroporation (Thermo Fisher Scientific).

Production of Recombinant AAV Vectors
AAV stocks were produced as previously described.[34] The AAV vector, serotype helper, and HgT1-adeno helper plasmids were transfected into HEK293T cells. Cells were harvested 48 hr later and lysed by 3 freeze-thaw cycles, and the cell lysate was treated with benzonase. Virions with recombinant AAV genomes were purified using an iodixanol density gradient. All MOI calculations were based on qPCR-based titers of AAV genomes using inverted terminal repeat (ITR)-specific primers and probe.[55] Details of the individual vectors are available in the Supplemental Materials and Methods.

Samples and Primary Human B Cell Genome Editing
PBMCs were collected from whole blood of consented donors and cryopreserved at the Fred Hutchinson Cancer Research Center. CD19+ B cells were subsequently isolated by negative selection (Pan-B cell kit, Miltenyi Biotec) and cultured in Iscove's modified Dulbecco's medium (IMDM) (Thermo Fisher Scientific) supplemented with 10% FBS and 55 μM beta-mercaptoethanol at 1×10^6 cell/mL to 1.5×10^6 cells/mL. B cells were activated with 100 ng/mL recombinant human MEGACD40L (Enzo Life Sciences), 1 μg/mL CpG oligodeoxynucleotide 2006 (Invitrogen), 50 ng/mL IL-2 (PeproTech), 50 ng/mL IL-10 (PeproTech), and 10 ng/mL IL-15 (PeproTech) for 2 days. Cells were then electroporated with Cas9 RNP complexes (see Supplemental Materials and Methods).

Flow Cytometry
Flow cytometric analysis was done on an LSR II flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star). Flow cytometry gating for fluorescent proteins (Figure S8A), viability (Figure S8B), and immunophenotyping (Figure S8C) are described in the Supplemental Materials and Methods.

Plasma Cell Differentiation Culture
Plasma cells were differentiated in vitro using a three-step culture system as previously described.[27] CD19+ B cells were activated and gene edited as outlined above and expanded for 5 days in the same cocktail. Following washing with 1X PBS, the cells were seeded in medium with IL-2 (50 ng/mL), IL-6 (50 ng/mL), IL-10 (50 ng/mL), and IL-15 (10 ng/mL) for 3 days. Next, the cells were washed with 1X PBS and seeded in medium with IL-6 (50 ng/mL), IL-15 (10 ng/mL), and human interferon-α 2B (15 ng/mL, Sigma-Aldrich) for 3 days to stimulate plasma cell differentiation.

ELISA
3 days prior to measuring culture protein levels, cells were collected, washed with PBS, and then resuspended in new culture medium at 1×10^6 cells/mL. After 3 days, the culture supernatant was collected and protein secretion levels were determined by ELISA for IgG and IgM (Ready-SET-GO, eBioscience, San Diego, CA), BAFF (BAFF/BLyS/Quantikine ELISA Kit, R&D Systems, Minneapolis, MN), and FIX (FIX Human ELISA Kit, Abcam, Cambridge, MA).

FIX Chromogenic Assay
Activity of recombinant FIX expressed by gene-edited B cells was assessed using a chromogenic assay (Rox Factor IX, 900020). The procedure was performed following the manufacturer's instructions. Absorbance values were measured using a VICTOR3 plate reader (PerkinElmer). A human normal pooled plasma standard was used to establish the calibration curve.

To assess FIX activity in edited plasma cell cultures, B cells were edited and differentiated in vitro. At day 8 post genome editing, FBS content was reduced from 10% to 2% of culture to reduce assay background. Insulin, transferrin, and sodium selenite (Thermo Fisher Scientific) were added to cultures to maintain cell survival in the reduced FBS environment. Finally, in specified cultures that were supplemented with vitamin K1 (Sigma-Aldrich), 5 μg/mL vitamin K1 was added to the medium for generating active FIX via vitamin-K-dependent carboxylation. 0.005% was used to increase solubility of vitamin K1.

