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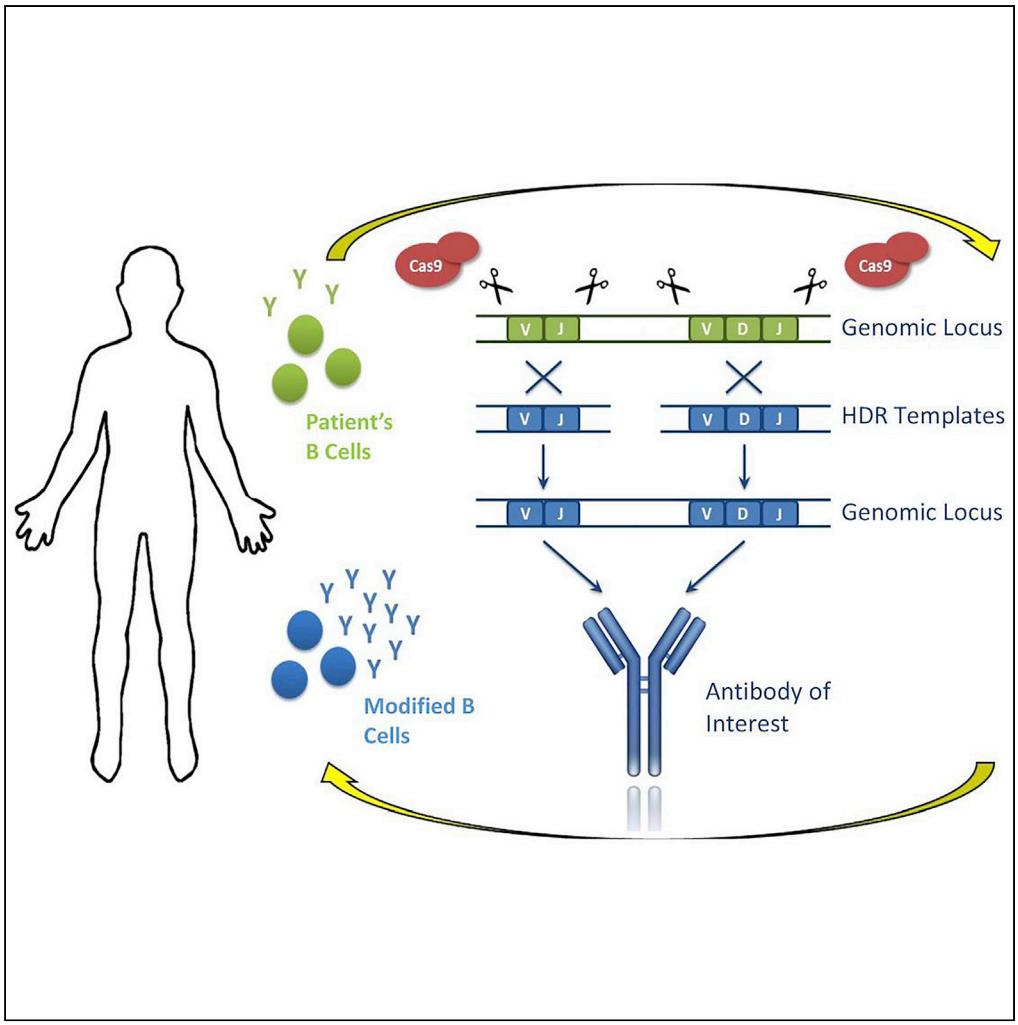

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HIGHLIGHTS

Methods to perform Cas9-mediated gene editing in primary human B cells are reported

Homologous recombination was confirmed by sequencing, PCR, and restriction digest

Replacement of B cell receptor loci in primary human B cells was achieved

Reprogramming the antibody specificity of B cells could have clinical importance

Greiner et al., iScience 12, 369–378
February 22, 2019
https://doi.org/10.1016/j.isci.2019.01.032

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CRISPR-Mediated Editing of the B Cell Receptor in Primary Human B Cells

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SUMMARY

Vaccination approaches have generally focused on the antigen rather than the resultant antibodies generated, which differ greatly in quality and function between individuals. The ability to replace the variable regions of the native B cell receptor (BCR) heavy and light chain loci with defined recombined sequences of a preferred monoclonal antibody could enable curative adoptive cell transfer. We report CRISPR-mediated homologous recombination (HR) into the BCR of primary human B cells. Ribonucleoprotein delivery enabled editing at the model *CXCR4* locus, as demonstrated by T7E1 assay, flow cytometry, and TIDE analysis. Insertion via HR was confirmed by sequencing, cross-boundary PCR, and restriction digest. Optimized conditions were used to achieve HR at the BCR variable heavy and light chains. Insertion was confirmed at the DNA level, and transgene expression from the native BCR promoters was observed. Reprogramming the specificity of antibodies in the genomes of B cells could have clinical importance.

INTRODUCTION

The CRISPR/Cas9 system enables rapid and precise genome engineering, and cell therapy is a particularly noteworthy application of this technology (Barrangou and Doudna, 2016). *Ex vivo* manipulation of cells is attractive because it obviates surmounting the formidable challenge of achieving efficient and cell-type-specific delivery *in vivo*; perhaps more importantly, *ex vivo* editing allows one to analyze and characterize edited cells before their adoptive transfer into patients (Barrangou and Doudna, 2016). Such quality control is very important, as *in vivo* editing by CRISPR/Cas9 may produce unexpected off-target mutations.

CRISPR-mediated *ex vivo* genome editing has been applied to correct the gene encoding hemoglobin in hematopoietic stem cells (HSCs) and/or progenitor cells, providing an innovative path to address β-hemoglobinopathies (Dever et al., 2016; Traxler et al., 2016). Genome engineering has also enabled deletion of *CCR5* in hematopoietic stem/progenitor cells (HSPCs) (Holt et al., 2010) or $CD4^+$ T cells (Perez et al., 2008), thereby protecting these cells from infection by HIV. Much effort has been made to edit HSCs and T cells, whereas far less attention has been given to the editing of B cells, despite the important role that they play in several immune processes, much of which is related to their ability to produce antibodies.

Monoclonal antibodies are the fastest growing class of therapeutic agents (Beck et al., 2010) and can be used to treat sundry pathologies, including autoimmune disease, cancer, and infectious disease. A main limitation associated with this therapeutic modality is the need for repeated administration—often for years or decades—which typically involves intravenous infusion at an ambulatory outpatient care center. Such logistics is very costly to the health care system and poses inconvenience to patients (Sylwestrzak et al., 2014) that may result in noncompliance. A second drawback of recombinant monoclonal antibodies is related to their production in cells of non-human origin (e.g., Chinese hamster ovary cells) or non-B-cell lineage (e.g., human embryonic kidney cells). The function of antibodies is strongly influenced by post-translational modifications (Li et al., 2015), which may differ between these cell lines and human B cells.

Harnessing the human antibody response is becoming increasingly feasible, as methodologies to isolate rare clones continue to improve (Wilson and Andrews, 2012; Sanjuan Nandin et al., 2017; Kwakkenbos et al., 2014; Franz et al., 2011). Primary human B cells have been transformed into stable cloned lines that secrete antibodies that neutralize respiratory syncytial virus *in vivo* (Kwakkenbos et al., 2010). The

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https://doi.org/10.1016/j.isci.2019.01.032

ability to induce the production of neutralizing antibodies to notable antigens by B cells *in situ* remains an unmet need, and repeated administration of recombinant products is not practical for several indications, particularly in the chronic therapeutic setting and for prophylaxis against infectious diseases.

The ability to replace the B cell receptor (BCR) heavy and light chains in an individual's B cells with sequences encoding a desired monoclonal antibody could lead to curative adoptive cell transfer. The antibody would be expressed dynamically and physiologically from its native enhancers and promoters in response to detection of antigen, resulting in the production of appropriate concentrations of antibody; such titrated dosing would be expected to ameliorate the undesirable side effects experienced by patients whose dose of recombinant product does not match their prevailing antigen concentration, which varies over time. In addition to defining specificity, this approach would generate autologous post-translational modifications. Such modifications can be optimized to program a preferred function (Lu et al., 2017), for example, by disrupting genes in other genomic loci that encode particular glycosyltransferases.

Although it has been shown that murine B cells (Cheong et al., 2016; Pogson et al., 2016; Chu et al., 2016) and primary human B cells (Hung et al., 2018; Wu et al., 2018) can be edited by CRISPR, homologous recombination (HR) at the BCR loci has been limited to hybridomas to date (Pogson et al., 2016). Herein, we sought to achieve HR at the BCR loci in primary human B cells for the first time. Such a demonstration would represent an important step toward achieving cellular humoral vaccines. Such cell therapies could replace repetitive administration of recombinant monoclonal antibodies, such as anti-tumor necrosis factor (TNF)-α, in the therapeutic setting. Knocking in sequences encoding broadly neutralizing antibodies against influenza or HIV (Walker and Burton, 2018) could also enable robust prophylaxis.

