Efficient Immune Cell Genome Engineering with Enhanced CRISPR Editing Tools

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Efficient Immune Cell Genome Engineering with Enhanced CRISPR Editing Tools

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ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR)-based methods have revolutionized genome engineering and the study of gene-phenotype relationships. However, modifying cells of the innate immune system, especially macrophages, has been challenging because of cell pathology and low targeting efficiency resulting from nucleic acid activation of intracellular sensors. Likewise, lymphocytes of the adaptive immune system are difficult to modify using CRISPR-enhanced homology-directed repair because of inefficient or toxic delivery of donor templates using transient transfection methods. To overcome these challenges and limitations, we modified existing tools and developed three alternative methods for CRISPR-based genome editing using a hit-and-run transient expression strategy, together with a convenient system for promoting gene expression using CRISPRa. Overall, our CRISPR tools and strategies designed to tackle both murine and human immune cell genome engineering provide efficient alternatives to existing methods and have wide application not only in terms of hematopoietic cells but also other mammalian cell types of interest. ImmunoHorizons, 2021, 5: 117–132.

INTRODUCTION

The bacterial innate defense system clustered regularly interspaced short palindromic repeats (CRISPR) was discovered more than three decades ago (1) but only harnessed for gene editing applications in a series of landmark studies published in 2013 (2–4). Since then, there has been an exponential increase of CRISPR-related publications, with many of the initial studies employing the Cas9 nuclease that can yield variable off-target effects in its unmodified form (5–7). Many groups have now developed new methods to minimize this problem (8–14). The Cas9 nickase technique is one useful approach with a strict pairing distance requirement that makes off-target action very unlikely. Another strategy to reduce CRISPR-Cas9 off-target effects is to limit the duration of Cas9 or guide-RNA (gRNA) expression in the target cells (13, 15). Although such temporal control can be achieved using transient transfection methods such as RNA electroporation, the on-target editing efficiency...
can be compromised (16) as compared with stable lentiviral transduction methods. In addition, transient transfection approaches do not permit easy regulation of Cas9 or gRNA expression duration, whereas cytotoxicity created by electroporation methods remains a concern for many sensitive cell types.

These issues are amplified for hematopoietic cells, with only a few reports using the Cas9 nickase strategy for immune cell (myeloid cell or lymphocyte) genome engineering. Macrophages have been especially difficult to modify using available CRISPR methods. All-in-one CRISPR-Cas9 techniques are inefficient (17), and although newer methods have yielded improved results (18–20), some of these approaches require antibiotic selection and clonal isolation that delay the phenotyping timeline and risk the loss of original cell properties. To enable high-throughput CRISPR-Cas9 genome-wide knockout (KO) library screens, the murine RAW264.7 macrophage (RAW) cell line has been modified to constitutively express Cas9 for efficient genome editing (21). However, constitutive Cas9 and gRNA expression can lead to the accumulation of off-target mutations. A doxycycline-inducible system pCW-Cas9 (22) has been reported to resolve both issues by controlling the genome editing time window to minimize long-term off-target effects and allowing immediate phenotyping before the onset of potential compensating mechanisms. Nonetheless, the lack of a live cell marker in current versions of the vectors used for such experiments makes it difficult to identify and isolate the Cas9-expressing population for further analysis.

Primary bone marrow–derived macrophages (BMDM) are particularly problematic to engineer because of their innate immune sensors. Lentiviral particles produced from packaging cells such as the HEK293T cell line are usually filtered or spun to remove cell debris. However, this process does not remove many small molecules and exosomes secreted from the exhausted and dying packaging cells. Lentiviral supernatant containing these uncharacterized materials is often directly applied to cell culture, engineer the RAW cell line. To deal with the issue of activation of immune sensors, we show that purified lentiviral particles enhance primary macrophage cell viability posttransduction. To control the duration of Cas9 expression and track cell with this enzyme present, we describe a modified method to enable doxycycline-induced Cas9 expression to be tracked by EGFP fluorescence in live single cells and use this system to effectively engineer the RAW cell line. To deal with the issue of activation of intracellular sensors, we show that purified lentiviral particles enhance primary macrophage cell viability posttransduction.

In contrast to macrophages, all-in-one lentiviral-mediated CRISPR-KO is highly efficient in human T lymphocytes (21, 22). However, this approach is not suitable for CRISPR–homology-directed repair (HDR) applications because of random genome integration of lentiviral vectors carrying the HDR donor template that can lead to undesired genomic instability. Transient transfection delivery methods such as lipid-based reagents and electroporation systems either yield low transfection efficiency or a high percentage of cell death. Furthermore, compared with the high targeting efficiency (80–90%) of CRISPR-induced nonhomologous end joining (NHEJ), the targeting efficiency of CRISPR-induced HDR is generally inefficient (10–20% for large fragment insertion) (23). Although the NHEJ inhibitor SCR7 (24) and the HDR stimulatory compound RS-1 (25) have been reported to enhance CRISPR-Cas9–induced HDR efficiency, the key to shifting the balance between the NHEJ and the HDR DNA repair mechanisms in favor of the latter outcome seems to rely on a high molar ratio of donor DNA to Cas9–gRNA ribonucleoprotein. This was demonstrated in human primary T cell engineering using $1 \times 10^6$ multiplicity of infection of adeno-associated virus (AAV) type 6 transduction to deliver a high copy number of HDR donor templates per target cell (26). AAV is a small ssDNA virus that infrequently integrates into the host genome, making it a safe delivery platform for the HDR donor template. However, the production and purification process of AAV particles is relatively labor intensive, increasing the cost and reducing the flexibility of preliminary experiments in the academic laboratory setting. In addition, the very limited payload of the AAV vector makes it impossible to fit Cas9, gRNA, and donor template in one AAV backbone. The alternative is the use of lentiviruses. Lentiviral particles are easy to produce and purify in most laboratories with relatively low cost. Furthermore, the payload of lentiviral particles is large enough to carry Cas9, gRNA, and donor template all in one. Despite these advantages, random integration of lentiviral DNA into the host genome makes it problematic as a platform to deliver the HDR donor template, leaving an opportunity for further improvement in vector design.