NSG Mouse Transplant
NSG mice were purchased from Jackson Laboratory and maintained in a designated pathogen-free facility at the Seattle Children's Research Institute (SCRI). All animal studies were performed according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) standards and were approved by the SCRI Institutional Animal Care and Use Committee (IACUC).

In our NSG transplant experiment, NSG mice were conditioned with 25 mg/kg Busulfan (Selleckchem) via intraperitoneal injection. 24 hr after conditioning, 10×10^6 in vitro differentiated plasma B cells, either mock or edited BAFF expressing, were delivered into each 6- to 8-week-old NSG recipient via retro-orbital infusion. Mice were bled at day 10 and sacrificed at day 21 post infusion. All peripheral blood samples were collected in serum separator tubes for serum collection.

Statistical Analysis and Data Availability
Statistical analyses were performed using GraphPad Prism 7 (GraphPad, San Diego, CA). Because there wasn't obvious skewing in any of the conditions and there was minimal variation within

conditions, we assumed all data followed a normal distribution. p values in multiple comparisons were calculated using one-way ANOVA with the Sidak correction; p values in comparisons between two groups were calculated using a paired two-tailed t test. Values from independent experiments are shown as mean ± SEM. The accession number for all sequencing data is NCBI Sequence Read Archive: SRP113557.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods, nine figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.ymthe.2017.11.012.

REFERENCES

1. Radbruch, A., Muehlinghaus, G., Luger, E.O., Inamine, A., Smith, K.G., Dörner, T., and Hiepe, F. (2006). Competence and competition: the challenge of becoming a long-lived plasma cell. Nat. Rev. Immunol. 6, 741–750.

2. Slifka, M.K., Antia, R., Whitmire, J.K., and Ahmed, R. (1998). Humoral immunity due to long-lived plasma cells. Immunity 8, 363–372.

3. Kim, E.K., Seo, H.S., Chae, M.J., Jeon, I.S., Song, B.Y., Park, Y.J., Ahn, H.M., Yun, C.O., and Kang, C.Y. (2014). Enhanced antitumor immunotherapeutic effect of B-cell-based vaccine transduced with modified adenoviral vector containing type 35 fiber structures. Gene Ther. 21, 106–114.

4. Hellebrand, E., Mautner, J., Reisbach, G., Nimmerjahn, F., Hallek, M., Mocikat, R., and Hammerschmidt, W. (2006). Epstein-Barr virus vector-mediated gene transfer into human B cells: potential for antitumor vaccination. Gene Ther. 13, 150–162.

5. Frecha, C., Costa, C., Lévy, C., Nègre, D., Russell, S.J., Maisner, A., Salles, G., Peng, K.W., Cosset, F.L., and Verhoeyen, E. (2009). Efficient and stable transduction of resting B lymphocytes and primary chronic lymphocyte leukemia cells using measles virus gp displaying lentiviral vectors. Blood 114, 3173–3180.

6. Serafini, M., Naldini, L., and Introna, M. (2004). Molecular evidence of inefficient transduction of proliferating human B lymphocytes by VSV-pseudotyped HIV-1-derived lentivectors. Virology 325, 413–424.

7. Levy, C., Fusil, F., Amirache, F., Costa, C., Girard-Gagnepain, A., Negre, D., Bernadin, O., Garaulet, G., Rodriguez, A., Nair, N., et al. (2016). Baboon envelope pseudotyped lentiviral vectors efficiently transduce human B cells and allow active factor IX B cell secretion in vivo in NOD/SCIDγ−/− mice. J. Thromb. Haemost. 14, 2478–2492.

8. Lévy, C., Amirache, F., Costa, C., Frecha, C., Muller, C.P., Kweder, H., Buckland, R., Cosset, F.L., and Verhoeyen, E. (2012). Lentiviral vectors displaying modified measles virus gp overcome pre-existing immunity in in vivo-like transduction of human T and B cells. Mol. Ther. 20, 1699–1712.

