Efficient CRISPR-Cas9-mediated mutagenesis in primary human B cells for identifying plasma cell regulators

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Human B lymphocytes are attractive targets for immunotherapies in autoantibody-mediated diseases. Gene editing technologies could provide a powerful tool to determine gene regulatory networks regulating B cell differentiation into plasma cells, and identify novel therapeutic targets for prevention and treatment of autoimmune disorders. Here, we describe a new approach that uses CRISPR-Cas9 technology to target genes in primary human B cells in vitro for identifying plasma cell regulators. We found that sgRNA and Cas9 components can be efficiently delivered into primary human B cells through RD114-pseudotyped retroviral vectors. Using this system, we achieved approximately 80% of gene knockout efficiency. We disrupted expression of a triad of transcription factors, IRF4, PRDM1, and XBP1, and showed that human B cell survival and plasma cell differentiation are severely impaired. Specifically, that IRF4, PRDM1, and XBP1 were expressed at different stages during plasma cell differentiation, IRF4, PRDM1, and XBP1-targeted B cells failed to progress to the pre-plasmablast, plasma cell state, and plasma cell survival, respectively. Our method opens a new avenue to study gene functions in primary human B cells and identify novel plasma cell regulators for therapeutic applications.

INTRODUCTION

Plasma cells (PCs) are known for their unique ability to secrete antibodies, which is the basis of humoral immunity. Recent studies in mice have shown that PCs can also regulate immunity through production of anti-inflammatory cytokines such as interleukin (IL)-10 and IL-35.1-4 Distinct PC subsets with protective and pathogenic functions have been identified by the expression of certain surface markers in both mouse and humans,1,3,5,6 providing possible selective depletion approaches of the pathogenic cells in various diseases. Nevertheless, this strategy is limited as those surface markers are also expressed by other immune cell lineages. Alternatively, key transcription factors (TFs) or/and intracellular regulators, which are essential for the generation of pathogenic PCs, hold promise as potential therapeutic targets. Such strategies have been developed for targeting TFs controlling T cell immunity in pre-clinical mouse models as well as clinical trials for autoimmune and malignant diseases.7-9 To date, a triad of TFs, IRF4, BLIMP1, and XBP1, has been identified as key regulators of mouse PC differentiation,10-12 meanwhile, the relevant molecules are poorly described in human PCs. In addition, molecular pathways regulating differentiation, survival, and antibody production of human PCs remain incompletely understood.

CRISPR-Cas9-mediated targeted mutagenesis can be harnessed for deciphering the gene regulatory networks governing PC differentiation. In mice, the CRISPR-Cas9 system has been successfully established to identify PC regulators in primary B cells isolated from Cas9 transgenic mice.13 Apart from that, several attempts have been undertaken to use CRISPR-Cas9 in studies of human B cell biology.14-16 Unlike Cas9-expressing mouse B cells requiring only single guide RNA (sgRNA) presence,13 gene editing of primary human B cells only happens when both sgRNA and Cas9 protein are present in the target cells. Synthetic sgRNAs and Cas9 protein can be delivered into primary human B cells as ribonucleoprotein (RNP) complexes using electroporation.17 However, this approach shows limitations in attempts to track the gene-edited cells or perform large-scale screening because of the lack of reporters and substantial costs, respectively. In contrast, the delivery of sgRNA and Cas9 by virus-based system could overcome those limitations.18 The biggest...
The hurdle of viral approaches is the large coding sequence of Cas9, which results in low infectious virus titers. Recently, Caeser et al. reported the delivery of Cas9 into primary human germinal center B cells isolated from tonsil tissue using Gibbon Ape Leukemia Virus (GaLV)-pseudotyped viruses. However, in this system, human germinal center B cells were transduced with Cas9 and oncogenic BCL2-BCL6 vectors, and followed by additional transduction with Cas9 vectors to reach an adequate transduction efficiency. Therefore, experimental workfolds that allow efficient delivery of CRISPR-Cas9 for primary human B cells and the study of molecules governing human B cell activation, B cell differentiation and antibody secretion are still needed.

Here, we developed a method that efficiently delivers Cas9 and sgRNA into primary human B cells using RD114-pseudotyped retroviral vectors with a gene knockout efficiency of approximately 80%. Remarkably, using this method, we showed that knockout of IRF4, PRDM1, and XBP1 leads to a marked abrogation of human PC differentiation from naive B cells. Collectively, we establish a robust and reliable method to efficiently screen for regulators controlling PC differentiation.