The all the above methods involve either gene inactivation or modification. There are numerous situations in which augmented expression of a given gene would be desirable in an experimental setting. Although methods for gene activation using the CRISPRa strategy have been reported (27–29), efficient induction of selective gene expression is not yet a commonly used method as we lack simple tools for this purpose.

In this study, we have addressed each of the problems outlined above and developed new vectors and strategies that enable more effective genetic modifications of macrophages and lymphocytes.

To control the duration of Cas9 expression and track cell with this enzyme present, we describe a modified method to enable doxycycline-induced Cas9 expression to be tracked by EGFP fluorescence in live single cells and use this system to effectively engineer the RAW cell line. To deal with the issue of activation of intracellular sensors, we show that purified lentiviral particles enhance primary macrophage cell viability posttransduction. To achieve efficient and more-precise genome modifications in human Jurkat T cells as a model for mature lymphocyte engineering, we adopted the Cas9 nickase strategy and describe an antibiotic selection strategy that helps enrich specifically for rare HDR clones following lipid-based plasmid cotransfection.

In addition, we further improved this method to minimize the genomic footprint created by the antibiotic resistance marker and make possible future rounds of genome editing in the same cells using the same strategy. We overcome the limitation associated with AAV and with integrating lentiviruses by the use of integrase-deficient lentiviral particles (30–32) and report two examples of this approach using all-in-one Cas9–gRNA–HDR-donor design in human Jurkat T cells. Finally, to enable gene activation rather than inactivation or modification, we modified an existing two-vector system (29) to create an all-in-one lentiviral-based CRISPRa system and show its utility as applied to the human Jurkat T cell model. These various new vectors and strategies constitute an effective suite of methods for the manipulation of both innate (macrophage) and adaptive
(lymphocyte) immune cells that offer benefits when applied to a wide variety of cell types.

**MATERIALS AND METHODS**

**Gibson assembly cloning of donor and lentiviral expression plasmids**

The pCW-Cas9-2A-EGFP (iCE) plasmid was constructed by replacing the FseI-BamHI fragment in the pCW-Cas9 plasmid (plasmid no. 50661; Addgene) (22) with the T2A-EGFP fragment. The LCv2B plasmid was constructed by replacing the BamHI-MluI fragment in the LentiCRISPRv2 plasmid (plasmid no. 52961; Addgene) (33) with the P2A-BSD fragment. The LentiGuide-TagBFP-2A-BSD (LGB) and the LentiGuide-TagRFP-2A-BSD (LGR) plasmids were constructed by replacing the XbaI-Cas9-BamHI fragment in the LCv2B plasmid with the TagBFP and the TagRFP-T fragment, respectively. The STAT3 HDR donor plasmid was constructed by replacing the NotI-XhoI fragment in the pcdNA3.1 plasmid with the indicated fragments. The BCL10 HDR donor plasmid was assembled based on a minimal PciI-linearized BCL10 backbone. The CD3E gRNA was linearized at the KpnI site upstream of the U6 promoter before the assembly of the donor template fragments. The LCv2S plasmid (Supplemental Fig. 3A) was constructed by adding the P2A-mScarlet-I fragment to the BamHI-linearized LCv2S backbone. The LCv2R plasmid was assembled by adding the P2A-TagRFP-T fragment via sticky-end ligation. To construct the LCv2S-HDR-tBFP-hRelA plasmid, the LCv2S plasmid (Supplemental Fig. 3A) was constructed by replacing the P2A-mScarlet-I fragment with the P2A-hRelA fragment. The Lenti-CMV-mCherry-2A-hCD3E plasmid, LCv2B plasmid containing the CD3ε gRNA was linearized at the KpnI site upstream of the U6 promoter before the assembly of the donor template fragments. The LCv2R plasmid was constructed by adding the P2A-mScarlet-I fragment to the BamHI-linearized LCv2S backbone. The LCv2E plasmid was assembled using Infusion cloning by adding the P2A-tEGFR fragment to the BamHI-linearized LCv2S backbone. The LES2A-CRE plasmid was assembled by replacing the XbaI-EcoRI fragment in the lentiCas9-Blast plasmid (plasmid no. 52962; Addgene) (33) with the mScarlet-I-P2A-CRE fragment. The Lenti-CMV-mCherry-P2A-CRE (also known as pLM-CMV-R-Cre) plasmid was a gift from M. Sadelain (plasmid no. 27546; Addgene) (34). The LentiSAMv2 plasmid was assembled by adding the P2A-MS2-p65-HSF1 fragment amplified from the lentimPH v2 plasmid (plasmid no. 89308; Addgene) (29) to the SsrG1-linearized lentiSAMv2 backbone (plasmid no. 75112; Addgene) (29). All constructs described in this article will be deposited with Addgene and become available to qualified investigators.

**CRISPR gRNA target design and cloning**

All gRNAs were designed based on the analysis results arising from use of a previously available online platform (crispr.mit.edu). For the dual-specificity phosphatase (DUSP) gene KO application, we selected the gRNA with the best score that targets downstream of the start codon of each DUSP gene. For the STAT3 HDR application, we selected a gRNA pair with the best score that targets near the start codon. For the BCL10 HDR application, we selected top five scoring gRNA pairs that target near the start codon. In addition, we selected a gRNA pair (D3) that confers a small offset distance of 3 bp. Both the CD3E gRNA and the RelA gRNA for the nonintegrating lentiviral (NIL) delivery applications were designed to overlap with the start codon in each case to avoid further editing following the HDR event. The RelA gRNA A1 was adopted from the human GeCKOv2 library A (33). The PX461 and PX462 plasmids were gifts from F. Zhang (plasmid nos. 48140 and 48141; Addgene) (35). The pKLV-BFP plasmid was a gift from K. Yusa (plasmid no. 50946; Addgene) (36). All lentiviral and other gRNA expression plasmids were prepared by sticky-end ligation before any further modification described. The PX462-hBCL10-gRNA-A1 plasmid was constructed by Gibson Assembly in the PciI-linearized PX461 backbone, followed by subcloning of the double gRNA expression fragment into the PX462 backbone with PvuI-XbaI sticky-end ligation. CRISPRa gRNAs were adopted from the Human CRISPR Activation Library (SAM–2 plasmid system) from F. Zhang (29).