9. Mock, U., Thiele, R., Uhde, A., Fehse, B., and Horn, S. (2012). Efficient lentiviral transduction and transgene expression in primary human B cells. Hum. Gene Ther. Methods 23, 408–415.

10. Hale, M., Mesojednik, T., Romano Ibarra, G.S., Sahni, J., Bernard, A., Sommer, K., Scharenberg, A.M., Rawlings, D.J., and Wagner, T.A. (2017). Engineering HIV-resistant, anti-HIV chimeric antigen receptor T cells. Mol. Ther 25, 570–579.

11. Dever, D.P., Bak, R.O., Reinisch, A., Camarena, J., Washington, G., Nicolas, C.E., Pavel-Dinu, M., Saxena, N., Wilkens, A.B., Mantri, S., et al. (2016). CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. Nature 539, 384–389.

12. Sather, B.D., Romano Ibarra, G.S., Sommer, K., Curinga, G., Hale, M., Khan, I.F., Singh, S., Song, Y., Gwiazda, K., Sahni, J., et al. (2015). Efficient modification of CCR5 in primary human hematopoietic cells using a megaTAL nuclease and AAV donor template. Sci. Transl. Med. 7, 307ra156.

13. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science 339, 823–826.

14. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., and Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823.

15. Chu, V.T., Graf, R., Wirtz, T., Weber, T., Favret, J., Li, X., Petsch, K., Tran, N.T., Sieweke, M.H., Berek, C., et al. (2016). Efficient CRISPR-mediated mutagenesis in primary immune cells using CrispRGold and a C57BL/6 Cas9 transgenic mouse line. Proc. Natl. Acad. Sci. USA 113, 12514–12519.

16. Pogson, M., Parola, C., Kelton, W.J., Heuberger, P., and Reddy, S.T. (2016). Immunogenomic engineering of a plug-and-(dis)play hybridoma platform. Nat. Commun. 7, 12535.

17. Cheong, T.C., Compagno, M., and Chiarle, R. (2016). Editing of mouse and human immunoglobulin genes by CRISPR-Cas9 system. Nat. Commun. 7, 10934.

18. Gwiazda, K.S., Grier, A.E., Sahni, J., Burleigh, S.M., Martin, U., Yang, J.G., Popp, N.A., Krutein, M.C., Khan, I.F., Jacoby, K., et al. (2016). High efficiency CRISPR/Cas9-mediated gene editing in primary human T-cells using mutant adenoviral E4orf6/E1b55k "helper" proteins. Mol. Ther. 24, 1570–1580.

19. Lin, S., Staahl, B.T., Alla, R.K., and Doudna, J.A. (2014). Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. eLife 3, e04766.

20. Heyer, W.D., Ehmsen, K.T., and Liu, J. (2010). Regulation of homologous recombination in eukaryotes. Annu. Rev. Genet. 44, 113–139.

21. Jiang, W., Lederman, M.M., Harding, C.V., Rodriguez, B., Mohner, R.J., and Sieg, S.F. (2007). TLR9 stimulation drives naïve B cells to proliferate and to attain enhanced antigen presenting function. Eur. J. Immunol. 37, 2205–2213.

22. Armitage, R.J., Macduff, B.M., Eisenman, J., Paxton, R., and Grabstein, K.H. (1995). IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. J. Immunol. 154, 483–490.

23. Armitage, R.J., Macduff, B.M., Spriggs, M.K., and Fanslow, W.C. (1993). Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. J. Immunol. 150, 3671–3680.

24. Rottman, J.B., Ganley, K.P., Williams, K., Wu, L., Mackay, C.R., and Ringler, D.J. (1997). Cellular localization of the chemokine receptor CCR5. Correlation to cellular targets of HIV-1 infection. Am. J. Pathol. *151*, 1341–1351.

25. Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. *8*, 2281–2308.