RESULTS

Efficient transduction of primary human B cells with RD114-pseudotyped retroviral vector

It has been shown that human hematopoietic stem and T cells are susceptible to RD114-pseudotyped retroviral vectors. To test whether primary human B cells are also susceptible to RD114-pseudotyped retroviral vectors, we produced these particles using 293Vec-RD114 cell line that stably and highly expresses retroviral structure proteins (gag, pol, and RD114 envelope). As controls, we included amphotropic and GaLV-pseudotyped retroviral vectors produced from Platinum-A and 293Vec-GaLV cell lines, respectively. Another GaLV-pseudotyped retroviral vector was produced from HEK-293T cell line by co-transfecting packaging (pHIT60) and envelope (GaLV WT) plasmids. All retroviral vectors were produced with the same main sgRNA plasmid harboring mCherry reporter (Figure 1A). Using the above-mentioned viral particles, we transduced untouched naive primary human B cells that are pre-activated with CD40 ligand in the presence of IL-4 and IL-21. Frequencies of mCherry+ cells were quantified by flow cytometry (Figure 1B). Consistent with a previous report, primary human B cells were efficiently transduced with GaLV-pseudotyped retroviral particles (Figure 1C). Interestingly, transduction efficiency was significantly higher with RD114-pseudotyped retroviruses in comparison with other tested viruses (Figure 1C). We conclude that RD114-pseudotyped retrovirus transduces primary human B cells with higher efficiency than other tested retroviral vectors.

Optimized protocols for efficient transduction of primary human B cells with RD114- and GaLV-pseudotyped retroviral vectors

Since primary human B cells can be efficiently transduced with RD114- or GaLV-pseudotyped retroviral particles, we next focused on establishing an optimal transduction protocol with these retroviral systems. First, we examined whether an optimal virus titer could be obtained with different molecular ratios of main/packaging/envelope plasmids. We used the common ratio at 3:1:1 and increased 2-fold stepwise of the main plasmid, in total 1.5 μg DNA. The amount of each plasmid was calculated based on their sizes and ratios in the plasmid mixture (Table S1). Second, we tested whether distinct modes of B cell activation might contribute to transduction efficiency of primary human B cells. Here, we activated primary human B cells either with human CD40L multimer or mouse CD40L-expressing cell line (40LB cells) (Figure 2A). Regarding plasmid ratios, we found variations in transduction frequency with various plasmid ratios. Remarkably, ratios of 12:1:1, 24:1:1, and 48:1:1 gave the highest transduction efficiency in primary human B cells, and viral particles from the 293Vec-RD114 cell line gave a better transduction in comparison with viral particles from the other cell lines tested (Figure 2B). For the B cell activation conditions, we found a significantly elevated transduction frequency of primary human B cells activated with the CD40L multimer construct compared with CD40L expressing cell line (Figure 2C). We conclude that primary human B cells activated with CD40L multimers are more susceptible to RD114-pseudotyped retroviral transduction than those activated with the CD40L expressing cell line. Highly efficient CRISPR-Cas9-mediated gene knockout of primary human B cells using RD114-pseudotyped retroviral vectors

To address whether RD114-pseudotyped retroviral vectors are able to deliver both sgRNA and Cas9 coding sequences to primary human B cells for gene knockout, we produced RD114-pseudotyped retroviral particles expressing either sgRNA or Cas9 to target the β2M (β2 microglobulin) gene. As for the sgRNA, we designed a sgRNA targeting the exon 1 of β2M gene and cloned it into the sgRNA plasmid carrying mCherry reporter (Figure 3A). It has been shown that the Furin recognition sequence allows a more efficient removal of T2A residues and that GSG linker enhances the ribosomal skip by T2A. Therefore, and since we had to substitute mCherry by GFP in the MSCV-Cas9-T2A-mCherry plasmid, we included these sequences to increase the overall efficiency (Figure 3B). To deliver sgRNA and Cas9 components, we co-transduced human pre-activated B cells with sgRNA and Cas9 retroviral particles. Four days post transduction, sgRNA/Cas9 (mCherry“GFP”) B cells were sorted and genomic DNA was extracted for analysis of insertion-deletion (Indel) frequencies. The remaining cells were examined for knockout efficacy at day 4 and day 8 through their β2M surface expression by flow cytometry (Figure 3C). Based on T7 endonuclease I (T7EI) assay and ICE analysis from purified co-transduced cells (Figure 3D), sgRNA targeting the β2M locus showed approximately 80% of gene knockout efficiency in primary human B cells (Figures 3E and 3F). FACs analysis at day 4 and 8 post transduction confirmed the gene editing efficiency with up to 80% of β2M− B cells (Figure 3G). Of note, only co-transduction with sgRNA and Cas9 give rise to editing, thus only in this condition we found a β2M− B cell population. Single transduction with only sgRNA or Cas9 did not show a β2M− B cell population (Figure S1).
Figure 1. RD114-pseudotyped retroviral vectors efficiently transduce primary human B cells

