Cell type-programmable genome editing with enveloped delivery vehicles

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Abstract

Therapeutic application of CRISPR-Cas9 is limited by the available approaches for delivering genome editing tools to specific cell types. Enveloped delivery vehicles (EDVs), such as virus-like particles, have the potential to enable targeted genome editing by deploying the cell-type specific membrane fusion activity of viral glycoproteins for transient delivery of CRISPR-Cas9 protein and guide RNA. We previously demonstrated that displaying the HIV-1 glycoprotein on enveloped Cas9-containing particles directs genome editing exclusively to CD4+ T cells within a mixed-cell population. Inspired by chimeric antigen receptors and their ability to link programmable targeting to T cell function, we show here that displaying antibody fragments on Cas9-enveloped particles can target genome editing tools to specific cells. These Cas9-packaging enveloped delivery vehicles (Cas9-EDVs) confer genome editing in target cells over bystander cells, even when target cells are rare (~2%) in the population. By swapping the displayed antibody, different cell types within a mixture can be edited selectively. Together, antibody-targeted EDVs have the potential to enable cell-selective genome editing for therapeutic applications both *ex vivo* and *in vivo*.

Introduction

CRISPR-Cas9 genome editing has led to transformative strategies for treating genetic diseases (*1–3*). To be successful, these therapies require the safe and effective delivery of genome editing tools into the nucleus of target cells (*4–6*). Currently, this has been successful (1) when target cells can be isolated from the body and modified *ex vivo (7, 8)*; (2) when genome editing tools are administered directly at the location of target cells inside the body (*9*); or (3) when targeting the liver *in vivo (10)*. Expansion of *in vivo* CRISPR-Cas9 applications will require delivery vehicles that safely and effectively target genome editors to specific cells or organs inside the body following systemic administration.

Retargeting the tropism of viruses or viral vectors has long been studied for oncolytic virus and gene therapy applications (*11–14*). Many of these approaches co-display a cell-selective targeting molecule alongside a viral glycoprotein that mediates cell entry, either by fusion at the plasma membrane or in the low-pH environment of the endosome. The vesicular stomatitis virus glycoprotein (VSVG) is a popular molecule for pseudotyping gene therapy vectors because it binds the ubiquitously-expressed LDL-R receptor and mediates effective target cell entry (*15*, *16*). Structural analysis of VSVG led to the identification of residues responsible for native receptor binding and the generation of a mutant form of VSVG that maintains endosomal fusion activity but lacks native receptor binding activity (*17*). Pairing the K47A R354A VSVG ("VSVGmut") with targeting molecules has recently been demonstrated as a robust strategy for retargeting lentiviral transgene delivery and has enabled the high-throughput screening of T and B cell receptor libraries to study receptor-antigen interactions (*18*, *19*).

Retroviral virus-like particles (VLPs) have been used for the transient delivery of Cas9 RNP complexes (*20–27*) or mRNA-encoded genome editors and single guide RNA (*28–31*).

Previously, we demonstrated that the viral HIV-1 envelope glycoprotein could be leveraged for cell-type-specific delivery of Cas9 RNPs to CD4+ T cells within a mixed cell population *ex vivo* (*21*). This suggests that the programmable display of targeting molecules on the outside of encapsulated vesicles could couple cell-specific targeting to the delivery of RNA, protein, or ribonucleoprotein cargos.

Here, we aimed to expand the cell-type-specific targeting of Cas9 RNPs beyond the tropism of naturally-occurring viral glycoproteins. By pairing the display of single-chain antibody fragments (scFvs) with VSVGmut, we demonstrate the selective delivery of genome editors based on antibody-antigen interactions. We show here that multiple different antibody-ligand pairs achieve targeted delivery of genome editors, with minimal genome editing of bystander cells *in vitro*. These enveloped delivery vehicles (EDVs) represent a new programmable platform for the delivery of genome editing cargo.