RESULTS

Cas9 RNP Is Required to Edit Primary Human B Cells

First, we aimed to determine how Cas9 should be delivered to primary human B cells to enable genome engineering. Transfection via electroporation is a common approach for delivering exogenous biologics into primary immune cells. Cutting and editing can be achieved in primary human T cells following electroporation of mRNA encoding Cas9 (Eyquem et al., 2017) or Cas9/guide RNA (gRNA) ribonucleoproteins (RNPs) (Schumann et al., 2015). In primary human B cells, RNPs were required to achieve editing, as delivery of Cas9 encoded by DNA or mRNA in combination with gRNAs targeting the model *CXCR4* locus did not produce any cutting, as evidenced by the T7E1 assay (Figure 1A, see Transparent Methods). This assay involves recognition and cleavage of mismatched DNA, which arises following non-homologous end joining.

These findings were validated by Tracking of Indels by DEcomposition (TIDE) analysis (Brinkman et al., 2014) of Sanger sequencing data (Figure 1B), which involves computational tracking of insertions and deletions by decomposition of the quantitative sequence trace data. It has been previously reported that use of RNP rather than DNA or mRNA increases cell viability substantially among human embryonic stem cells (Kim et al., 2014). RNPs also lead to substantially more specific genome modification than does DNA (Zuris et al., 2015). We next confirmed that disruption of this locus at the genomic level translated to decreased protein production. Flow cytometry revealed a 70% reduction in the levels of surface CXCR4 expression following RNP delivery (Figure 1C). Having demonstrated that gene disruption was possible, we sought to make use of the cell-intrinsic HR-directed DNA repair pathway to insert a DNA template of interest. During the previous cutting experiments, cells were cultured with interleukin-4, which was sufficient to maintain cell viability; however, to perform HR-mediated DNA repair, cells must proliferate as well. Thus the determination of suitable culturing conditions for primary human B cells was paramount to further investigation.

HR Is Achieved in Primary Human B Cells

Specifically, we had to identify a suitable activation protocol to perform HR on primary B cells. *In vivo*, activation of B cells can lead to differentiation into plasmablasts that perish within 1 week. *In vitro*, activated B cells were viable for more than 2 weeks, unless electroporation was performed in conjunction with the activation protocol, in which case viability was greatly decreased. We assessed various combinations of cytokines, Toll-like receptor agonists, and/or CD40 ligation (>30 conditions evaluated) and found that the CellXVivo Human B Cell Expansion Kit (R&D Systems) yielded the best activation and viability (data not shown). Locus-specific genome sequencing revealed that activation of B cells was required to achieve HR, as determined by CRISPR-GA analysis (Guell et al., 2014) of MiSeq data (Table 1). CRISPR-GA maps deep sequencing reads, estimates and locates insertions and deletions, and computes the allele

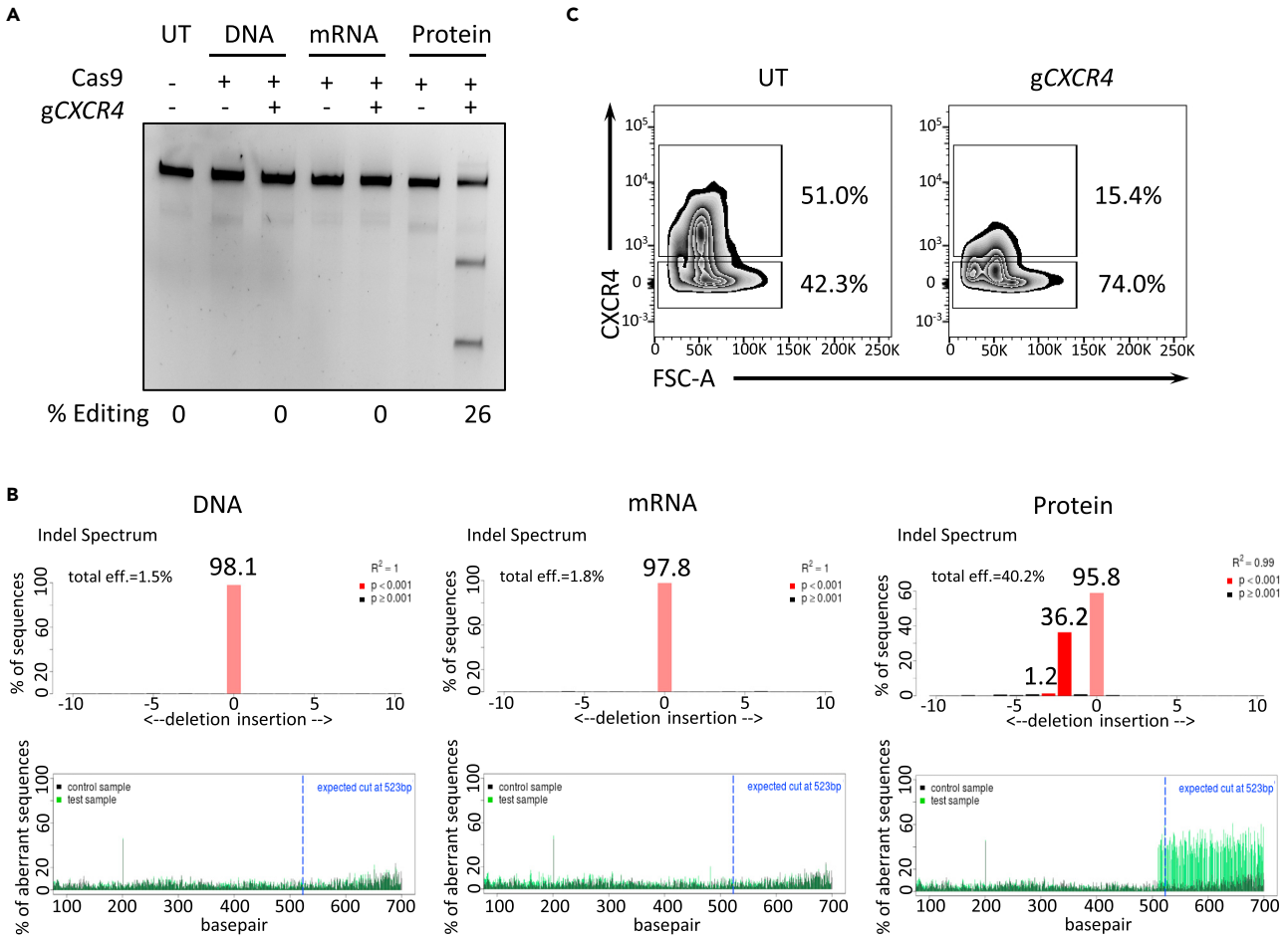

Figure 1. Cas9 RNP Enables Efficient Genome Editing of Primary Human B Cells

(A) A T7E1 assay shows efficient cutting of the model *CXCR4* locus following electroporation with Cas9 RNP, but not following electroporation with gRNA in conjunction with DNA or mRNA encoding Cas9. DNA was isolated 3 days post-transfection.

(B) TIDE analysis following electroporation of primary human B cells with DNA encoding Cas9 plus gRNA, mRNA encoding Cas9 plus gRNA, or Cas9 RNP targeting the model *CXCR4* locus. DNA was isolated 3 days post-transfection.

(C) Flow cytometry analysis of cell surface CXCR4 expression confirms that target protein expression is greatly reduced following electroporation of Cas9 RNP. Cells were analyzed 6 days post-transfection. UT, untransfected.

replacement efficiency. Pre-activation of cells for 5 days led to more efficient editing than pre-activation for 3 days. Activation of cells pre- and post-transfection was required to achieve HR. We varied additional transfection conditions and determined that a Cas9:gRNA:HR template ratio of 100:580:100 pmol was optimal.

To analyze HR efficiency in a timely manner, we used a restriction digest assay that involved use of a single-stranded oligonucleotide containing a HindIII restriction site targeting *CXCR4* (Schumann et al., 2015) (Figure 2A). This oligonucleotide was included in the transfection media along with an RNP targeting *CXCR4*, and editing was confirmed by TIDE analysis of Sanger sequencing data (Figure 2B) and by CRISPR-GA analysis of MiSeq data (Figure 2C). Although encouraging as a proof of concept, the MiSeq data indicated that the efficiency of editing was rather low, as was cell viability following activation and electroporation.