**Production, purification, and concentration of lentiviral particles**

Lentiviral particles were produced in HEK293T cells following transient transfection of the packaging plasmids pVSVG (plasmid no. 8454; Addgene) and psPAX2 (plasmid no. 12260; Addgene) and the lentiviral expression plasmid in a 1:10:10 ratio using Lipofectamine 3000. Lentiviral supernatant was harvested 44–52 h posttransfection and spun at 800 × g for 5 min to clear cell debris. NIL particles were produced the same way, except with the use of the psPAX2-D64V plasmid (plasmid no. 63586; Addgene) (30) instead of the psPAX2 plasmid. All lentiviral particles other than those used in the RAW cell transductions and Fig. 2B (top panel) were further purified and concentrated with the Lenti-X Concentrator reagent (Takara) according to the manufacturer’s instructions.

**Cell culture, transfection, and antibiotic selection conditions**

HEK293T, RAW, and Jurkat cell lines were purchased from the American Type Culture Collection. Cell culture was performed in a humidified environment with 5% CO₂ at 37°C. Both HEK293T and RAW cell lines were cultured in DMEM supplemented with 10% FBS, penicillin/streptomycin, and 50 μg/ml of kanamycin. Jurkat cell lines were cultured in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, and 50 μM 2-ME (complete RPMI medium). For plasmid transfections of HEK293T and Jurkat cells, Lipofectamine 3000 (Thermo Fisher Scientific) was used according to the manufacturer’s protocol. Puromycin (0.5–3 μg/ml) or blasticidin (0.5–3 μg/ml) was added to cell culture medium 1–2 d after transduction of both RAW and Jurkat cell lines.

**Genomic DNA extraction, T7EN assay, and sequencing**

Genomic DNA was extracted from RAW, HEK293T, or Jurkat cells using the QIAmp DNA Mini Kit (Qiagen) following the
manufacturer’s protocol. Genomic regions of interest were then amplified by PCR for follow-up analysis. For the T7EN assay, equal amounts of genomic PCR products were used within each comparison group for the hybridization reaction in a thermocycler programmed to 95°C for 10 min, −2°C per second ramp to 85°C, −0.1°C per second ramp to 25°C, and then held at 4°C until a subsequent T7 endonuclease I (T7EN) (New England BioLabs) treatment at 37°C for 30–90 min. Twenty-five millimolar final concentration of EDTA was added to each reaction before agarose gel electrophoresis analysis. Insertion/deletion (indel) percentage was determined as described previously (8). For the genomic DNA sequencing, each DUSP gene locus surrounding the CRISPR gRNA target site (~500 bp to +500 bp) was amplified and cloned into a XhoI-NotI linearized pcDNA3.1 backbone by Gibson Assembly. Sequencing was performed with the BGH-R (Table I) primer on 20 clones per gene.

Bone marrow isolation and intracellular Ki67 staining
Mice were maintained in specific pathogen–free conditions, and all procedures were approved by the National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Use Committee (National Institutes of Health [NIH], Bethesda, MD, ASP LISB-4E). Bone marrow progenitors isolated from C57BL/6 mice (The Jackson Laboratory) and Rosa26-Cas9 B6J mice (no. 026179; The Jackson Laboratory) were differentiated into BMDM during a 7-d culture in complete DMEM (10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES) supplemented with 60 ng/ml recombinant mouse M-CSF (R&D Systems). For analysis of proliferation, cells were harvested at the indicated day of the BMDM culture, processed using BD Cytofix/Cytoperm reagents as directed, blocked, and stained with anti–Ki-67 (BD Pharmingen).

Macrophage TLR4 activation and intracellular phospho-p38 staining
BMDM or RAW cells were stimulated with TLR4 ligand Kdo2-Lipid A (Avanti Polar Lipids). Ligand addition was staggered so that for all time points, cells were fixed at the same time by addition of paraformaldehyde to cell cultures at a final concentration of 1.6% (10 min). After one wash with PBS 1% FBS, cells were gently harvested from plates by scraping, permeabilized overnight using ice-cold MeOH at −20°C, blocked using 5% goat serum and Fc receptor–specific Ab, and stained for 1 h at room temperature with anti-p38 (phospho-Thr180/Tyr182, 36/p38; BD) Ab. For both BMDM and RAW macrophages, two independent time-course experiments were pooled using normalized area under the curve (AUC). AUC was calculated using GraphPad Prism, and the AUC for each condition was normalized to the empty vector (EV) control from the same experiment.

Cell sorting, FACS analysis, and live cell confocal imaging
Cell sorting was conducted by the Flow Cytometry Section, Research Technologies Branch of NIAID. FACS analysis data were collected using a BD LSRII or BD Fortessa Cell Analyzer and further processed with FlowJo. Human PDCD1 or PDL1 cell surface protein staining was analyzed using anti–PD-1 (clone MIH4; Thermo Fisher Scientific) or anti–PD-L1 (clone MIH1; Thermo Fisher Scientific) Ab, respectively. Live cell confocal time-lapse imaging data were collected using a Leica SP8 microscope with a 63x NA 1.4 oil objective (Biological Imaging Section, Research Technologies Branch of NIAID). The imaging chamber (IS5411; Thermo Fisher Scientific) was coated with poly-D-lysine (P7280; Sigma-Aldrich) for 1 h at 37°C and washed twice with PBS. Cells were imaged in a heated 37°C environment with 5% CO2. Imaging data were processed by Imaris (Bitplane).