Results

To test whether antibody fragments could direct the delivery of Cas9 by EDVs (Cas9-EDVs), we first cloned a CD19 antibody as a single-chain variable fragment (scFv) fused to the stalk and transmembrane domain of CD8α (**Figure 1A, Supplementary Figure 1**). Cas9-EDVs bud from the plasma membrane of transfected producer cells; we, therefore, anticipated that we could decorate the outside of Cas9-EDVs with both CD19 scFv targeting molecules and VSVGmut (**Figure 1B**) by co-expressing both during Cas9-EDV production. To simplify the study of cell-targeted genome editing by Cas9-EDVs, we generated a 293T cell line coexpressing the B-cell ligand CD19 and EGFP (**Supplementary Figure 2**), enabling the assessment of genome editing in on-target, EGFP+ cells, and off-target, EGFP- bystander cells (**Figure 1C**).

We produced *B2M*-targeted Cas9-EDVs surface-labeled with either VSVG, CD19 scFV/VSVGmut, or a control scFv/VSVGmut. We treated a ~3:1 mixture of 293T and CD19 EGFP 293T cells and used flow cytometry to assess genome editing by B2M expression. While the VSVG Cas9-EDVs mediated genome editing in both CD19+ and CD19- populations, treatment with CD19-scFv Cas9-EDVs resulted in the knockout of B2M specifically in CD19+ cells (**Figure 1D**). On-target genome editing activity was specific to the CD19-scFv, as a mismatched control scFv did not result in detectable B2M knockout in either the CD19+ or CD19- cells. Antibody-targeted Cas9-EDV activity was titratable, with up to 70% of target cells exhibiting B2M knockout with no editing detected in bystander, non-target cells (**Figure 1E**). As it was possible that genome editing was occurring at levels below the sensitivity of flow cytometry analysis, we isolated Cas9-EDV-treated CD19+ and CD19- cells by fluorescence-activated cell sorting (FACS) for next-generation amplicon sequencing analysis. For cells treated with the CD19-scFv Cas9-EDVss, we observed ~74% of modified reads in on-target EGFP+ cells compared to ~3% in bystander cells (**Figure 1F**). We speculate that bystander genome editing may be attributable to non-specific cellular uptake of EDVs, where VSVGmut may mediate endosomal disruption in the absence of targeted cell uptake.

Systemic administration of genome editing tools may require selective delivery to rare cell types inside the body. Therefore, we assessed the ability of antibody-targeted Cas9-EDVs to mediate cell-type-specific genome editing when target cells were either abundant or rare within a mixed cell population. CD19+ target cells were diluted with unmodified cells to generate cell mixtures with target cell frequencies ranging from ~2 to 92%. Antibody-targeted Cas9-EDV treatment resulted in genome-edited target cells across all mixtures tested, even when target cells were as rare as 2% of the population (**Figure 1G**).

We next investigated the modularity and programmability of antibody-targeted Cas9-EDVs to direct genome editing to cells expressing ligands besides CD19. We constructed a panel of 293T cells displaying various plasma membrane proteins expressed by human immune cells: CD20, CD4, and CD28 (**Figure 2A**). Ligand-expressing 293T cells were generated through lentiviral transduction, generating cell mixtures where <25% of cells expressed the cognate ligand (**Supplementary Figure 2**). In parallel, we constructed scFv versions of targeting molecules for CD20, CD4, CD28, and an additional scFv targeting molecule for CD19. Cas9-EDVs displaying targeting molecules were produced and tested on cell mixtures expressing cognate ligands. Genome editing was titratable and selective for ligand+ over bystander cells (**Figure 2B**). In all engineered cell mixtures, ligand+ and ligand- cells were similarly susceptible to VSVG-displaying Cas9-EDV genome editing activity. Lastly, we tested whether antibody-targeted Cas9-EDVs' cell selectivity depended on antibody-antigen interactions. We produced a panel of CD19, CD20, and CD4 antibody-targeted EDVs and tested each on cell mixtures expressing either CD19, CD20, or CD4 ligands. Antibody-targeted Cas9-EDVs only mediated genome editing in cells expressing their matched ligand and not in mismatched ligand-expressing cells (**Figure 2C**).