Caspase Inhibition Improves Cell Viability and HR Efficiency

To increase cell viability following electroporation, the pan-caspase inhibitor Q-VD-OPH ("OPH") was added to the culture media, conferring a nearly 3-fold increase in the proportion of viable cells (Figure 2D). Addition of 10 μM OPH to the culture media improves cell viability following activation and

	Pre-activation	Post-activation	HR Efficiency (%)
Untransfected controls	–	–	0.01
Donors	5 days	3 days	0.33
Donors	5 days		0.24
Donors	3 days	5 days	0.16
Donors	3 days		0.19
Donors		5 days	0.05

Table 1. Pre-activation of Primary Human B Cells Is Required to Achieve Homologous Recombination
CRISPR-GA was used to analyze MiSeq data from at least two donors per group.

electroporation, thereby leading to an increased amount of HR per cell input, as evidenced by HindIII restriction digest (Figure 2E).

HR Is Achieved at the BCR Heavy and Light Chain Loci

Although there are many loci of potential relevance for genome editing in primary human B cells, the BCR variable heavy and light chain loci are of particular interest, especially for adoptive cell therapy applications (Figure 3A). Top-performing gRNAs were identified by performing T7E1 assays after transfecting cells with RNPs targeting various sequences in the V_H3-23, J_H5, V_K3-20, and J_K4 loci (see Figures S1A–S1D). These loci were chosen because they are among the most frequently recombined loci (Prabakaran et al., 2012; Glanville et al., 2009; Boyd et al., 2010) and even co-recombine preferentially (Boyd et al., 2009; Xiao et al., 2013). Combinatorial cutting of both V and J loci was apparently achieved without loss of efficiency at either targeted locus (see Figures S1E and S1F). Although CRISPR is a tool that is known to enable multiplex editing in a given cell, these data do not rule out the remote possibility that the gRNAs cut individually in separate cells. We next established that a defined sequence could be inserted into a specific V region following a single cut or across V(D)/J following dual cutting (Figure 3B). Insertion was confirmed by cross-boundary PCR, which involved the use of a forward primer that is specific to the genome and a reverse primer that is specific to the inserted HR template (Figure 3C).

Sequences Encoding Monoclonal Antibodies Can Be Inserted into Endogenous Loci

Replacement of the endogenously recombined BCR with a sequence encoding a defined monoclonal antibody of interest could allow for programmed lifelong immunity. Cutting at the V locus only—rather than at both V and J—allows one to reduce the number of gRNAs (and hence potential off-target editing) by 2-fold. It also allows one to insert a specific constant region with embedded functionality of choice (e.g., IgG1 versus IgG4; introduction of mutations into Fc to confer extended half-life, Zalevsky et al., 2010; or antibody-dependent cellular cytotoxicity versus complement-dependent cytotoxicity, Hessell et al., 2007) rather than rely on the class switch recombination that has occurred or will occur in the cell being edited. We thus focused on this approach, despite the fact that we confirmed that insertion across V(D)/J is possible (Figure 3C). Sequences encoding a nanobody (ozoralizumab) or a monoclonal antibody (adalimumab) that neutralizes TNF-α were inserted into the heavy and/or light (kappa) loci of primary human B cells isolated from donors, as confirmed by cross-boundary PCR (Figures 3D–3F, see Figures S2A–S2C).

Nanobody and Antibody Are Expressed from Endogenous Promoters

Next, we sought to demonstrate that it was possible to achieve intrinsic expression of an inserted sequence driven by the native BCR promoter(s). RT-PCR was performed to demonstrate expression from the endogenous heavy chain promoter at the mRNA level (see Figure S2D). Flow cytometry was used to assess expression from this locus of the FLAG epitope, which was fused to the C terminus of the nanobody (Figures 4A and 4B). Finally, it was observed that B cells could express both the heavy and light chains of a defined monoclonal antibody, wherein the variable chains were fused to defined constant regions and epitope tags (FLAG and hemagglutinin, respectively) (Figures 4C and 4D). Moving forward, marker-free co-selection might be used in tandem to enrich for edited cells (Agudelo et al., 2017).

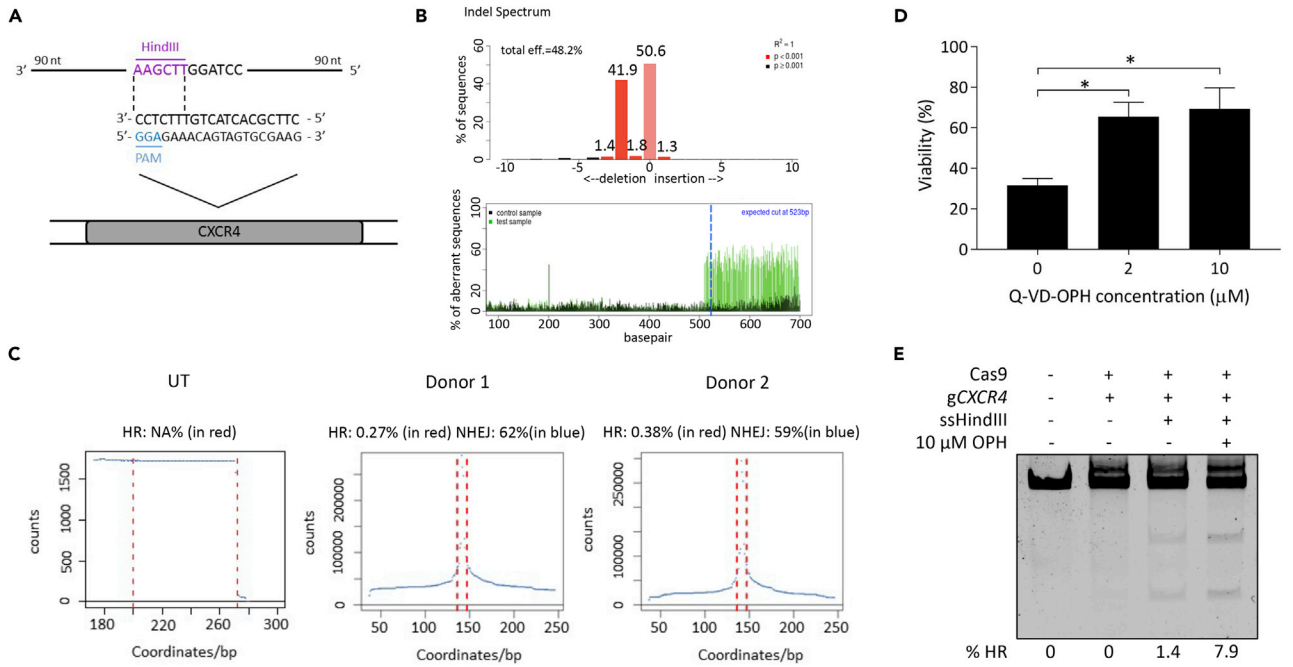

Figure 2. Site-Specific Introduction of a Restriction Site Is Achieved by Homologous Recombination in Primary Human B Cells

(A) A schematic of homologous recombination (HR) to insert a HindIII restriction site into the *CXCR4* locus.

(B) TIDE analysis confirms editing in primary human B cells upon inclusion of a single-stranded DNA template.

(C) CRISPR-GA analysis of MiSeq data confirms insertion of the HindIII restriction site into the *CXCR4* locus of primary human B cells, illustrating that HR is also possible. Cells were collected and analyzed 5 days post-transfection.

(D) Addition of the pan-caspase inhibitor Q-VD-OPH ("OPH") improves cell viability, which was quantified by flow cytometry 5 days post-transfection. $n = 3$ independent experiments, mean ± SEM. *$p < 0.05$, calculated by unpaired Student's t test.

(E) A HindIII restriction digest shows that insertion is possible in activated primary human B cells and is improved by the addition of OPH. DNA was isolated 6 days post-transfection.

DISCUSSION

To negate the need for repeated administration of recombinant monoclonal antibodies, several groups have strived to develop gene therapy-based approaches (Guijarro-Munoz et al., 2013). *Ex vivo* approaches involve transduction of allogeneic or autologous cells before adoptive cell transfer, typically as subcutaneous organoids. *In vivo* approaches include viral and non-viral transfection, typically via intramuscular injection. Viral vectors have been limited by safety concerns and clearance of cells expressing viral antigens; non-viral vectors have been limited by low transfection efficiency.

Vectored immunoprophylaxis represents a particularly interesting strategy to enable expression of broadly neutralizing antibodies to protect against, for example, influenza (Balazs et al., 2013) or HIV (Balazs et al., 2014), but the limitations associated with adeno-associated virus (AAV) vectors are considerable. These include the limited carrying capacity of the vector, modest expression levels of the transgene, a lack of regulation over such expression, and pre-existing and/or rapidly developing immunity to AAV (Brady et al., 2017). Human HSPCs have been engineered to express a broadly neutralizing anti-HIV antibody after *in vitro* maturation to B cells (Luo et al., 2009), although this was done using lentivirus-mediated gene therapy and therefore did not involve spatial addressing of the transgenes to the relevant loci. Although many cell types have been explored as *in vivo* bioreactors, the degree of success has been modest using orthogonal cells to produce monoclonal antibodies, which are naturally expressed by B cells. In non-B cells, transgene expression is typically driven by a constitutive promoter, which may not be physiologically appropriate.