Chemical reagents, lysis buffer, and Western blots
Doxycycline hydrochloride (D3447; Sigma-Aldrich) was reconstituted in DMSO at 50 mg/ml and stored at −20°C. Jurkat T cells were stimulated with 50 ng/ml PMA (Santa Cruz Biotechnology) and 1 μM ionomycin (Sigma-Aldrich) (37). RAW cells were lysed in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% Igepal. Western blots were analyzed using anti-DUSP3 Ab (clone EPR5492; Abcam), anti-DUSP1 Ab (07-535; Millipore), and anti–FLAG Ab (sc-166355; Santa Cruz Biotechnology).

Human PBMC isolation and transduction
Human whole blood from healthy anonymous volunteer donors was purchased from an NIH blood bank. This was exempted from the need for informed consent and Institutional Review Board review, as determined by the NIH Office of Human Subjects Research Protection. Human PBMCs were isolated from whole blood by Ficoll density gradient separation, cultured in complete RPMI medium, and stimulated with 1 μg/ml anti-CD3 (300334; BioLegend) and 1 μg/ml anti-CD28 (302944; BioLegend) soluble Abs for 18 h. Purified and concentrated lentiviral particles were then added to the human PBMC culture, with 8 μg/ml Polybrene (TR-1003; Millipore) and 100 U/ml recombinant human IL-2 (TECIN [teceleukin]). These cells were then spun at 400 x g for 90 min in a prewarmed centrifuge at 34°C.

RESULTS
Dual-color inducible CRISPR-Cas9 editing system in RAW cells
To achieve a controllable balance between on-target and off-target mutations induced by CRISPR, we adopted the doxycycline-inducible pCW-Cas9 system to enable precise manipulation of the genome editing time period. In pCW-Cas9–transduced RAW macrophages, a range of Cas9 protein expression was induced by doxycycline dose titration (Supplemental Fig. 1A). We modified this existing system to enable single-cell flow cytometric analysis and developed the iCE lentiviral expression plasmid (Fig. 1A). Doxycycline titration in the iCE-transduced and purmorpholin-selected RAW cells (RAWiCE) revealed saturating Cas9-2A-EGFP protein expression at 1.0 μg/ml doxycycline (Fig. 1B). Single clone isolation from the RAWiCE line yielded cells that fully responded to doxycycline induction (Fig. 1C), indicating the digital nature of

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FIGURE 1. Dual-color inducible CRISPR strategy efficiently modifies DUSP genes in RAW cells.

(A) Lentiviral vector encoding inducible Cas9-T2A-EGFP under the control of the tTRE promoter. (B) EGFP expression by RAWiCE cells treated with doxycycline. (C) EGFP expression by RAWiCE clones treated with doxycycline. (D) Lentiviral vector for gRNA expression with TagBFP fluorescent marker and blasticidin resistance gene BSD. (E) Analysis of DUSP3, EGFP, and tBFP by RAWiCE cells transduced with the indicated LGB lentiviral particles and selected with blasticidin after doxycycline treatment. Results are representative of three independent experiments. (F) DUSP protein expression by RAWiCE cells transduced with the indicated LGB targeting DUSP2 or DUSP3. Data are representative of three independent experiments. (G) Indel analysis of genomic DNA from RAWiCE lines. (H) Sequence analysis of the genomic DNA from the RAWiCE lines.

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TABLE I. Summary of oligonucleotide sequences and gRNA targeting sequences

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence Related to Fig.</th>
<th>Target Gene gRNA Targeting Sequence Related to Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mDUSP1-E1F</td>
<td>5'-CGCTTTTGGCTTTTGGCCGGCGCCCCAGCTCGGTAGG-3'</td>
<td>mDUSP1</td>
</tr>
<tr>
<td>mDUSP1-E1R</td>
<td>5'-GTTGACCTGTTTCTGCACAGCTGAGGAGACCAG-3'</td>
<td>mDUSP2</td>
</tr>
<tr>
<td>mDUSP2-E1F</td>
<td>5'-GCTTGAAAGAATGGAAAAGTGGGAAGCTGAGCAGTAC-3'</td>
<td>mDUSP3</td>
</tr>
<tr>
<td>mDUSP2-E1R</td>
<td>5'-GAGCTGCAGAGGAGATGGGGG-3'</td>
<td>mDUSP4</td>
</tr>
<tr>
<td>mDUSP3-E1F</td>
<td>5'-GTTTGGGGTGAATGAATGGATGCCCACTCTGCGTGAT-3'</td>
<td>hSTAT3</td>
</tr>
<tr>
<td>mDUSP3-E1R</td>
<td>5'-GAGATGTAAGCATTTGATTAAGTGCTG-3'</td>
<td>hCD3E</td>
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<tr>
<td>mDUSP4-E1F</td>
<td>5'-GGTGGACTGTTTGCTGCACAGCTCAGGGCAGGAAGCCGAA-3'</td>
<td>hRelA</td>
</tr>
<tr>
<td>mDUSP4-E1R</td>
<td>5'-GAGATGTAAGCATTTGATTAAGTGCTG-3'</td>
<td>hCD3E-R</td>
</tr>
</tbody>
</table>

Target Gene gRNA Targeting Sequence Related to Fig.

| mDUSP1 | 5'-ATCGTGGCCGCCGCCGCCCAA-3' | 1, 2, S1, and S2 |
| mDUSP2 | 5'-TTGGACCTGTTTCTGCACAGCTGAGGAGACCAG-3' |
| mDUSP3 | 5'-GCTTGAAAGAATGGAAAAGTGGGAAGCTGAGCAGTAC-3' |
| mDUSP4 | 5'-GAGCTGTGATTATCCAAGGTGGGATTGATAGTGGG-3' |
| hSTAT3 (gRNA-A) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hSTAT3 (gRNA-1) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hBCLI10 (gRNA-A) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hBCLI10 (gRNA-B) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hBCLI10 (gRNA-C) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hBCLI10 (gRNA-D) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hBCLI10 (gRNA-1) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hBCLI10 (gRNA-2) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hBCLI10 (gRNA-3) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hCD3E | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hRelA (gRNA-A1) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hPCDC1 (gRNA-1) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hPCDC1 (gRNA-2) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |
| hPCDC1 (gRNA-3) | 5'-GAGATGTATGTAAGCATTTGATTAAGTGCTG-3' |

This inducible expression system. To track single cells that constitutively express a targeting gRNA with a different set of antibiotic selection and live cell fluorescent markers, we developed the LGB plasmid (Fig. 1D) based on the LentiCRISPRv2 backbone, which had been optimized to produce high-titer lentiviral particles. For applications that require simultaneous tracking of two targeting gRNAs, we also developed the LGR plasmid (Supplemental Fig. 1B) to offer a different color option and enhance the overall flexibility of this inducible CRISPR-Cas9 editing system.