(A) Experimental design. Human naive B cells were negatively selected from buffy coat and activated with human CD40L multimer in presence of IL-4 and IL-21 for 2 days. Simultaneously, retroviral packaging cell lines were plated for 20 h before transfection. Two days post transfection, retroviral supernatants were harvested and used for human B cell transduction. Transduced B cells were harvested on day 4 after transduction and analyzed by flow cytometry. (Created with BioRender.com). (B) Representative FACS plots show gating strategy to identify mCherry+ B cells at day 4 post transduction. (C) Representative FACS plots (top) and the graph (bottom) show the percentages of mCherry+ B cells 4 days post transduction. Non-transduced cells were used to define mCherry+ gate. HEK-293T was co-transfected with sgRNA plasmid, packaging plasmid (pHIT60), and envelope plasmid (GaLV WT); Platinum-A, 293Vec-GaLV, and 293Vec-RD114 were transfected with sgRNA plasmid only. Data were pooled from eight donors, shown as mean ± SEM. Statistical significance was calculated using one-way ANOVA test, only significant p values are shown: **p < 0.01; ****p < 0.0001.
A. Workflow

Human naive B cell isolation → Transduction → FACS analysis

Plating
1. HEK-293T
2. Platinum-A
3. 293Vec-GalV
4. 293Vec-RD114

Day
-3 -2 0 4

B. picomol ratio of sgRNA:Packaging:Envelope in total 1.5μg of plasmid DNA

Non-transduced

CD19
mCherry

Packaging and envelope plasmids
sgRNA plasmid

(i) CD40L multimer
(ii) 40LB feeder cells

C. 293Vec-RD114

mCherry B cells (%)
CRISPR-Cas9-mediated IRF4, PRDM1, and XBP1 knockout impairs plasma cell differentiation in vitro