It was recently shown that inserting a receptor of interest into its physiologically native locus can greatly improve function. Specifically, directing an HR template encoding a chimeric antigen receptor (CAR) to the T cell receptor (TCR) α constant locus leads to uniform CAR expression in primary human T cells isolated

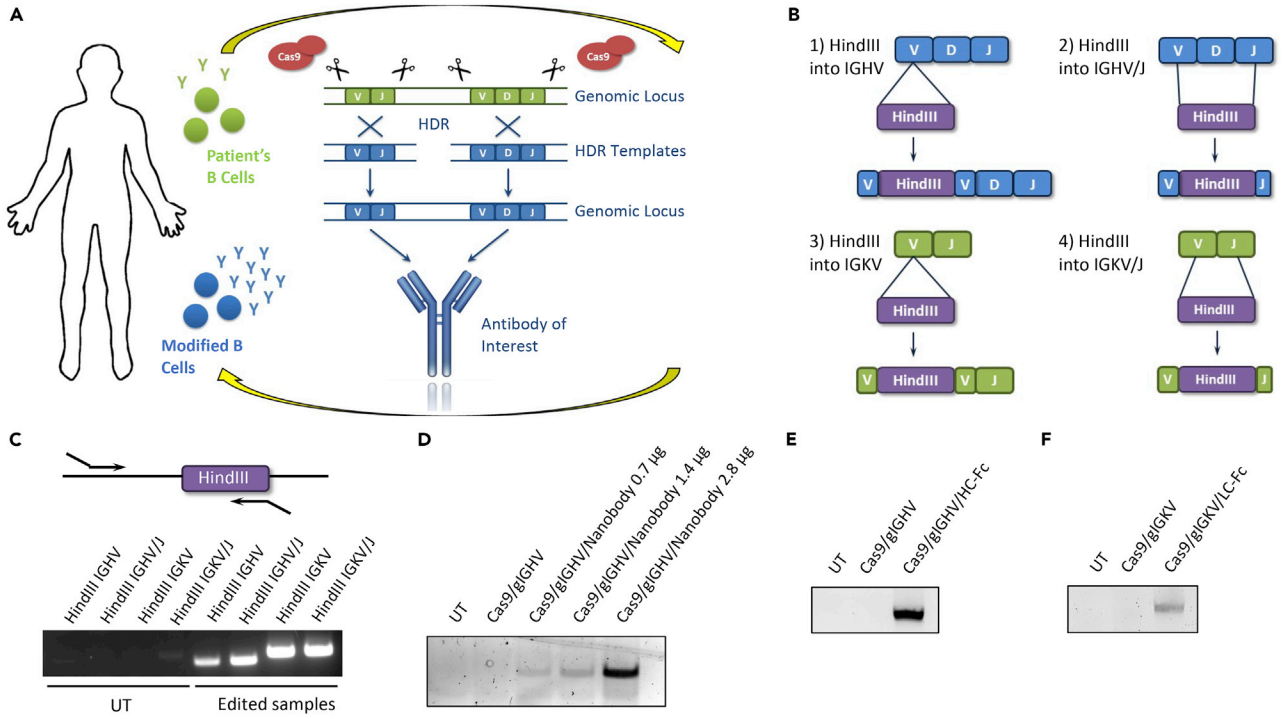

Figure 3. Defined Sequences Can Be Successfully Inserted into the BCR Heavy and Light Chain Loci of Primary Human B Cells

(A) A schematic of the conceptual framework of BCR reprogramming: an individual's B cells are recovered, modified *ex vivo* to express a monoclonal antibody of interest, and reinfused into the individual.

(B) A schematic of HR template insertion into or substitution across the BCR loci. A HindIII restriction site was introduced into the heavy and light chains, either by insertion into the V region or by replacement of a region spanning V/J.

(C) Site-specific insertion is confirmed by cross-boundary PCR. An amplicon is observed only if insertion into the defined genomic locus has occurred.

(D–F) Confirmation of insertion into the BCR loci by cross-boundary PCR. An amplicon is observed only if insertion has occurred. Cells were analyzed 5 days post-transfection. (D) Insertion efficiency into the heavy chain (V_H3-23) locus occurs in a dose-dependent manner for increasing amounts of Nanobody HR template. (E) An HR template encoding the heavy variable and constant regions of adalimumab was successfully inserted into the heavy chain (V_H3–23) locus of primary human B cells. (F) An HR template encoding the light variable and constant regions of adalimumab was successfully inserted into the light chain (V_K3–20) locus of primary human B cells. Untransfected (UT) cells were used as unedited controls.

See also Figures S1 and S2.

from peripheral blood (Eyquem et al., 2017). Moreover, the potency of these T cells is enhanced, as they confer vastly superior antitumor efficacy to CAR T cells that were generated by conventional retroviral methods or whose CAR was inserted into other loci (or regulated by non-native promoters). These data reveal that HR-mediated reconstitution of the native lymphocyte receptor locus yields optimal regulation of cell surface receptor expression. More recently, it was shown that the specificity of primary human T cells could be reprogrammed by replacing the endogenous TCR with a defined one that targets a known tumor antigen, NY-ESO-1 (Roth et al., 2018). Following recognition of the antigen, the TCR-engineered T cells produced effective antitumor responses both *in vitro* and *in vivo*, leading to substantial killing of cancer cells and delayed tumor progression.

Previous studies involving genome editing of B cells have been demonstrative, albeit distinct in scope from this work. The first report involved delivery of Cas9 and gRNA via retro- or lentivirus rather than RNP (Cheong et al., 2016). The authors demonstrated the ability to induce class switch recombination to a desired subclass by cutting in the constant regions, but did not perform HR or modify the variable regions that encode the antibody specificity. The work was performed in primary murine B cells, murine hybridomas, and human B cell lines. The first description of HR in B cells involved engineering of murine hybridomas to express a fusion construct encoding both a light chain and a variable heavy chain, resulting in full-length antibody expression (Pogson et al., 2016). These cells naturally proliferate, rendering HR readily feasible relative to resting B cells.

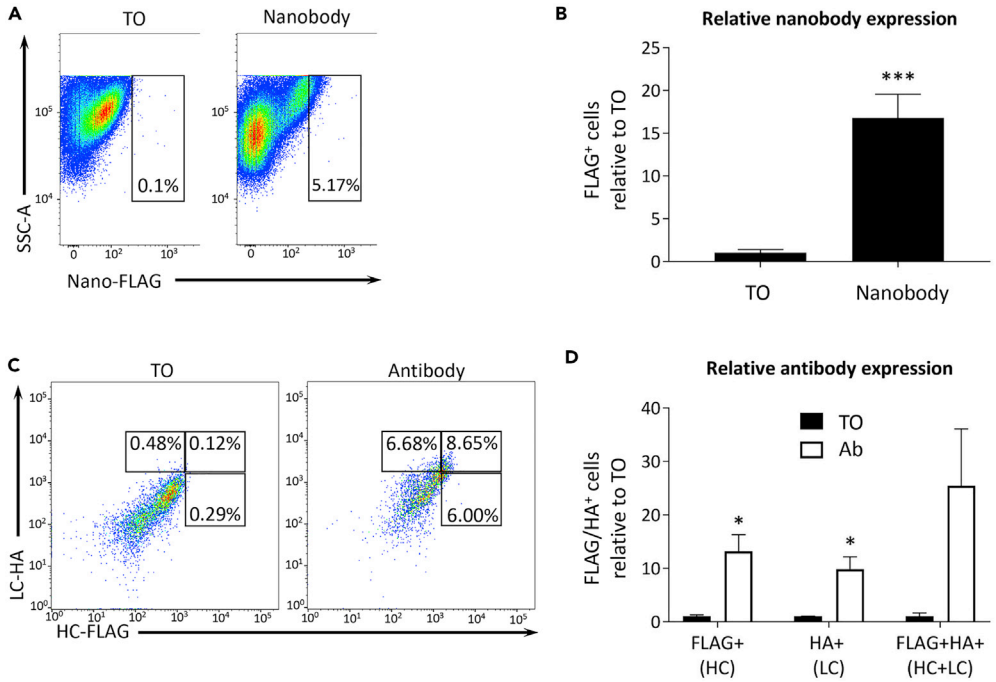

Figure 4. Native Expression of Defined Sequences from the Heavy and Light Chain BCR Loci

(A) Insertion of an HR template encoding a nanobody yields expression from the heavy chain locus. A FLAG epitope tag was fused to the C terminus of ozoralizumab to enable detection by intracellular flow cytometry.

(B) Quantitation of the proportion of cells isolated from healthy donors expressing ozoralizumab by intracellular flow cytometry.

(C) Multiplexed insertion of the HR templates encoding the heavy chain (HC) and light chain (LC) of adalimumab yields expression of both chains, which were, respectively, fused to C-terminal FLAG and hemagglutinin (HA) epitope tags for detection by intracellular flow cytometry.