To test this method, we targeted members of the DUSP family in RAW cells. The RAWiCE cells transduced with LGB targeting each chosen DUSP genomic locus were first selected by blasticidin to maximize the gRNA-expressing (tBFP+) population. Following doxycycline induction at 10 μg/ml for 6 d, we analyzed DUSP3 expression by intracellular staining and EGFP−tBFP+ gating (Fig. 1E), choosing this specific protein for analysis among the set targeted because of the availability of a well-characterized Ab for single-cell analysis by flow cytometry. We observed substantial (>95%) reduction in total DUSP3 protein expression (Fig. 1F) within the CRISPR-targeted EGFP−tBFP+ population, indicating that the majority of targeted cells (Fig. 1E) were indeed complete KO of DUSP3 expression. Because specific Abs were not available for direct single-cell protein level analysis of the other targeted DUSP loci, we performed T7EN assays to confirm the presence of indel mutations at the targeted DUSP genomic loci (Fig. 1G, Table I). Because the T7EN assay does not yield the actual DUSP KO percentage, we performed genomic DNA sequencing to better

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estimate the CRISPR-editing outcomes of all five DUSP genes. The sequencing results revealed that most if not all targeted DUSP alleles had been mutated at the expected loci surrounding the protospacer adjacent motif (PAM) sites (Fig. 1H, Supplemental Fig. 1C). Overall, these results strongly suggest that our dual-color inducible CRISPR strategy confers high genome editing efficiency in an extremely sensitive, albeit transformed, innate immune cell type.

Upon stimulating the various DUSP-KO RAW cells with TLR4 ligand, we did not observe sustained p38 phosphorylation in DUSP1-KO RAW cells (Supplemental Fig. 2A), an expected phenotype based on a previous study using primary BMDM derived from DUSP1-KO mice (38). Thus, we examined the effects of such a KO in BMDM using the same gRNA used to target DUSP1 in RAW cells. Although lentiviruses can transduce nondividing cells, transduction efficiency is optimized in cells undergoing active replication. Based on the Ki67 proliferative marker expression profile of bone marrow cells cultured with M-CSF (Supplemental Fig. 2B), we developed a lentiviral gRNA transduction protocol (Fig. 2A) to create target gene KO BMDM for further analysis. Unlike RAW cells, BMDM are more sensitive to crude lentiviral supernatant from HEK293T packaging cells seen as a dramatic posttransduction decline in the live BMDM percentage (Fig. 2B, top panel). To optimize BMDM survival, we further purified and concentrated lentiviral particles to remove potentially toxic materials from the crude supernatant. This approach improved the live BMDM percentage as the LGB titration reached a peak transduction efficiency (Fig. 2B, bottom panel). Using this improved transduction method for LGB expression in Cas9’ EGFP’ BMDM, we performed intracellular protein staining and validated the editing efficiency of the method using a DUSP3 gRNA, again because of reliable Ab availability (Fig. 2C). Consistent with previous report using BMDM from DUSP1-KO mice (38), when examining BMDM with the DUSP1 loci targeted in this manner, we observed sustained TLR4-induced p38 phosphorylation, as compared with EV and the DUSP4 controls (Fig. 2D). Increased p38 phosphorylation phenotype at the 80-min timepoint generally correlated with the LGB-DUSP1 gRNA expression level, as indicated by tBFP fluorescence in Cas9’ EGFP’ BMDM (Fig. 2E). Our results suggest that TLR4-induced p38 is differentially regulated between primary BMDM and the transformed RAW macrophages and illustrate the utility of our method for study of these otherwise hard-to-modify cells.

An efficient selection strategy for CRISPR-induced HDR clones in Jurkat T cells

We next turned to development of efficient strategies for editing the genome of lymphocytes. To minimize off-target effects, we designed a pair of gRNAs for targeting with the Cas9-D10A nickase (Cas9n) strategy, choosing the human STAT3 locus as an exemplar (Fig. 3A). This pair of gRNAs together with Cas9n expression in HEK293T cells was confirmed to create indel mutations at the targeted STAT3 loci, as measured by the T7EN assay (Fig. 3B), following cotransfection of the PX461 and the pKLV-BFP plasmids. However, our goal was more ambitious than just achieving gene expression loss in these cells. In addition to expression of Cas9n and a pair of gRNAs lying in close proximity, a DNA donor template is required to induce precise knock-in modification via the HDR repair mechanism. A STAT3 HDR donor plasmid (Fig. 3C) was designed with 2000-bp homology arms flanking the insert fragment containing the PuroR gene, followed by the T2A sequence and the mNeonGreen (mNG) fluorescent protein-coding gene. Following cotransfection of PX461-STAT3-gRNA-A, pKLV-BFP-STAT3-gRNA-I, and STAT3 HDR donor plasmids, ~6 × 10^5 total (~0.5% cotransfection efficiency) Jurkat T cells were selected with puromycin and separated by dilution cloning before further validation (Fig. 3D). Based on the low HDR efficiency for large fragment insertion (~10%), it is technically challenging to isolate this tiny (~<0.05% or ~<300 cells) population of successful HDR cell clones without using any selection method.