IRF4, PRDM1, and XBP1 are known as the triad of PC master regulators in the mouse. Taking advantage of our CRISPR-Cas9 RD114-pseudotyped retroviral vectors, we targeted these loci and assessed the outcome on human B cell survival and PC differentiation. We designed sgRNAs to target IRF4, PRDM1, and XBP1 at the exon 1, exon 7, and exon 2, respectively (Figure S2A). Then, we co-transduced human pre-activated B cells with Cas9 and sgRNA vectors. Four days after transduction, mCherry^GFP^ B cells were sorted for T7EI assay and Indel analysis. As a result, gene-specific targeted human B cells showed at least 80% of gene editing efficiency at the targeted loci (Figures 4A, 4B, and S2B). To answer whether CRISPR-Cas9-mediated IRF4, PRDM1, and XBP1 deficiencies lead to impaired survival and block of PC differentiation, we determined frequencies of mCherry^GFP^ B cells and CD27^CD38^ PCs at day 8 post transduction (day 4 post differentiation) using flow cytometry. Knocking out IRF4 and PRDM1 in primary human B cells resulted in a significant reduction of mCherry^GFP^ B cells in comparison with non-targeted B cells, while XBP1 gene knockout showed a slight reduction (Figure 4C). Consistent with data in the mouse,11 we found a reduced frequency of CD27^CD38^ PCs among viable IRF4^−/−, PRDM1^−/−, and XBP1^−/−-deficient human B cells compared with non-targeted control cells (Figure 4D). Nonetheless, within the targeted cells, we were able to detect few CD27^CD38^ PCs, therefore further analysis addressed if these co-transduced CD27^CD38^ cells was edited. Thus, we sorted from IRF4-targeted and non-targeted samples mCherry^GFP^, mCherry^GFP^, mCherry^GFP^CD27^CD38^, mCherry^GFP^CD27^CD38^, and mCherry^GFP^CD27^CD38^ cells (Figure 4E). We analyzed those cell populations by T7EI assay and quantified Indel rate by Sanger sequencing combined with ICE analysis. Remarkably, the T7EI assay and Indel rate showed that the majority of differentiated PCs (mCherry^GFP^CD27^CD38^) and pre-plasmablasts (pre-PBs) (mCherry^GFP^CD27^CD38^) were not edited, while mCherry^GFP^CD27^CD38^ were edited properly for the target alleles (Figure 4F). Of note, total mCherry^GFP^ were wild type (Figure 4G). These results demonstrated that at the time of PC analysis (day 4 after differentiation) edited cells are absent from the PC population in contrast to co-transduced but not edited PCs. Intra-nuclear staining of IRF4, PRDM1, and XBP1 in sgIRF4, sgPRDM1, and sgXBP1-targeted samples confirmed the reduction of TF-expressing PC populations in total viable cells (Figure S2C). To further characterize that these TF-expressing PC cells were derived from non-targeted cells, we performed IRF4 intra-nuclear staining using mCherry^GFP^ and mCherry^GFP^ sorted from the same IRF4-targeted samples at day 2 after differentiation (day 6 after transduction). Remarkably, we detected very few IRF4-expressing PCs in the sorted mCherry^GFP^ compared with mCherry^GFP^ (Figure 4F), demonstrating that the TF-expressing PC populations in total viable cells are mainly from non-targeted mCherry^GFP^ cells. We also found that IRF4-targeted cells failed to progress to pre-PB (CD27^CD38^) state, PRDM1-targeted cells failed to differentiate into PC (CD27^CD38^) state, while the survival of XBP1-targeted cells was substantially impaired (Figure 4D). Thus, we performed intra-nuclear staining of IRF4, PRDM1, and XBP1 in wild-type primary human B cells differentiated in vitro to examine at which state of the B cell differentiation these TFs were expressed. We found that activated B cells (CD27^CD38^) and pre-PBs expressed similar levels of IRF4 while PBs/PCs expressed a notable increased level (Figure 4G). Additionally, PRDM1 started being expressed in pre-PBs and was increased in PB/PCs, while XBP1 was only expressed by PB/PCs (Figure 4G). These could explain that IRF4, PRDM1, and XBP1-targeted B cells failed to progress to the pre-PB, PC state and PC survival, respectively. We also found a down-regulated expression of PRDM1 in IRF4-targeted cells compared with non-targeted cells (Figure 4H) as reported by prior studies in mouse.27,28 Collectively, we demonstrated that IRF4, PRDM1, and XBP1 are required for human B cell survival and PC differentiation.

DISCUSSION

The delivery of CRISPR-Cas9 into cells is a crucial step in gene editing technology. In the mouse, to extend the utility of CRISPR-Cas9 technology, several Cas9 transgenic mice have been generated.13,29–31 These mouse strains simplify and allow gene editing with high efficiency both in vivo and ex vivo as only sgRNAs are required for a successful gene knockout in Cas9-expressing cells. Previously, Cas9-expressing B cells transduced with sgRNA retroviral particles have been used to identify molecules involved in mouse B cell activation and PC differentiation.13 However, the same systems are not available for human B cells. Thus, to apply such gene editing technology in human B cells, it is required to deliver both sgRNA and Cas9 components.

Generally, CRISPR-Cas9 can be delivered to human cells by three main approaches, namely (1) plasmid DNA expressing sgRNA and Cas9 protein delivered by viral vectors, (2) sgRNA and mRNA coding for Cas9 protein, and (3) ribonucleoprotein (RNP) containing sgRNA and Cas9 protein. Despite insertional mutagenesis and slow onset of editing, viral vectors are still the most appropriate delivery method for large-scale screening of human PC differentiation regulators ex vivo.