(D) Quantitation of the proportion of cells isolated from healthy donors expressing HC, LC, or both by intracellular flow cytometry. Cells were analyzed 6 days post-transfection. In the case of adalimumab insertion, only viable cells were analyzed. TO, transfection only (no RNP or HDR template). n = 3 independent experiments, mean ± SEM. *$p < 0.05$, ***$p < 0.005$ calculated by unpaired Student's t test.

Editing of primary B cells has also been reported. Using a transgenic Cas9-expressing mouse, it was shown that primary murine B cells could be edited with high efficiency, thereby enabling a small-scale CRISPR-mediated screen to identify genes essential for B cell activation and plasma cell differentiation (Chu et al., 2016). The ability to achieve editing in primary human B cells, necessitating the introduction of Cas9 in addition to gRNA, was recently described (Hung et al., 2018). The method was applied to promote differentiation into plasma cells *ex vivo* by disrupting genes known to be involved in the regulation of development. The inclusion of either a single-stranded DNA oligonucleotide or AAV containing HR templates afforded HR. The resultant expression of BAFF from the *CCR5* locus promoted the engraftment of such edited B cells into immunodeficient mice.

Herein, we sought to focus on the reprogramming of loci of particular interest within B cells, namely, the BCR heavy and light chain loci. Although previous efforts have focused on editing of these loci in murine hybridomas or on other loci in primary murine or human B cells, this work is the first to demonstrate the possibility of defining an antibody among a polyclonal population of cells that comprised heterogeneously recombined V and J segments. We demonstrate that PCR products are suitable HR templates, potentially obviating AAV constructs in primary human B cells. Future work will involve functional studies to confirm the neutralizing ability of the antibodies expressed from these cells, both *in vitro* and *in vivo*. Future efforts will also seek to improve editing efficiency. As mentioned above, selection of appropriate V or J segments influences the efficiency of editing, as some are preferentially recombined over others. The observed editing efficiency may also be influenced by cell viability, as cells whose BCR loci are cut but not corrected may not survive in the absence of tonic signaling (Yasuda et al., 2017).

In summary, we have demonstrated for the first time the ability to perform HR-mediated integration in the BCR variable loci of primary human B cells using the CRISPR/Cas9 system. Specifically, we have determined how to improve cell viability following activation and electroporation, and we have shown that a clinically used therapeutic monoclonal antibody can be inserted into and expressed from the endogenous BCR heavy and light chain loci. This method creates opportunities in the study of basic B cell biology as well as the development of cellular humoral vaccines, enabling one to consider vaccination from the perspective of the antibody rather than the antigen. Rather than accept that antibody repertoires are guided by unique germlines, V(D)/J recombination events, and somatic hypermutation in a given individual, it would be desirable to provide an optimal sequence to any person in need of a particular response. Memory B cells (Tangye and Tarlinton, 2009; Kurosaki et al., 2015) could be adoptively transferred (Li et al., 2011), leading to lifelong production of the antibody from long-lived plasma cells (Nutt et al., 2015). Although naturally a stochastic process, differentiation into plasma cells could be promoted by exposure to particular cytokines (Hasbold et al., 2004) or via transfection with appropriate transcription factors (Nutt et al., 2015). A safety switch such as inducible apoptosis, which has been demonstrated to be effective in patients (Di Stasi et al., 2011), may be included to prevent suboptimal outcomes. Clinical translation of genome-edited cellular therapies, whose function and safety can be controlled through synthetic biology (Fischbach et al., 2013), heralds an emerging era in the treatment of genetic, infectious, and acquired diseases (Cornu et al., 2017). Programming memory humoral immunity could have important clinical implications.

Limitations of the Study

The work described herein is proof of concept but would benefit greatly from additional studies. Although demonstration of genome editing at the endogenous BCR heavy and light chain variable regions—leading to expression from the native promoters—is exciting, future work that examines the function of the expressed antibodies will be elucidating. Although nanobody/antibody expression was suggested by preliminary proof-of-concept data, further validation is needed. Unfortunately, such experiments cannot be performed by the authors at this time. Future studies will include quantification of secreted antibody by ELISA with soluble target antigen. Because the antigen selected (TNF-α) has a natural receptor expressed on the surface of primary human B cells, certain studies were not possible. Investigation of antibodies that target orthogonal antigens (e.g., influenza or HIV) would enable assessment of target neutralization. Glycomic profiling will also be informative.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and two figures and can be found with this article online at https://doi.org/10.1016/j.isci.2019.01.032.

ACKNOWLEDGMENTS

We thank Hye-Jung Kim, Sabrina Haag, Nathan Mathewson, and Soumya Badrinath for helpful suggestions. This work was supported by the Ovarian Cancer Research Alliance (M.S.G. is a Liz Tilberis Scholar) and the Claudia Adams Barr Program in Innovative Basic Cancer Research. R.B.P. was supported by a Grifols-Fulbright Grant.

AUTHOR CONTRIBUTIONS

V.G., R.B.P., S.L., C.H., E.M.C., and M.S.G. designed the experiments. V.G., R.B.P., and S.L. performed the experiments. V.G., R.B.P., S.L., and M.S.G. analyzed the data. V.G., R.B.P., S.L., and M.S.G. wrote the manuscript.

DECLARATION OF INTERESTS

V.G. and M.S.G. are listed as inventors on two patent filings relating to this technology. M.S.G. is a consultant for ProgramAble Cell Therapies, which has licensed the intellectual property. R.B.P., S.L., C.H., and E.M.C. declare no competing interests.

Received: September 12, 2018
Revised: January 25, 2019
Accepted: January 29, 2019
Published: February 22, 2019

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Supplemental Information

CRISPR-Mediated Editing of the B Cell Receptor in Primary Human B Cells

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SUPPLEMENTAL FIGURES

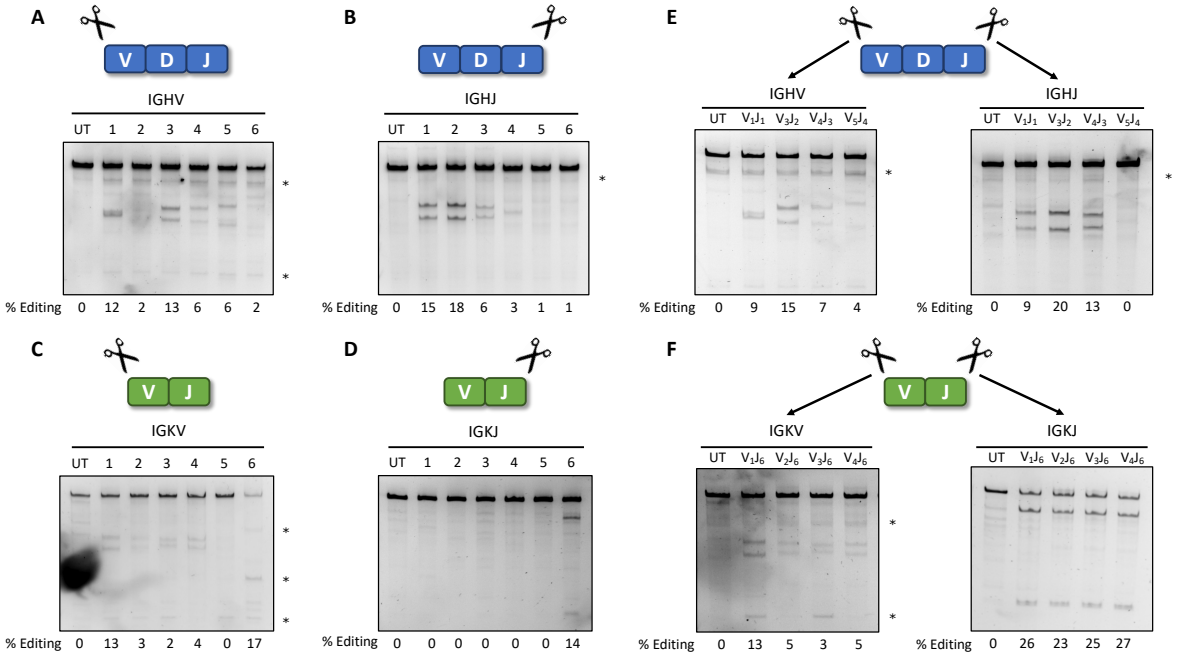

Figure S1. ***Related to Figure 3.*** **Identification and validation of gRNAs targeting the BCR loci of interest.** (**A-D**) Identification of gRNAs that cut at IGHV (V_H3-22/23), IGHJ (J_H5/6), IGKV (V_K3-19/20), and IGKJ (J_K3/4) with high efficiency. Single cutting experiments were performed to examine cutting within the heavy (**A,B**) or light (**C,D**) chain. (**E,F**) The premier gRNAs identified from **A-D** were combined in multiplexed cutting experiments that co-targeted both V and J of the heavy (**E**) or light (**F**) chain. Percent editing was assessed for each of the four loci. Note that templates can be inserted either into the V locus or a region spanning the V/J loci of the heavy chain and light chain loci. The former must include a constant region and stop codon, while the latter would be spliced to the constant region to which the B cell has or will class-switch. Asterisks denote non-specific bands.