After puromycin selection, flow cytometric analysis of 40 puromycin-resistant Jurkat cell clones revealed that most of the selected population had showed mNG expression (Fig. 3E). Further genomic PCR analysis of seven clones that express medium to high level of mNG showed that six out these seven clones were indeed the expected knock-in for all STAT3 alleles (Fig. 3F). Based on these results, we estimated the clonal distribution of the parental mNG-STAT3 Jurkat cell line (Fig. 3G). The high percentage (82.5%) of partial or complete mNG-STAT3 knock-in clones suggests that this type of antibiotic selection strategy efficiently enriches the desired HDR cell clones.

We extended studies of this method by targeting the human BCL10 locus to express a fluorescent protein-tagged fusion protein, using six nickase gRNA pairs (Fig. 4A) and measuring the targeting efficiency by T7EN assay (Fig. 4B) as in Fig. 3B. Despite the higher indel frequency induced by the D3 gRNA combination (Fig. 4A, 4B), we selected the A1 gRNA combination to minimize potential off-target effects, based on a then-available algorithm (crispr.mit.edu). To enhance cotransfection efficiency, we created a double gRNA expression plasmid (Fig. 4C) based on the PX462 backbone. Furthermore, we attempted to minimize the genome editing footprint by incorporating loxP sites (39) flanking the PuroR-T2A selection cassette in the BCL10 HDR donor plasmid (Fig. 4D). Upon CRE-mediated excision of the loxP-PuroR-T2A-loxP fragment, further genome editing with the same strategy can be applied because puromycin sensitivity is restored (Fig. 4E). After plasmid cotransfection and puromycin selection, most Jurkat cells had acquired mNG-BCL10 expression (Fig. 4F). After NIL-CRE (see Fig. 5A for details) treatment, we observed enhanced mNG expression (Fig. 4G) and a substantially higher frequency of mNG-BCL10 aggregate formation upon Ag receptor stimulation, in accord with previous observations (40). Puromycin retreatment killed ~99.8% of the NIL-CRE–treated mNG-BCL10 Jurkat cells but did not affect the survival of the original puromycin-resistant mNG-BCL10 line. Finally, based on this NIL-CRE–treated or Puro-T2A–excised mNG-BCL10 Jurkat parental cell line, we repeated STAT3 genome editing as in Fig. 3 but using a modified STAT3 HDR donor plasmid (Fig. 4H) in which the mScarlet element replaces the mNG element (Fig. 3C). Following plasmid cotransfection and puromycin selection, more than 75% of mNG-BCL10+ Jurkat cells

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gained targeted mScarlet-STAT3 expression (Fig. 4I). With minimal off-target editing concerns, these results demonstrated a highly efficient yet sustainable selection method to enrich targeted knock-in cell population from very few recombinant clones created by CRISPR-mediated HDR.

**FIGURE 2.** LGB-mediated CRISPR in BMDM recapitulates reported role for DUSP1 in control of TLR4-p38 signaling.

(A) Workflow diagram for generating CRISPR-KO BMDM for TLR4 signaling pathway analysis. (B) Lentiviral titration in BMDM for cell toxicity. (C) DUSP3 protein expression following LGB-DUSP3 transduction of Cas9⁺ BMDM. (D) Phospho-p38 induced by TLR4 engagement of DUSP1-KO BMDM as compared with the EV and the DUSP4-KO controls. Representative data (left) and AUC pooled from two independent time-course experiments (right); AUC was normalized to the EV control from the same experiment. (E) Examination of TLR4-induced phospho-p38 signaling together with LGB-DUSP1 gRNA expression as indicated by tBFP fluorescence.

**All-in-one nonintegrating lentiviral delivery of the CRISPR-HDR blueprint**

As an alternative approach to achieve targeted engineering of cells, we sought to deliver Cas9, gRNA, and the HDR donor template to the entire T cell culture in a transient manner. We therefore turned
to the use of integrase-deficient lentiviral particles. We packaged the Lenti-CMV-mCherry-P2A-CRE viral vector either with the wild-type integrase in the psPAX2 plasmid to produce integrating lentiviral particles or with the integrase-deficient mutant in the psPAX2-D64V plasmid to produce NIL particles. Although the integrating lentiviral transduction created stable mCherry expression in Jurkat T cells, the NIL delivery failed to integrate into the genome and led to transient mCherry expression (Fig. 5A).

To test whether NIL delivery of the entire CRISPR-HDR instruction can generate targeted knock-in cells, we assembled the LentiCRISPRv2B-HDR-mCherry-2A-hCD3E viral vector (Fig. 5B) with a gRNA targeting the human CD3E locus (Fig. 5C). Seven days after the NIL-CRISPR-Cas9-HDR delivery, we indeed observed a distinct population of mCherry+ Jurkat cells by flow cytometry (Fig. 5D). Using primers that distinguish the CD3E locus from randomly integrated HDR template sequences, genomic PCR analysis confirmed the presence of targeted knock-in alleles within the mCherry-sorted Jurkat population (Fig. 5E).

Using NIL delivery of the LentiCRISPRv2S-HDR-tBFP-hRelA viral vector (Fig. 5F), we attempted to generate TagBFP-RelA fusion knock-in Jurkat cells, employing a gRNA targeting the human RelA locus (Fig. 5G). This HDR attempt yielded a distinct

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FIGURE 4. CRE-loxP excision strategy minimizes genomic footprint of the PuroR selection cassette and allows for multiple rounds of genome editing.