Discussion

Chimeric Antigen Receptors (CARs) ushered in a new age of cellular immunotherapies due to their ability to link antibody-directed programmable targeting to T cell function (*32*, *33*). Inspired by this, we show here that antibody fragments enable Cas9-EDV-mediated delivery of genome editing tools to specific target cells. Tropism can be programmed by co-displaying an antibody fragment alongside VSVGmut (a form of VSVG defective for native receptor binding activity but

capable of endosomal fusion) on the Cas9-EDV envelope. Antibody-targeted Cas9-EDVs selectively mediate genome editing in target cells over bystander, non-target cells.

We observed that not all scFv-based targeting molecules resulted in equivalent levels of genome editing in target cells. For example, CD20 scFv-2 outperformed CD20 scFv-1 for genome editing of CD20-expressing cells. This difference may be attributable to the affinity of the scFv for its target, with the lower-performing scFv binding less efficiently. Alternatively, it could be that some scFvs enable antigen binding but do not trigger effective endocytosis, a requirement for VSVGmut-mediated cell entry. Lastly, differences in scFv-delivery may result from suboptimal targeting molecule display on Cas9-EDVs.

Demonstrating cell-targeted genome editing of primary cells, both *ex vivo* and *in vivo*, will be critical for further evaluating the usefulness of antibody-targeted Cas9-EDVs for therapeutic applications. As it has been shown that enveloped virus-like particles can deliver genome editing tools *in vivo* and that CARs function robustly to target T cell activity to specific cells, we anticipate this approach will be broadly useful for programmable, cell-selective delivery inside the body.

Methods

Plasmid construction

VSVGmut (K47A R354A VSVG) sequence was human codon-optimized and synthesized as a gBlock (Integrated DNA Technologies) and cloned into the pCAGGS expression plasmid. To generate the CD19 scFv-1 expression plasmid, the sequence encoding the CD8α signal peptide, myc epitope tag, scFv, and CD8α stalk and transmembrane domain of α-CD19-4-1BBζ-P2A-mCherry (*21*, *34*, *35*) was subcloned into pCAGGS. This plasmid was subsequently used as an entry plasmid for cloning all other scFv antibody fragments: the CD8α signal peptide, myc tag, and scFv sequences were dropped out by EcoRI/Esp3I restriction digest (New England Biolabs) and new DNA sequences encoding CD8α signal peptide and scFv were inserted. This cloning strategy resulted in the removal of the n-terminal myc epitope tag and the addition of a serine amino acid residue between the scFv and CD8α hinge domains. A flexible linker (GGGGSGGGGSGGGGSS) was used to link VH and VL domains of source monoclonal antibody sequences. If the antibody source sequence was already a scFv, the linker from the source sequence was used. Except for CD19 scFv-1, all antibody fragment sequences were human codon optimized and synthesized as eBlock Gene Fragments (Integrated DNA Technologies). InFusion cloning (Takara Bio) was used to generate all plasmids. Additional information on the scFv targeting molecules and source sequences can be found in **Supplementary Table 1**.

A second- generation lentiviral transfer plasmid encoding expression of EF1α promoter - CAR-P2A-mCherry (*21*) was digested with XbaI & MluI (New England Biolabs) to drop out the CAR-P2A-mCherry transgene. Human CD19 (Uniprot #Q71UW0) DNA was ordered as a gBlock (Integrated DNA Technologies) and IRES-EGFP (amplified from the Xlone TRE3G MCS-TEV-Halo-3XF IRES EGFP-Nuc-Puro plasmid, a gift from the Darzacq/Tijan Lab) sequences were