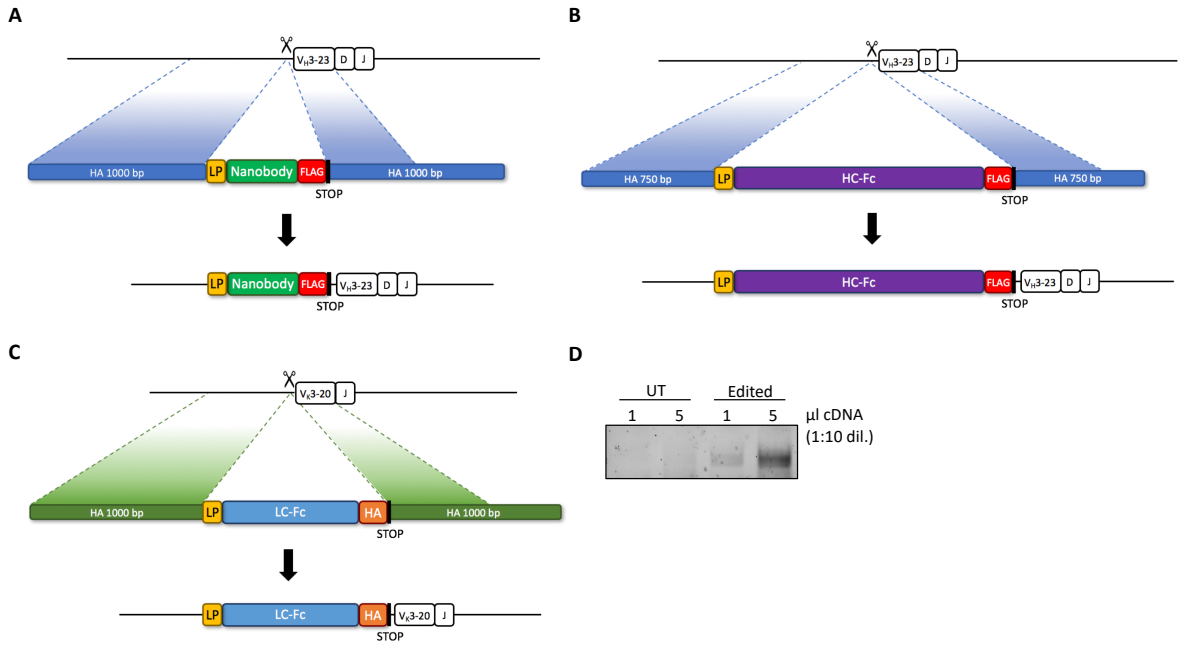

Figure S2. ***Related to Figure 3.*** **Site-specific introduction of HR templates into the BCR loci.** (**A-C**) Schematic of cross-boundary PCR at the heavy and light chain loci for a (**A**) nanobody, (**B**) antibody heavy chain, or (**C**) antibody light chain. One primer is specific to the genome and one primer is specific to the HR template. An amplicon is observed only if insertion has occurred. (**D**) RT-PCR confirms expression of the adalimumab heavy chain transgene from the native heavy chain locus at the mRNA level. mRNA was isolated 6 days post-transfection. UT, untransfected.

TRANSPARENT METHODS

gRNA synthesis. To prepare templates for *in vitro* transcription, the following oligonucleotides were annealed and assembled using the NEBuilder® HiFi DNA Assembly Master Mix (NEB) according to the manufacturer's instructions: Common gRNA oligo: 5'-CAC CGA CTC GGT GCC ACT TTT TCA AGT TGA TAA CGG ACT AGC CTT ATT TTA ACT TGC TAT GCT GTT TCC AGC ATA GCT-3', T7-gRNA oligo: 5'-TAA TAC GAC TCA CTA TAG **N20** GTT TTA GAG CTA TGC TGG AAA CAG C-3'. N20 represents the specific gRNA sequences that were used: CXCR4: GGA GAA ACA GTA GTG CGA AG, IGHV: GAA AAC ACC TGA AAA TCC CA, IGHJ: GTC CTC GGG GCA TGT TCC GA, IGKV: TTA GGA CCC AGA GGG AAC CA, IGKJ: CTG TGG CTC ACT TTC GGC GG. The resulting DNA fragment was amplified with Q5® High-Fidelity 2X Master Mix (NEB) and the primers gRNA_for: 5'-TAA TAC GAC TCA CTA TAG GA-3', gRNA_rev: 5'-CAC CGA CTC GGT GCC ACT TT-3' according to the manufacturer's instructions. The PCR product was purified with a QIAquick PCR Purification Kit (Qiagen), and the veracity of the size was confirmed by agarose gel electrophoresis. *In vitro* transcription was performed using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB) according to the manufacturer's instructions with a 16 h incubation at 37 °C. Remaining DNA was removed with RNase-free DNase I (NEB), RNA was purified with the GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Fisher), and product size as well as purity were confirmed by MOPS gel electrophoresis.

Primary B cell isolation and culture. Blood collars for human T cells were obtained from the Brigham and Women's Hospital Blood Donor Center with informed consent obtained from all donors. All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the Dana-Farber Cancer Institute Biohzazrd Control Committee. Primary human B cells were isolated from fresh leukoreduction collar blood by using 1 ml of RosetteSep™ Human B Cell Enrichment Cocktail (Stemcell Technologies) for 10 ml collar blood. Isolation was performed according to the manufacturer's protocol and was combined with Ficoll-Paque PLUS (GE Healthcare) in a SepMate™-50 (Stemcell Technologies). ACK Buffer (Gibco) was used to lyse red blood cells during the washing steps. Cells were then cultured in RPMI-1640 (Gibco), supplemented with 10% FBS (Gemini Bioproducts), 100 U/ml Penicillin / 100 µg/ml Streptomycin (Gibco), 10 mM HEPES (Gibco), 1 mM Sodium Pyruvate (Gibco), and 2 mM L-Glutamine (Gibco) at a concentration of 1-2 x 10^6 cells/ml in straight standing 25 cm^2 tissue culture flasks (Corning). Cells were either cultured in the presence of 5 ng/ml IL-4 (BioLegend) or activated with CellXVivo Human B Cell Expansion Kit (R&D Systems, 5 µl/ml of each expander).

Cas9 RNP assembly and nucleofection. 15 µg Cas9 (~100 pmol) were incubated with 20 µg gRNA (~580 pmol) for 20 min at room temperature (RT). 100 pmol of single-stranded HR template was added right before nucleofection. The HR templates used are provided in the Supplementary Information.

B cells were nucleofected with the Amaxa® Human B Cell Nucleofector® Kit (Lonza). 2-5 x 10^6 cells per transfection were collected, washed once with PBS, and resuspended in 100 µl Nucleofector solution. The cell suspension was added to the Cas9 RNP/HR template mix, and cells were nucleofected with program V-015 in a Nucleofector™ 2b device (Lonza). 0.5 ml

media were added immediately after nucleofection, and cells were allowed to recover for 30 min in the cuvette inside the incubator at 37 °C. Cells were then transferred to FACS tubes and cultured in 2 ml culturing/activation media supplemented with 10 µM Q-VD-OPH (Cayman Chemical) and 1 µM Scr7 (XcessBio) until analysis. For nucleofection of Cas9 as plasmid or mRNA, 2-5 µg lentiCas9-eGFP (Addgene, #63592) or Cas9 mRNA 5meC, Ψ (Trilink) were mixed with 20 µg gRNA (~580 pmol) and nucleofected under the same conditions.