Figure 2. Establishing efficient transduction conditions for primary human B cells with RD114- and GalV-pseudotyped retroviral vectors

(A) Experimental design. Negatively selected human naive B cells were activated either with (i) human CD40L multimer in the presence of IL-4 and IL-21, or with (ii) 40LB feeder cells, in the presence of IL-2 and IL-21. 40LB feeder cells were irradiated (30 Gy) and plated 1 day prior to the B cell culture. (Created with BioRender.com). (B) Representative FACS plots (top) show the percentage of mCherry^− B cells at day 4 post transduction using different plasmid conditions as indicated. Picomole of sgRNA plasmid increased as opposed of packaging and envelope plasmids, respectively. The graphs (bottom) show frequencies of mCherry^+ B cells obtained from three donors for condition (i) and (ii). (C) The graph shows the percentage of mCherry^− B cells activated with condition (i) compared with condition (ii) at day 4 post transduction with retrovirus produced by the 293Vec-RD114 cell line. Groups were compared using unpaired t test, only significant p values are shown: "p < 0.1; "p < 0.01; ""p < 0.001. Data shown as mean ± SEM.
due to stable expression of sgRNA and Cas9, easy tracking of knockout cells, and cost advantages when compared with mRNA and RNP methods.32 Unfavorably, Cas9 requires a large insert in plasmid DNA, which hinders the viral particle packaging process, resulting in low virus titers, and eventually impeding the delivery efficacy of Cas9 into target cells. Thus, a viral vector with superior transduction efficiency for primary human B cells would compensate for virus titer challenges.

Here, we documented a highly efficient delivery method of CRISPR-Cas9 that uses RD114-pseudotyped retroviral vectors to knockout candidate genes in primary human B cells and evaluate whether they abrogate PC differentiation. IRF4, PRDM1, and XBPI-targeted primary human B cells failed to differentiate into PCs, highlighting the crucial roles of these genes in human PC generation. Importantly, these findings show a shared involvement of IRF4, PRDM1, and XBPI in PC differentiation between mouse and human.10,13,33 Nevertheless, there are reports suggesting that XBPI does not play a major role in controlling mouse PC differentiation.24 In this study, we validated the use of ICE online program (https://ice.synthego.com/, Synthego) to quantify the Indel rate in the target samples by comparing the levels of β2M-knockout using flow cytometry and quantification Indel rate using ICE online program. The Indel rate was approximately 80% by ICE analysis (Figure 3F), in agreement with the phenotyping data by FACS analysis (Figure 3G). Collectively, our system has demonstrated feasibility for functional investigation of molecules expressed in human PCs, which may provide novel insights into biology and function of human PCs in health and disease.

While interesting, the system may have some limitations. For instance, due to a slow onset of gene editing by virus-based systems,2 this approach may be inappropriate for identifying regulators of PCs differentiated from memory B cells, as these cells often rapidly differentiate into PC before the target gene is knocked out. Therefore, RD114-pseudotyped retroviral vectors should be tested on resting, non-activated or shortly activated B cells, to examine whether virus-mediated delivery of CRISPR-Cas9 can be used for identification of regulators of PC differentiation from memory B cells. Sequential transduction protocols for delivering of sgRNA and Cas9 could be more efficient; however, this would reduce the sensitivity to identify PC regulators, because (1) viral supernatant contains viral RNA, DNA, and proteins that can induce differentiation of B cells into PCs, and (2) sequential transduction protocols would require maintenance of B cells under activation conditions longer and this would induce the PC differentiation before the CRISPR-Cas9-mediated mutagenesis becomes effective. We considered that the all-in-one plasmid containing both Cas9 and sgRNA might be advantageous in terms of transduction and editing efficiency. However, with this approach we achieved a lower transduction and knockout efficacy compared with co-transduction (data not shown). We believe that the size of the all-in-one plasmid exceeds the retroviral particle packaging capacity, resulting in a very low virus titer.

In conclusion, our approach allows knockout of molecules up-regulated in activated B cells, plasmablasts, and PCs in order to gain better understanding of the molecular mechanisms governing B cell activation, and survival, PC differentiation, and antibody secretion.

**MATERIALS AND METHODS**

**Human buffy coats**

Buffy coats of three female and 11 male healthy donors, age from 18 were provided by DRK-Blutspendedienst Nord-Ost (https://www.blutspende-nordost.de/). This study was reviewed and approved by the ethics committee of Charité Universitätsmedizin Berlin in accordance with Declaration of Helsinki.