(A) Cas9 nickase gRNA pair design targeting the human BCL10 locus. (B) T7EN assay for locus-specific editing in HEK293T cells. (C) Double gRNAs and Cas9-D10A nickase expression plasmid derived from the PX462 backbone. (D) BCL10 HDR donor plasmid design. (E) Strategy for creating puromycin-selected Jurkat T cells with postselection excision of the Puro-T2A cassette. (F) mNG expression by puromycin-selected mNG-BCL10 knock-in Jurkat line (G) treated with or without NIL-CRE. (H) Modified STAT3 HDR donor plasmid. (I) mNG and mScarlet coexpression by puromycin-selected mScarlet-STAT3/mNG-BCL10 double knock-in Jurkat line.
FIGURE 5. All-in-one NIL-CRISPR-Cas9-HDR delivery enables targeted genome modifications in human Jurkat T cells. (A) Time course of mCherry-P2A-CRE expression after NIL versus integrating lentiviral transduction of Jurkat T cells. (B) All-in-one lentiviral design for mCherry-P2A-CD3E knock-in via CRISPR-Cas9-HDR. (C) Human CD3E locus showing the gRNA target site used in (B). (D) mCherry expression by Jurkat cells transduced with NIL-LCv2B-HDR-mCherry-2A-hCD3E. Results are representative of two independent experiments. (E) Genomic PCR analysis of the human CD3E locus using primers outside both homology arms. (F) All-in-one lentiviral design for tBFP-RelA knock-in via CRISPR-Cas9-HDR. Start codon and the original second codon are labeled as highlighted in (G). (G) Human RelA locus showing (Continued)
and stable tBFP * Jurkat population that was further purified by FACS (Fig. 5H). We used live cell confocal time-lapse imaging to visualize the RelA nuclear translocation response to PMA-ionomycin stimulation (Supplemental Video 1). As expected, in the steady-state, tBFP-RelA was mostly excluded from the H2B-sfCherry–defined nuclear region. Upon NF-κB pathway activation, a rapid but transient increase in the nuclear to cytoplasmic ratio of tBFP-RelA was observed in ~85% of the Jurkat population (Fig. 5I). To distinguish targeted genomic knock-in from random lentiviral integration, we transduced the original tBFP-RelA knock-in Jurkat line with LentiCRISPRv2B (Fig. 5J) carrying RelA gRNA-A1 (Fig. 5K) that can inactivate up to 75% of the endogenous RelA expression based on our previous data. After 5 d of blasticidin selection, ~70% of the gated population had lost tBFP expression (Fig. 5L), indicating that most of the tBFP * cells were indeed created by the HDR mechanism at the targeted human RelA locus and did not result from random genomic integration of the tBFP-coding sequence. Taken together, these results provide proof-of-concept data to support the all-in-one NIL-Cas9-gRNA–donor-template delivery approach as a powerful alternative to the existing CRISPR-HDR methods in transformed human T cells.

Selective gene expression induction with all-in-one lentiviral CRISPRa system

The vectors and methods described above are useful for either eliminating expression of a particular gene or modifying an already-expressed gene in a given cell type, but there are numerous situations in which it would be useful to induce expression of an otherwise silent locus in a specific cell type. To achieve this end, we created an all-in-one lentiviral CRISPRa system that we call LentisAMPHv2 (Fig. 6A), which is based on an existing two-plasmid system (29). LentisAMPHv2 also contains blasticidin resistance marker for efficient selection of transduced cells. With this system, we tested six gRNAs from an existing CRISPRa library (29) targeting the promoter region of the human PDCD1 or PDL1 genomic locus. These gRNAs induced various levels of target gene expression (Fig. 6B), as compared with the corresponding no gRNA control condition. For the gRNA that induces the highest level of PDCD1 expression, we also tested the specificity of this induction by measuring the cell surface expression level of PDL1, which was not increased under these conditions (Fig. 6C).

DISCUSSION

Immunologists have had more difficulty than investigators in other areas of biology using CRISPR in attempts to modify cells of interest, such as macrophages and lymphocytes, because of several unique features of these hematopoietic elements. To our knowledge, in this study, we have developed new or modified versions of existing reagents or methods to improve such cell perturbation, providing new tools that enable more-efficient KO of genes in macrophages, HDR targeting in lymphocytes, or gene activation in these cells.

We have encountered substantial challenges in our initial attempts to use commonly available CRISPR-KO methods to engineer the widely-studied RAW macrophage cell line. Using the LentiCRISPR system, we consistently observed high amounts of cell death following lentiviral transduction and puromycin selection, likely because of a combinatorial effect of low viral titer and cytotoxic materials from the crude lentiviral supernatant. In contrast, the high transduction efficiency of our LGB gRNA expression system ensures that a majority of the transduced RAW cells survive under blasticidin selection, providing targeted cells that are a good representation of the original cell line. Inducible Cas9 expression allows more-precise control of the genome editing time window and therefore minimizes long-term off-target effects that cannot be avoided by constitutive Cas9 expression in many lentiviral-based CRISPR expression systems. For gRNAs that efficiently KO essential cell survival genes or oncogenes, percentage changes in EGFP * tBFP * (Cas9* gRNA*) cells over doxycycline treatment time help quantify the impacts of these gRNAs. The high genome editing efficiency and the rapid single-cell phenotyping option of our dual-color inducible CRISPR system in RAW cells makes this platform suitable for high-throughput genome-wide gRNA library screening. Inducible CRISPR system also allows for stable uninduced cell lines to be frozen for long-term storage and future repeat analysis.

Lentiviral or retroviral supernatant produced from HEK293T packaging cells often influences target cell growth rate and morphology, especially when a high-volume ratio of viral supernatant to cell culture medium is applied to the target cell line. Our data illustrate that further purification of lentiviral particles helps minimize BMDM cell death. Thus, our methods achieve high transduction efficiency without sacrificing cell health in culture. With these approaches, we were able to identify Cas9 and gRNA double-positive macrophages from both the RAW murine cell line and primary BMDM, allowing us to focus on those CRISPR-targeted populations for downstream analysis. Interestingly, these studies revealed distinct p38-MAPK regulation in immortalized versus primary macrophages. CRISPR deletion of the phosphatase DUSP1 in BMDM resulted in sustained p38 phosphorylation, consistent with a previous report using BMDM derived from DUSP1-deficient mice (38). In contrast, DUSP1 deletion in RAW cells, using the same gRNA, did not lead to substantial changes in the target site of gRNA used in (F). (H) tBFP expression of Jurkat cells untreated or treated with LCv2S-HDR-tBFP-hRelA, before or after tBFP * cell sorting. Results are representative of two independent experiments. (I) Live cell confocal time-lapse imaging of tBFP-RelA knock-in Jurkat cells stimulated with PMA-ionomycin. Pre-existing H2B-sfCherry expression created by lentiviral transduction was used as nuclear marker. (J) Modified LentiCRISPRv2 plasmid LentiCRISPRv2B replacing PuroR with blasticidin resistance gene BSD. (K) Human RelA locus showing the target site of gRNA A1 near exon 3. (L) tBFP expression by the Jurkat tBFP-RelA knock-in line either untreated or transduced with LCv2B carrying RelA gRNA-A1 to KO endogenous RelA expression.