inserted using InFusion cloning (Takara Bio). This cloning strategy inserted a MluI restriction digest site 3' of the CD19 stop codon and removed the MluI restriction digest site 3' of the EGFP stop codon. Human CD4 (Uniprot #P01730), CD20 (Uniprot #P11836), and CD28 (Uniprot #P10747) amino acid sequences were human codon optimized for synthesis and ordered as an eBlock (CD28) or gBlocks (CD20, CD4) (Integrated DNA Technologies). Ligand-encoding sequences were subsequently cloned by restriction digest removal of CD19-encoding sequence from the EF1α-CD19 IRES-EGFP lentiviral plasmid using XbaI & MluI (New England Biolabs) and inserted with InFusion cloning (Takara Bio). VSVGmut and scFv targeting plasmids were prepared using the HiSpeed Plasmid Maxi or Plasmid Plus Midi kits (QIAGEN). Lentiviral plasmids were prepared with the QIAprep Spin Miniprep Kit (QIAGEN). All plasmids were sequence-confirmed (UC Berkeley DNA Sequencing Facility, Quintara Bio, or Primordium Labs) prior to use.

Tissue culture and cell line generation

Lenti-X and 293T cells (UC Berkeley Cell Culture Facility) were cultured in DMEM (Corning) supplemented with 10% fetal bovine serum (VWR) and 100 U/ml penicillin-streptomycin (Gibco). To generate lentiviruses encoding EF1α-ligand IRES-EGFP, 3.5-4 million Lenti-X cells were plated in a 10 cm tissue culture dish (Corning) and transfected with 1μg pCMV-VSV-G (Addgene plasmid #8454), 10μg psPax2 (Addgene plasmid #12260), and 10μg of EF1α-ligand IRES-EGFP lentiviral transfer plasmid using polyethylenimine (Polysciences Inc.) at a 3:1 PEI:plasmid ratio. Lentiviral-containing supernatants were harvested two days post-transfection and passed through a 0.45μm PES syringe filter (VWR). Ligand-expressing 293T cells were generated by transducing 293T cells (100,000 per well in a 12-well dish) with lentivirus (0.15-1ml) in a total well volume of 1ml. Four days post-transduction, flow cytometry was used to identify cell mixtures where <25% of cells were expressing EGFP. Following expansion, CD19

EGFP 293T cells were additionally sorted for EGFP expression using an SH800S cell sorter (Sony Biotechnology) to generate a population of cells ~100% CD19+/EGFP+ cells.

Cas9-EDV production

VSVG Cas9-EDVs (formerly known as “Cas9-VLPs”) were produced as previously described (*21*) packaging *B2M*-targeted Cas9 RNPs with the guide RNA spacer sequence 5’-GAGTAGCGCGAGCACAGCTA . Briefly, 3.5-4 million Lenti-X cells (Takara Bio) were seeded into 10 cm tissue culture dishes (Corning) and transfected the next day with 1µg pCMV-VSV-G (Addgene plasmid #8454), 6.7µg Gag-Cas9 (Addgene plasmid #171060), 3.3µg psPax2 (Addgene plasmid #12260), and 10µg U6-B2M (Addgene plasmid #171635) using polyethylenimine (Polysciences Inc.) at a 3:1 PEI:plasmid ratio. Two days post-transfection, Cas9-EDV-containing supernatants were harvested and passed through a 0.45µm PES syringe filter (VWR) and concentrated with Lenti-X Concentrator (Takara Bio) according to the manufacturer’s instructions. Concentrated Cas9-EDVs were resuspended in Opti-MEM (Gibco) at a final concentration of 10x unless otherwise noted in the figure legend. Cas9-EDVs were either stored at 4°C or frozen at -80°C within an isopropanol-filled freezing container until use.

Antibody-targeted Cas9-EDVss were produced in the same way as VSVG Cas9-EDVs, except that the pCMV-VSV-G plasmid was omitted and 7.5µg of the scFv targeting plasmid and 2.5µg of VSVGmut were included during transfection.