For HR of sequences encoding antibodies, 3 µg of dsDNA was added. The sequences of the HR templates for the ozoralizumab nanobody (fused to FLAG), adalimumab variable heavy chain-Fc (fused to FLAG), and adalimumab variable light chain-Fc (fused to HA) are provided:

CXCR4_HindIII: 5′-GGG CAA TGG ATT GGT CAT CCT GGT CAT GGG TTA CCA GAA GAA ACT GAG AAG CAT GAC GGA CAA GTA CAG GCT GCA CCT GTC AGT GGC CGA AAG CTT GGA TCC CAT CAC GCT TCC CTT CTG GGC AGT TGA TGC CGT GGC AAA CTG GTAC TTT GGG AAC TTC CTA TGC AAG GCA GTC CAT GTC ATC TAC ACA GT-3′, IGHV_HindIII: 5’-TAT ATA GTA GGA GAT ATG CAA ATA GAG CCC TCC GTC TGC TGA TGA AAA CCA GCC CAG CCC TGA CCC TGC AGC TCT GAG AGA GGA GCC CAG AAG CTT GGA TCC CAG GTG TTT TCA TTT GGT GAT CAG GAC TGA ACA GAG AGA ACT CAC CAT GGA GTT TGG GCT GAG CTG GCT TTT TCT TGT GGC TAT TTT AAA AGG TAA TT-3’, IGHV/J_HindIII: 5’-TAT ATA GTA GGA GAT ATG CAA ATA GAG CCC TCC GTC TGC TGA TGA AAA CCA GCC CAG CCC TGA CCC TGC AGC TCT GAG AGA GGA GCC CAG AAG CTT GGA TCC CAG GTG TTT TCG GAC CTG GGC GGA CTG GCC AGG AGG GGA TGG GCA CTG GGG TGC CTT GAG GAT CTG GGA GCC TCT GTG GAT TTT CCG ATG CCT TTG GA-3’, IGKV_HindIII: 5’-CCC AGC TGC TTT GCA TGT CCC TCC CAG CCG CCC TGC AGT CCA GAG CCC ATA TCA ATG CCT GGG TCA GAG CTC TGG AGA AGA GCT GCT CAG TTA GGA CCC AGA AGC TTG GAT CCA AAC CCC AGC GCA GCT TCT CTT CCT CCT GCT ACT CTG GCT CCC AGG TGA GGG GAA CAT GGG ATG GTT TTG CAT GTC AGT GAA AAC CC-3’, IGKV/J_HindIII: 5’- CCC AGC TGC TTT GCA TGT CCC TCC CAG CCG CCC TGC AGT CCA GAG CCC ATA TCA ATG CCT GGG TCA GAG CTC TGG AGA AGA GCT GCT CAG TTA GGA CCC AGA AGC TTG GAT CCG ACC AAG GTG GAG ATC AAA CGT AAG TGC ACT TTC CTA ATG CTT TTT CTT ATA AGG TTT TAA ATT TGG AGC GTT TTT GTG TTT GAG AT-3’.

The sequences of the HR templates for the ozoralizumab nanobody (fused to FLAG), adalimumab variable heavy chain-Fc (fused to FLAG), and adalimumab variable light chain-Fc (fused to HA) are provided as follows (homology arms of 1,000 bp, 750 bp, and 1,000 bp were included, respectively, and are in upper case):

Ozoralizumab nanobody-FLAG

GGATACACAGGTGAGCACACGTGAAATTATGTTTTTTTTCCTTCGGAGACATGGTTTTGCTTTGTCGTCCAGGCTGGA
GCTCCGTGGTGAGATCATAGCTCTCTGCAGCCTCAGACCCTGGGTCTCCAGCAATCCTCCTCCCTCTGCCCTATGTAGC
TGGGACTACAGGGGTGCACCACCATGCTCGGCTTCAGTGCGTATTAAACCGCACACTTCAATTATGCAGTATTTATTA
TATAGCAATAATGCCTCAATAAGGGTATTACAAATAAGTGGATAATTTGTTAAAGATTGATGGAAAGATAGATACCA
ACATGAGAAATGTATGACACTCAAGAAAATAAAACTGTAGGAAACTTGCTTTTCTTTATATTTGTTAGGTAATCACC
ACAGTGTGTACACATCACACCATGTTCCCATTACAGAGAAAAGGTTCTGCGAACCTCACGAGCTGTGACCCCTGTGTG
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TAC AAA GAC GAT GAC GAC AAG tga
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CTGGTGACTCTTCCGCAATCAGTCCTGGGACAGAAACCTCAGGGACAGCCTTTATGAGTTTTGATCACATCTAACAGA
GAGGATGGGCCAGGGCCAGAGTCATGTAGAACCTCACAGGTTCCACGTCTGACCCTTCTCCTGACACTA

Adalimumab variable heavy chain-Fc-FLAG
TCAATAAGGGTATTACAAATAAGTGGATAATTTGTTAAAGATTGATGGAAAGATAGATACCAACATGAGAAATGTA
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tacaagaccacccccccccgtgctggacagcgacggcagcttcttcctgtacagcaagctgaccgtggacaagagcagatggcagcagggcaacgtgttcagct
gcagcgtgatgcacgaggccctgcacaaccactacacccagaagagcctgagcctgagccccggcaag GAC TAC AAA GAC GAT GAC GAC
AAG tga
ATTTGGTGATCAGGACTGAACAGAGAGAACTCACCATGGAGTTTGGGCTGAGCTGGCTTTTTCTTGTGGCTATTTTA
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CAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCC
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ACGGCCGTATATTACTGTGCGAAAGACACAGTGAGGGGAAGTCATTGTGAGCCCAGACACAAACCTCCCTGCAGGAAC
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Adalimumab variable light chain-Fc-HA

CAATGGGTGCCCAAAGAGGTAGTAGTCTCAGAACAGGGAGAGACAGACTGAGTCCCTCCACTAACTCAGCCCAACACC
TGTGGATGAGTAGGGGATGCCTGAGATCCTGGGGTGGATGGGAGGTGGGGCACTGATCTGTCAATCTGCTTTTTCTTC
AAGGATCAGGCAGCAGAGACCCAGAAGCTTCATGTCTTTGTAAGGCTCTTCCAAGCCAACACAGATAATTTGACAAA
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agcagcaccctgaccctgagcaaggccgactacgagaagcacaaggtgtacgcctgcgaggtgacccaccagggcctgagcagccccgtgaccaagagcttc
aacagaggcgagtgc TAC CCA TAC GAT GTT CCA GAT TAC GCT tga
AAACCCCAGCGCAGCTTCTCTTCCTCCTGCTACTCTGGCTCCCAGGTGAGGGGAACATGGGATGGTTTTGCATGTCAG
TGAAAACCCTCTCAAGTCCTGTTACCTGGCAACTCTGCTCAGTCAATACAATAATTAAAGCTCAATATAAAGCAATAA
TTCTGGCTCTTCTGGGAAGACAATGGGTTTGATTTAGATTACATGGGTGACTTTTCTGTTTTATTTCCAATCTCAGAT
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TATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACC
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ACAGAGAAACTACTAAAATTGACATGAGAAATAGAAAATCATTAGATTCAAGATCAATTTATTAAT

Amplification of targeted genomic region. Genomic DNA was isolated from cells using the DNeasy® Blood & Tissue Kit (Qiagen). 100 ng DNA was used to amplify the targeted genomic region using the Q5® High-Fidelity 2X Master Mix (NEB). To this end, the following primers were used: CXCR4_for: 5′-AGA GGA GTT AGC CAA GAT GTG ACT TTG AAA CC-3′, CXCR4_rev: 5′-GGA CAG GAT GAC AAT ACC AGG CAG GAT AAG GCC-3′, IGHV_for: 5’- GTG AGC ACT GGG GAC ATT GTA AAA CCC ACC -3’, IGHV_rev: 5’-CTG CCG CTG ATT TCC CCC CCA TCG TTC CTG-3’, IGHJ_for: 5’-ATG GGA ACC CAG CCT GTC CTC CCC AAG TCC-3’, IGHJ_rev: 5’- GAG GTC CTG GAG CCT CCC TAA GCC CCT GTC-3’, IGKV_for: 5’-GCA GTT GTG AAA GTC CTC ACA CCC ACA GTG-3’, IGKV_rev: 5’-GGC

CCT GCT GGA TGC ACC ATA GAT GAG GAG-3', IGKJ_for: 5'-GGT GAC CCA GAA GTA AAT AGC AGG ACA CCA-3', IGKJ_rev: 5'-TGA GCT TTT GGG AAT AAC CAC CTT TCC ACC-3'. PCR was performed using the following cycle conditions: one cycle of 98 °C for 30 s; 35 cycles of 98 °C for 5 s, 72 °C for 10 s, 72 °C for 20 s; one cycle of 72 °C for 2 min. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) for further analysis.

Cross-boundary PCR. To confirm insertion of the HR template into the targeted BCR loci by PCR, we used forward primers located in the endogenous genomic region (IGHV_for, IGKV_for) and reverse primers binding to the inserted HindIII site sequences specific for insertion into IGHV, IGHV/J, IGKV and IGKV/J: HindIII_HV_rev: 5'-ACC AAA TGA AAA CAC CTG GGA TCC AAG CTT-3', HindIII_HVJ_rev: 5'-CAG GTC CGA AAA CAC CTG GGA TCC AAG CTT-3', HindIII_KV_rev: 5'-GGG GTT TGG ATC CAA GCT TCT GGG TCC TAA-3', HindIII_KVJ_rev: 5'-CTT GGT CGG ATC CAA GCT TCT GGG TCC TAA-3'.