**Human naive B cell culture**

Human PBMCs were isolated from buffy coats of healthy donors by Ficoll Density Gradient method and cryo-preserved at −80 °C. Human naive B cells were isolated from frozen PBMCs with naïve B cell isolation kit II (Miltenyi Biotec, Cat. #130-091-150). In brief, frozen PBMCs were washed with PBS/BSA/EDTA, then incubated with Biotin-antibody Cocktail for 15 min. Cells were washed again with PBS/BSA/EDTA and incubated with Anti-Biotin MicroBeads.

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**Figure 3. Efficient CRISPR-Cas9-mediated knockout of β2M housekeeping gene in primary human B cells**

(A) β2M-specific targeting sequence was inserted into Bsil restriction site of sgRNA plasmid (left). The scheme shows the targeted site of β2M exon 1 and two primer sites for amplifying the flanking region of β2M-targeted site by PCR (right). (B) The sequence of T2A-GFP was amplified from plasmid MSCV-Cas9-2A-GFP-sgRNA and Furin-GS Gel sequence was included in the forward primer. HindIII restriction sites were added in both forward and reverse primers. The amplified fragment was digested with HindIII enzyme and replaced for T2A-mCherry sequence in plasmid MSCV-Cas9-T2A-mCherry. The reconstructed plasmid carries sequences of Cas9-Furin-GSG-T2A-GFP. (C) Experimental design. Negatively selected human naive B cells were activated with CD40L, multimer in presence of IL-4 and IL-21 for 2 days. In parallel, 293TVec-RD114 retroviral packaging cells were plated for 20 h until transfection. Two days post transfection, retroviral supernatant was harvested for transduction. Day 4 post transduction, a half of B cells was harvested for FACS analysis or cell sorting and the other half was remained in culture with IL-10 and IL-21 for another 4 days. (Created with BioRender.com). (D) Representative FACS plots show co-transduced B cells before (top) and after (bottom) sorting at day 4 post transduction. (E) Agarose gel from T7endonuclease I digestion of sgRNA (mCherry) and Cas9 (GFP) (left) and β2M− B cells (right) at day 4 and 8 post transduction. mCherry”GFP” B cells were gated based on non-transduced sample, β2M− B cells were gated based on non-targeted sample (transduced with sgRNA and Cas9 plasmid, but sgRNA has no targeted sequence). The graph (right) shows the percentage of mCherry”GFP” and surface β2M− B cells in mCherry”GFP” cells at day 0, 4, and 8 post transduction. Statistical significance was calculated using the unpaired t test: **p < 0.01; ****p < 0.0001. Data were pooled from three donors (F) and six donors (G), shown as mean ± SEM.
for 15 min. Finally, cells were washed and transferred onto magnetically applied LS column (Miltenyi Biotec, Cat. #130-042-401) for separation. Untouched naïve B cells were collected from negative fraction with purity > 95% according to CD19+/CD3−. Human naïve B cells (5 × 10^4 cells/well in 96-well plate) were cultured in RPMI-1640 GlutaMAX medium (Gibco, Cat. #61870-010) supplied with 10% (v/v) FBS (Corning, Cat. #35-079-CV), 1 mM Sodium Pyruvate (Gibco, Cat. #11360-039), 100 U/mL Penicillin (Gibco, Cat. #15140-122), 100 μg/mL Streptomycin (Gibco, Cat. #15140-122), 50 μM 2-mercaptoethanol (Gibco, Cat. #31350-010), and 25 mM HEPES (Gibco, Cat. #15630056). Human naïve B cells were activated either with (1) human CD40L multimer (1 μg/mL, 2-mercaptoethanol (Gibco, Cat. #31350-010), and 25 mM HEPES (Gibco, Cat. #15630056)).