26. Richardson, C.D., Ray, G.J., DeWitt, M.A., Curie, G.L., and Corn, J.E. (2016). Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat. Biotechnol. *34*, 339–344.

27. Jourdan, M., Caraux, A., De Vos, J., Fiol, G., Larroque, M., Cognot, C., Bret, C., Duperray, C., Hose, D., and Klein, B. (2009). An in vitro model of differentiation of memory B cells into plasmablasts and plasma cells including detailed phenotypic and molecular characterization. Blood *114*, 5173–5181.

28. Ochiai, K., Maienschein-Cline, M., Simonetti, G., Chen, J., Rosenthal, R., Brink, R., Chong, A.S., Klein, U., Dinner, A.R., Singh, H., and Sciammas, R. (2013). Transcriptional regulation of germinal center B and plasma cell fates by dynamical control of IRF4. Immunity *38*, 918–929.

29. Shaffer, A.L., Lin, K.I., Kuo, T.C., Yu, X., Hurt, E.M., Rosenwald, A., Giltnane, J.M., Yang, L., Zhao, H., Calame, K., and Staudt, L. (2002). Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity *17*, 51–62.

30. Muto, A., Ochiai, K., Kimura, Y., Itoh-Nakadai, A., Calame, K.L., Ikebe, D., Tashiro, S., and Igarashi, K. (2010). Bach2 represses plasma cell gene regulatory network in B cells to promote antibody class switch. EMBO J. *29*, 4048–4061.

31. Nera, K.P., Kohonen, P., Narvi, E., Peippo, A., Mustonen, L., Terho, P., Koskela, K., Buerstedde, J.M., and Lassila, O. (2006). Loss of Pax5 promotes plasma cell differentiation. Immunity *24*, 283–293.

32. Muto, A., Tashiro, S., Nakajima, O., Hoshino, H., Takahashi, S., Sakoda, E., Ikebe, D., Yamamoto, M., and Igarashi, K. (2004). The transcriptional programme of antibody class switching involves the repressor Bach2. Nature *429*, 566–571.

33. Kozmik, Z., Wang, S., Dörfler, P., Adams, B., and Busslinger, M. (1992). The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. Mol. Cell. Biol. *12*, 2662–2672.

34. Khan, I.F., Hirata, R.K., and Russell, D.W. (2011). AAV-mediated gene targeting methods for human cells. Nat. Protoc. *6*, 482–501.

35. Hirata, R., Chamberlain, J., Dong, R., and Russell, D.W. (2002). Targeted transgene insertion into human chromosomes by adeno-associated virus vectors. Nat. Biotechnol. *20*, 735–738.

36. Russell, D.W., and Hirata, R.K. (1998). Human gene targeting by viral vectors. Nat. Genet. *18*, 325–330.

37. Challita, P.M., Skelton, D., el-Khoueiry, A., Yu, X.J., Weinberg, K., and Kohn, D.B. (1995). Multiple modifications in cis elements of the long terminal repeat of retroviral vectors lead to increased expression and decreased DNA methylation in embryonic carcinoma cells. J. Virol. *69*, 748–755.

38. Liu, R., Paxton, W.A., Choe, S., Ceradini, D., Martin, S.R., Horuk, R., MacDonald, M.E., Stuhlmann, H., Koup, R.A., and Landau, N.R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell *86*, 367–377.

39. Simioni, P., Tormene, D., Tognin, G., Gavasso, S., Bulato, C., Iacobelli, N.P., Finn, J.D., Spiezia, L., Radu, C., and Arruda, V.R. (2009). X-linked thrombophilia with a mutant factor IX (factor IX Padua). N. Engl. J. Med. *361*, 1671–1675.

40. Mackay, F., and Browning, J.L. (2002). BAFF: a fundamental survival factor for B cells. Nat. Rev. Immunol. *2*, 465–475.

41. Nathwani, A.C., Reiss, U.M., Tuddenham, E.G., Rosales, C., Chowdary, P., McIntosh, J., Della Peruta, M., Lheriteau, E., Patel, N., Raj, D., et al. (2014). Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N. Engl. J. Med. *371*, 1994–2004.