Flow cytometry

Cells were stained with anti-human B2M-APC (316312, Biolegend) or human B2M-PE (316306, Biolegend) in PBS containing 1% bovine serum albumin and an Attune NxT flow cytometer with 96-well autosampler (Thermo Fisher Scientific) was used for flow cytometry analysis. Ligand expression was confirmed for engineered 293T cells using anti-human CD28-PE (302907,

Biolegend), anti-human CD20-PE (302306, Biolegend), anti-human CD4-PE/Cyanine7 (300512, Biolegend) and anti-human CD19-PE (302208, Biolegend). Data analysis was performed using FlowJo v10 10.7.1 (FlowJo, LLC, Ashland OR).

Amplicon sequencing

Next-generation sequencing was used for detection of on-target genome editing in EGFP+ and EGFP- sorted 293T cells. Genomic DNA was extracted using QuickExtract (Lucigen) as previously described (*21*). PrimeStar GXL DNA polymerase (Takara Bio) was used to amplfy the *B2M* Cas9-RNP target site using primers 5'- GCTCTTCCGATCTaagctgacagcattcgggc and 5'- GCTCTTCCGATCTgaagtcacggagcgagagag. The resulting PCR products were cleaned up using magnetic SPRI beds (UC Berkeley DNA Sequencing Facility). Library preparation and sequencing was performed by the Innovative Genomics Institute Next-Genration Sequencing Core using MiSeq V2 Micro 2x150bp kit (Illumina). Reads were trimmed and merged (Geneious Prime, version 2022.0.1) and analyzed with CRISPResso2 (http://crispresso.pinellolab.partners.org/login).

Statistical analysis

Statistical analysis was performed using Prism v9. Statistical details for all experiments, including value and definition of N, and error bars can be found in the Figure Legends.

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The Regents of the University of California have patents issued and/or pending for CRISPR technologies (on which J.A.D. is an inventor) and delivery technologies (on which J.A.D. and J.R.H are co-inventors). J.A.D. is a cofounder of Caribou Biosciences, Editas Medicine, Scribe Therapeutics, Intellia Therapeutics, and Mammoth Biosciences. J.A.D. is a scientific advisory board member of Vertex, Caribou Biosciences, Intellia Therapeutics, Scribe Therapeutics, Mammoth Biosciences, Algen Biotechnologies, Felix Biosciences, The Column Group, and Inari. J.A.D. is Chief Science Advisor to Sixth Street, a Director at Johnson & Johnson, Altos and Tempus, and has research projects sponsored by AppleTree Partners and Roche. All of the other authors have no competing interests.

Supplementary materials

Supplementary Figure 1 - Single-chain variable fragment (scFV) targeting molecule schematic

Supplementary Figure 2 - Validation of cell-surface ligand expression in engineered 293T cells

Supplementary Table 1 - Sequence information for scFv targeting molecules

Figure legends

Figure 1. Cell-specific genome editing with antibody-targeted Cas9-EDVs.

Figure 2. Antibody-targeted Cas9-EDVs are a programmable, modular strategy for mediating genome editing of specific cells