Cell lines

Platinum-A cell line was a kind gift from Dr. Toshio Kitamura’s laboratory (The University of Tokyo, Tokyo, Japan). 293Vec-GaLV and 293Vec-RD114 cell lines were kind gifts from BioVec Pharma (Canada), and 40LB cell line expressing mouse CD40 ligand was a kind gift from Dr. Daïsuke Kitamura’s laboratory (Tokyo University of Science, Tokyo, Japan). HEK-293T, Platinum-A, 293Vec-GaLV, 293Vec-RD114, and 40LB cell lines were cultured in DMEM GlutaMAX medium (Gibco, Cat. #61965-026) supplemented with 10% (v/v) FBS (Corning, Cat. #35-079-CV), 1 mM Sodium Pyruvate GlutaMAX medium (Gibco, Cat. #61870-010) supplied with 1.5% (v/v) FBS (Corning, Cat. #35-079-CV), 1 mM Sodium Pyruvate (Gibco, Cat. #11360-039), 100 U/mL Penicillin (Gibco, Cat. #15140-122), 100 μg/mL Streptomycin (Gibco, Cat. #15140-122), 50 μM 2-mercaptoethanol (Gibco, Cat. #31350-010), and 25 mM HEPES (Gibco, Cat. #15630056).

293Vec-GaLV and 293Vec-RD114 cell lines were cultured in complete DMEM medium before transfection. Day 4 post transduction, the transduced B cells were further cultured in PC differentiation medium containing IL-10 (10 ng/mL) and IL-21 (10 ng/mL) for 2 days before retroviral transduction. Day 4 post transduction, the transduced B cells were further cultured in PC differentiation medium containing IL-10 (10 ng/mL) and IL-21 (10 ng/mL). B cells were analyzed by flow cytometry in different time points. All recombinant human cytokines were purchased from Peprotech. *Figure 4. IRF4, PRDM1, and XBP1 are crucial for B cell survival and PC differentiation in vitro*

(A) Agarose gel from T7 endonuclease I assay shows digested bands from sgIRF4, sgPRDM1, and sgXBP1-targeted compared to non-targeted samples, (−): non-targeted; sgIRF4, sgPRDM1, and sgXBP1: sgRNA plasmid with targeted sequence of each gene, respectively. Red asterisks mark the corresponding digested DNA bands. (B) The graph shows the percentage of Indel mutations at IRF4, PRDM1, and XBP1-specific targeted site compared with non-targeted samples. Alignment and the percentage of Indel mutations were performed by ICE analysis online program (Synthego). (C) Representative FACS plots (left) and graph (right) show the percentage of co-transduced B cells gated on CD19+ B cells from non-transduced, non-targeted, sgIRF4, sgPRDM1, and sgXBP1-targeted samples. (D) Representative FACS plots (left) and graph (right) show the percentage of the PC population gated on co-transduced B cells from pre-differentiation and post-differentiation, non-targeted, sgIRF4, sgPRDM1, and sgXBP1-targeted samples. (E) FACS plots (top) show gating strategy to identify populations for cell sorting and cell purity post sorting at day 6 post transduction (day 2 post differentiation). P1–5 are mCherry+/GFP+, mCherry+/GFP+, mCherry+/GFP+/CD27−/CD38−, mCherry+/GFP+/CD27−/CD38+, and mCherry+/GFP+/CD27+CD38−, respectively. Agarose gel from T7 endonuclease I assay (left bottom) shows digested bands for mCherry+/GFP+, mCherry+/GFP+, mCherry+/GFP+/CD27+CD38−, and mCherry+/GFP+/CD27−CD38+ sorted from IRF4-targeted cells compared with non-targeted cells. Red asterisks mark the corresponding digested DNA bands. The graph (right bottom) shows the percentage of Indel mutations at IRF4-targeted site compared with non-targeted sample of indicated cell populations. Alignment and the percentage of Indel mutations were performed by ICE analysis online program (Synthego). (F) Representative FACS plots (left) and the graph (right) show the percentage of IRF4+/CD27− cells in mCherry+/GFP+ and mCherry+/GFP+ cells sorted from the same IRF4-targeted samples. The color coding indicates samples from the same donor. (G) Representative histograms show the expression of IRF4, PRDM1, and XBP1 in CD27− CD38−, CD27− CD38+, and CD27+ CD38− WT cells. (H) Representative histogram shows expression of PRDM1 in sorted mCherry+/GFP+ cells from IRF4-targeted compared to non-targeted samples. Cells were analyzed or used for cell sorting at day 4 post transduction (prior differentiation) (A and B), day 8 post transduction (day 4 post differentiation) (C and D), and day 6 post transduction (day 2 post differentiation) (E–H). Data were representative or pooled from three donors (A and E), five donors (C and D), and four donors (F–H), shown as mean ± SEM. Statistical significance was calculated using one-way ANOVA test (C and D), only significant p values are shown: **p < 0.01; ***p < 0.001; ****p < 0.0001. Each gene was independently targeted by three different sgRNAs. Data for one sgRNA are shown.
before adding to the cell lines. Six hours later, Lipofectamine-contained medium was replaced with fresh medium for retroviral production.