To confirm the insertion of the nanobody, heavy chain (HV), and light chain (KV) into the targeted BCR loci, the following primers were used: Nano_for1: 5'-CAG TAT GGT GAG AGG GAA ATA ATG GGG AGG-3', Nano_rev1: 5'-CAG TAC ATC CAG TAG TCG CTG AAG GTG AAG-3', Fc-HV_for1: 5'-CAT GCT CGG CTT CAG TGC GTA TTA AAC CGC-3', Fc-HV_rev1: 5'-CAG TGC ATG GCG TAG TCG TCG AAG GTG AAG-3', Fc-KV_for1: 5'-CCT GCC TTC TAG AAC TGG ACA ACC GAG GGT-3', Fc-KV_rev1 5'-CCA GGT AGT TTC TGA TGC CCT GGC TGG CTC-3'. PCR was performed using the Q5® High-Fidelity 2X Master Mix (NEB) with following cycle conditions: one cycle of 98 °C for 30 s; 35 cycles of 98 °C for 5 s, 72 °C for 10 s, 72 °C for 20 s; one cycle of 72 °C for 2 min.

T7E1 assay and HindIII digest. In order to determine cutting efficiency, 200 ng of purified PCR product were used for T7E1 digestion using the T7 Endonuclease I (NEB). To this end, duplex formation of 19 µl total sample volume including 2 µl Buffer 2 (NEB) was performed with the following cycle program: 95 °C (5 min), 95-85 °C (-2 °C/s), 85-25 °C (0.1 °C/s), 4 °C (hold). 1 U of T7 Endonuclease I was added, and the reaction was stopped after 15 min at 37 °C by adding 1.5 µl 0.25 M EDTA. 12 µl of the digested sample was mixed with 3 µl 5x Novex® Hi-Density TBE Sample Buffer (Thermo Fisher) and loaded on a 6% Novex® TBE Gel (Thermo Fisher) together with 0.25 µl 100 bp ladder (NEB). Vertical DNA electrophoresis was run for 40 min at 150 V and stained with SYBR Safe DNA Gel Stain (Thermo Fisher) for 30 min at RT in the dark. DNA band intensity was determined by ImageJ or Image Lab from Bio-Rad, and cutting efficiency was calculated as follows: % cutting = 100 x $(1-(1-\text{fraction cleaved})^{1/2})$. To confirm insertion of the HindIII site, 200 ng of purified PCR product was incubated with 2 U of HindIII-HF® (NEB) and CutSmart Buffer (NEB) in a total volume of 20 µl for 2 h at 37 °C. 12 µl of the digested sample was run on a 6% TBE gel and analyzed as before. HR efficiency was calculated as follows: % HR = (cleavage products/(DNA substrate + cleavage products)) x 100.

TIDE analysis. For Sanger sequencing of the targeted and edited genomic region in the CXCR4 locus, we used the primers CXCR4_for: 5′-AGA GGA GTT AGC CAA GAT GTG ACT TTG AAA CC-3′ and CXCR4_rev: 5′-GGA CAG GAT GAC AAT ACC AGG CAG GAT AAG GCC-3′. The TIDE analysis was performed using the TIDE calculator developed by the Netherlands Cancer Institute (Brinkman *et al.*, *Nucl Acids Res*, 2014) (Brinkman et al., 2014).

MiSeq deep sequencing. The genomic target regions were first amplified with the Q5® High-Fidelity 2X Master Mix (NEB) using the following primers (resulting in ~300 bp regions): Illumina_CXCR4_for: 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTT CCT GCC CAC CAT CTA CTC CAT CAT CTT CTT AAC TG-3', Illumina_CXCR4_rev: 5'-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TCA GGT AGC GGT CCA GAC TGA TGA AGG CCA GGA TGA GGA C-3', Illumina IGHV_for: 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGC TCT GAG CCC CAC TAT CTC CAA AGG CCT-3', Illumina IGHV_rev: 5'-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TCA CTG TTT CTC TCA CTC TTA TCC ATT CAC A-3', Illumina IGHJ_rev: 5'-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TAA ATG TGG CTC CCC AAG CCC CCA GGC TCA G-3', Illumina IGKV_for: 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ACC TGG GCA TGG GCT GCT GAG AGC AGA AAG-3', Illumina IGKV_rev: 5'-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TGC AGA GTT GCC AGG TAA CAG GAC TTG AGA G-3', Illumina IGKJ_for: 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AAG TTA ACA CTG TGG ATC ACC TTC GGC CAA-3', Illumina IGKJ_rev: 5'-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TCT TTT GCT CCT TAG AAA TAC AAG GGT TCC T-3'.

100 ng of genomic DNA was amplified with the following cycling conditions: CXCR4: one cycle of 98 °C for 30 s; 35 cycles of 98 °C for 5 s, 72 °C for 10 s, 72 °C for 20 s; one cycle of 72 °C for 2 min; BCR: one cycle of 98 °C for 30 s; 4 cycles of 98 °C for 5 s, 65 °C for 10 s, 72 °C for 20 s; 29 cycles of 98 °C for 5 s, 72 °C for 10 s, 72 °C for 20 s; 1 cycle of 72 °C 2 min. PCR fragments were purified using the QIAquick PCR Purification Kit (Qiagen), and size and purity were confirmed by agarose gel electrophoresis. In a second PCR step, Illumina Universal Adapters (5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T-3′) and the PCR barcode sequence (5′-CAA GCA GAA GAC GGC ATA CGA GAT-Index- GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T-3′) were attached to the PCR fragment. The NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1&2, NEB) were used with the following PCR conditions: one cycle of 98 °C for 30 s; 5 cycles of 98 °C for 10 s, 65 °C for 75 s; one cycle of 65 °C for 5 min. PCR products were purified again with the QIAquick PCR Purification Kit (Qiagen), and size and purity were confirmed by agarose gel electrophoresis. Concentrations were equalized to 80 ng/µl, pooled, and run on a MiSeq Sequencing System (Illumina). Deep sequencing data were analyzed using the CRISPR Genome Analyzer (CRISPR-GA, Guell M, Yang L, Church G (2014) Genome Editing Assessment using CRISPR Genome Analyzer (CRISPR-GA) Bioinformatics).

RNA isolation and RT-PCR. RNA was isolated 6 days post-transfection using TRIzol® Reagent (Life Technologies) according to the manufacturer's instructions. Isolated RNA was treated with RNase-free DNase I (NEB) and purified using the GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Fisher). First-strand cDNA was synthesized using the ProtoScript® First Strand cDNA Synthesis Kit (NEB) and the primer cDNA-Fc-HV-rev1: 5'-CTT GTA GTT GTT CTC GGG CTG GCC GTT GCT-3'. Briefly, 500 ng of RNA were incubated with 2.5 µL of 20 µM primer in a total volume of 8 µL at 70 °C for 5 min. 10 µL of M-MuLV Reaction Mix and 2 µL of M-MuLV Enzyme Mix were added, and samples were incubated for 1 h at 42 °C and 5 min at 80 °C. For the nested PCR, 1 or 5 µL of the synthesized

cDNA (diluted 1:10) were used as template. PCR amplification was performed using the Q5® High-Fidelity 2X Master Mix (NEB) and the following primers: mRNA-Fc-HV_for: 5'-CCT GAG CAC CGC CAG CAG CCT GGA CTA CTG-3' (forward) and mRNA-Fc-HV_rev: 5'-CCA GTC CTG GTG CAG CAC GGT CAG CAC GCT-3' (reverse). The following cycle conditions were used: one cycle of 98 °C for 30 s; 35 cycles of 98 °C for 5 s, 72 °C for 10 s, 72 °C for 20 s; one cycle of 72 °C for 2 min.

Flow cytometry. Cell surface staining was performed with PE anti-human CD19 Antibody (HIB19, BioLegend) and APC anti-human CD184 (CXCR4) Antibody (12G5, Biolgend) for 20 min at RT in the dark following a prior 10 min incubation with Human TruStain FcX™ (Fc Block, BioLegend). For intracellular staining, cells were fixed and permeabilized using a BD Cytofix/Cytoperm™ Kit (BD Biosciences) according to the manufacturer's instructions. FLAG-HV was stained with either PE or APC anti-DYKDDDDK (L5, BioLegend) and HA-KV was stained with PE anti-HA-Tag (C29F4, Cell Signaling Technology) for 1 h at RT in the dark for each antibody. Viability was analyzed in living cells with 7-AAD Viability Staining Solution (BioLegend) and in fixed cells using the Zombie NIR™ Fixable Viability Kit (BioLegend) or Zombie Aqua™ Fixable Viability Kit (BioLegend) prior to fixation according to the respective manuals. Stained cells were analyzed on a BD LSRFortessa™ Cell Analyzer (Becton Dickinson).

Statistical analysis. The unpaired Student's *t*-test was used to determine statistically significant differences between two groups. Data were analyzed using GraphPad Prism 7 software. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Data and materials availability statement. The datasets and materials generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.