42. Nathwani, A.C., Tuddenham, E.G., Rangarajan, S., Rosales, C., McIntosh, J., Linch, D.C., Chowdary, P., Riddell, A., Pie, A.J., Harrington, C., et al. (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N. Engl. J. Med. *365*, 2357–2365.

43. Calcedo, R., Morizono, H., Wang, L., McCarter, R., He, J., Jones, D., Batshaw, M.L., and Wilson, J.M. (2011). Adeno-associated virus antibody profiles in newborns, children, and adolescents. Clin. Vaccine Immunol. *18*, 1586–1588.

44. Li, C., Narkbunnam, N., Samulski, R.J., Asokan, A., Hu, G., Jacobson, L.J., Manco-Johnson, M.J., and Monahan, P.E.; Joint Outcome Study Investigators (2012). Neutralizing antibodies against adeno-associated virus examined prospectively in pediatric patients with hemophilia. Gene Ther. *19*, 288–294.

45. Boutin, S., Monteilhet, V., Veron, P., Leborgne, C., Benveniste, O., Montus, M.F., and Masurier, C. (2010). Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. Hum. Gene Ther. *21*, 704–712.

46. Skupsky, J., Zhang, A.H., Su, Y., and Scott, D.W. (2010). B-cell-delivered gene therapy induces functional T regulatory cells and leads to a loss of antigen-specific effector cells. Mol. Ther *18*, 1527–1535.

47. Lei, T.C., and Scott, D.W. (2005). Induction of tolerance to factor VIII inhibitors by gene therapy with immunodominant A2 and C2 domains presented by B cells as Ig fusion proteins. Blood *105*, 4865–4870.

48. Melo, M.E., Qian, J., El-Amine, M., Agarwal, R.K., Soukhareva, N., Kang, Y., and Scott, D.W. (2002). Gene transfer of Ig-fusion proteins into B cells prevents and treats autoimmune diseases. J. Immunol. *168*, 4788–4795.

49. Yu, H., Borsotti, C., Schickel, J.N., Zhu, S., Strowig, T., Eynon, E.E., Frleta, D., Gurer, C., Murphy, A.J., Yancopoulos, G.D., et al. (2017). A novel humanized mouse model with significant improvement of class-switched, antigen-specific antibody production. Blood *129*, 959–969.

50. Villaudy, J., Schotte, R., Legrand, N., and Spits, H. (2014). Critical assessment of human antibody generation in humanized mouse models. J. Immunol. Methods *410*, 18–27.

51. Chen, Q., He, F., Kwang, J., Chan, J.K., and Chen, J. (2012). GM-CSF and IL-4 stimulate antibody responses in humanized mice by promoting T, B, and dendritic cell maturation. J. Immunol. *189*, 5223–5229.

52. Lang, J., Kelly, M., Freed, B.M., McCarter, M.D., Kedl, R.M., Torres, R.M., and Pelanda, R. (2013). Studies of lymphocyte reconstitution in a humanized mouse model reveal a requirement of T cells for human B cell maturation. J. Immunol. *190*, 2090–2101.

53. Cheng, Q., Mumtaz, I.M., Khodadadi, L., Radbruch, A., Hoyer, B.F., and Hiepe, F. (2013). Autoantibodies from long-lived 'memory' plasma cells of NZB/W mice drive immune complex nephritis. Ann. Rheum. Dis. *72*, 2011–2017.

54. Manz, R.A., Thiel, A., and Radbruch, A. (1997). Lifetime of plasma cells in the bone marrow. Nature *388*, 133–134.

55. Aurnhammer, C., Haase, M., Muether, N., Hausl, M., Rauschhuber, C., Huber, I., Nitschko, H., Busch, U., Sing, A., Ehrhardt, A., and Baiker, A. (2012). Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. Hum. Gene Ther. Methods *23*, 18–28.