Two days post transfection, retroviral supernatant was harvested and centrifuged at speed 500 × g for 10 min at room temperature to remove cell debris. Polybrene (Sigma-Aldrich, Cat. #TR-1003-G) (final concentration 10 µg/mL) was added to retroviral supernatant prior to transduction. Medium in each well of 2-day-activated B cells was replaced with 200 µL of viral supernatant, followed by 1,500 × g centrifugation for 3 h at 32°C. Volume of sgRNA and Cas9 retroviral supernatant was mixed 100µL:100µL for co-transduction. Immediately after centrifugation, retroviral supernatant was replaced with complete RPMI medium (Gibco, Cat. #61870-010) supplied with 800 mM DMSO (Sigma-Aldrich, Cat. #400425, RRID: AB_893689).

PCR, gel purification, and DNA sequencing
KOD polymerase (Sigma-Aldrich, Cat. #71086-3) was used for all PCR reactions; 2X KOD buffer was premixed as follows: 80 µL H₂O, 1,600 µL 5M Betaine (Sigma-Aldrich, Cat. #B0300-1VL), 400 µL DMSO (Sigma-Aldrich, Cat. #D2438), 800 µL 10X KOD buffer, 800 µL 2 mM dNTPs, and 320 µL 25 mM MgSO₄. Each 20 µL of PCR master mix consisted of 7.6 µL H₂O, 10 µL 2X KOD buffer, 0.5 µL 10 µM each primer, 0.4 µL KOD polymerase, and 1 µL DNA template. The PCR cycler was set as follows: 95°C for 5 min, 40 cycles of (95°C 20s, 58–62°C 20s, 70°C 30 s/1 kb), 70°C for 5 min, and 4°C pause. The PCR products were separated on 1% agarose gel. The corresponding DNA bands were excised and purified using NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Cat. #740.609.250), and dispatched to Eurofins Genomics for sequencing.

T7 endonuclease I assay
Flanking regions of CRISPR-Cas9 targeted sites were amplified by PCR and purified as described above. The purified PCR products were re-annealed as follows: 95°C for 5 min, ramp down to 85°C at 2°C/s rate, ramp down to 25°C at 0.1°C/s rate, and pause at 4°C. The re-annealed PCR products were incubated with T7 Endonuclease I (NEB, Cat. #M0302L) at 37°C for 15 min, the reaction was stopped by adding 0.25M EDTA. The digested PCR products were analyzed on 1% agarose gel for visualization.

Insertion-deletion analysis
The sequencing results of purified PCR products at CRISPR-Cas9 targeted regions were analyzed with Inference of CRISPR Edits (ICE) analysis online program (https://ice.synthego.com/). Data quantification and statistical significance was performed using GraphPad Prism 9 (https://graphpad.com). Groups were compared using one-way ANOVA test or unpaired or paired t test; considered p values were indicated for each figure: *p < 0.1; **p < 0.01; ***p < 0.001; ****p < 0.0001.

DATA AVAILABILITY STATEMENT
This study did not generate/analyze any datasets/code. All other data will be made available upon written request to the corresponding author.
SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.11.016.

ACKNOWLEDGMENTS
We thank J. Kirsch and T. Kaiser (Flow Cytometry Core Facility of the DRFZ); Manuela Frese-Schaper, Christin Kabus, and Katrin Lehmann (laboratory managers of the DRFZ) for technical help. This work was supported by grants from the German Research Foundation (laboratories of the DRFZ); Manuela Frese-Schaper, Christin Kabus, and Katrin Lehmann (laboratories of the DRFZ mass spectrometry laboratories). The authors read and approved the submitted version of the article.

AUTHOR CONTRIBUTIONS
T.A.L., V.T.C., A.C.L., K.R., T.D., and V.D.D. developed the study concept. T.A.L., V.T.C., A.C.L., E.S., C.K., D.H., K.R., T.D., and V.D.D. performed experiments and contributed to project development, data discussions, and preparation of the manuscript. All authors read and approved the submitted version of the article.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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