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p38 activation dynamics. This adds to our previous report of dysregulated TLR4-induced MAPK activity in RAW cells compared with BMDM (41).

The Cas9 nickase strategy requires two gRNA targets to be in close proximity to efficiently induce DNA double-strand break repair mechanisms (8, 9). Therefore, it is extremely unlikely to find

FIGURE 6. Lentiviral-mediated CRISPRa with the LentiSAMPHv2 System.

(A) The all-in-one LentiSAMPHv2 (LSv2) plasmid for CRISPR-based gene activation applications. (B) Flow cytometric analysis of human PDCD1 (top panel) or PDL1 (bottom panel) cell surface protein expression after LSv2 transduction with EV control or the indicated CRISPRa targeting gRNA. (C) Flow cytometric analysis of human PDCD1 (left panel) or PDL1 (right panel) cell surface protein expression in untransduced control cells versus cells transduced with LSv2 targeting the human PDCD1 promoter region.

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another off-target pairing site in the genome. Our data support previous reports that the pair of Cas9 nickases is optimized with minimized offset of the gRNA pairs (8, 9). By omitting an upstream promoter in the donor template design, our PuRoR selection strategy reduces the probability of selecting randomly integrated clones. As a potential drawback, this strategy relies on targeting genomic loci that are well expressed to generate sufficient expression of the drug resistance gene. Among the target genes that we have tested, we observed that this strategy worked best with the N-terminal PuRoR-2A but not the C-terminal 2A-PuRoR fusion expression. When combined with a CRE-lox or FLP-FRT recombination design, our PuRoR selection strategy can be further optimized to sustain many rounds of genome editing, enabling sequential engineering of multiplex reporters, significantly reducing the genomic footprint to a short single recombination site.

Following the NIL-mediated mCherry-P2A-CRE or mScarlet-P2A-CRE (Supplemental Fig. 3D) delivery, target cells that transiently express CRE recombine can be isolated from the untreated population via FACS. For relatively small proteins such as BCL10, the protein expression level is dramatically reduced by the genomic insertion of a PuRoR selection cassette together with a fluorescent protein. Our data suggest that reduced BCL10 protein expression can lead to the loss of BCL10 oligomerization upon Ag receptor signaling, which justifies the need to remove PuRoR selection cassettes from the targeted genomic loci to achieve more functional BCL10 reporter Jurkat T cells.

Lentiviruses such as HIV have evolved to efficiently infect human T cells, making them perfect delivery vehicles for human T cell genome engineering. In Jurkat cells, lentiviral delivery is much more efficient than any existing transfection method including electroporation, which often leads to substantial cell death upon electric shock and reduces cell growth and overall protein production for the surviving population. In addition, lentiviral particles are easy to prepare by most laboratories, carry double to triple the payload as compared with the AAV system, and therefore enable all-in-one design and delivery of Cas9, gRNA expression, and HDR donor template together. Although there is an existing report (42) that demonstrates the feasibility of NIL delivery of the HDR donor template, in this study, we demonstrate the utility of this method using an all-in-one Cas9-gRNA–donor HDR blueprint in an especially difficult-to-engineer type of cell, T lymphocytes. Our experiments using human primary T cells showed that the NIL-LCv2R (Supplemental Fig. 3B) particles efficiently delivered a payload of over 10,000 bp to more than 80% of the total population (Supplemental Fig. 3E).

As one of the first studies, to our knowledge, to report the use of NIL as delivery vehicle for CRISPR-HDR applications, we suggest many optimization possibilities for this method. First of all, sensitive marker expression, such as mScarlet in the LCv2R backbone (Supplemental Fig. 3B) or TEGFR in the LCv2E backbone (Supplemental Fig. 3C), could be included to eliminate residual random integration events from NIL particles. Viral titers could be enhanced by incorporating two copies of the Sp1 binding site upstream of the U6 promoter (32). In addition, various NHEJ-inhibiting chemicals such as SCR7 (43), HDR-stimulating chemicals such as RS-1 (25), and cell cycle inhibitors for G2/M phase arrest (44) could be tested to enhance knock-in efficiency. Furthermore, eSpCas9 (14) or more-specific Cas9 variant could be used instead of Cas9 nuclease to minimize off-target effects. Finally, the use of both NIL and AAV systems could be combined to deliver few copies of NIL-Cas9-gRNA particles but high copy number of AAV-HDR donor template (26) per target cell to maximize HDR efficiency and donor template accuracy.

Although inactivation or mutation of a target gene is the predominant focus of CRISPR editing to date, the selective induction of expression of a particular molecule using this method has many applications. To this end we also introduce an all-in-one vector system for activation of selected gene loci in immune cells. Using a single lentiviral vector containing gRNA that targets either the human PDCD1 or PDL1 genomic locus, we demonstrate high-level expression of a previously silent locus in Jurkat cells and the value of this induced expression in assays of immune function. This strategy builds on pre-existing methods for large-scale gene induction (45) by providing experimentalists with a simple method for selective, one-vector-at-a-time alteration of cell phenotype in a positive rather than negative manner.

In summary, to our knowledge, this report provides a set of tools and methods for immunologists seeking to engineer hematopoietic cell types such as macrophages and lymphocytes that are more difficult to modify using CRISPR methods than the epithelial or mesenchymal cells studied in other fields. Our improvements to available vectors and methods enhance the ease and efficiency of achieving targeted gene inactivation, selective gene editing, and precise gene activation and should prove valuable in a wide range of investigations.

DISCLOSURES

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