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Figure 1

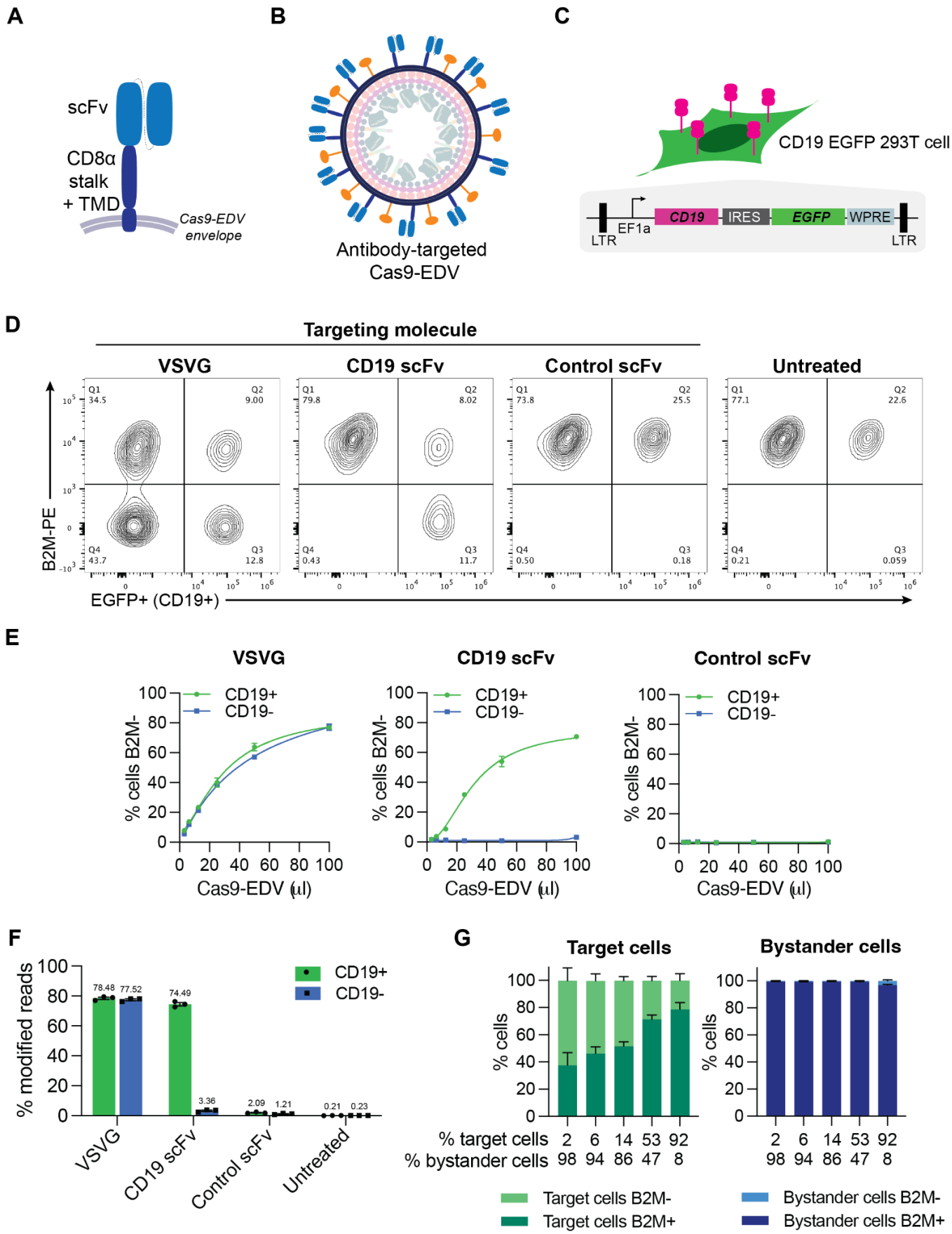
A
scFv
CD8α
stalk
+ TMD
Cas9-EDV
envelope
B
Antibody-targeted
Cas9-EDV
C
CD19 EGFP 293T cell
LTR
EF1a
CD19
IRES
EGFP
WPRE
LTR
D
Targeting molecule
VSVG
CD19 scFv
Control scFv
Untreated
B2M-PE
EGFP+ (CD19+)
Q1 34.5
Q2 9.00
Q4 43.7
Q3 12.8
Q1 79.8
Q2 8.02
Q4 0.43
Q3 11.7
Q1 73.8
Q2 25.5
Q4 0.50
Q3 0.18
Q1 77.1
Q2 22.6
Q4 0.21
Q3 0.059
E
VSVG
CD19 scFv
Control scFv
CD19+
CD19-
% cells B2M-
Cas9-EDV (µl)
F
% modified reads
CD19+
CD19-
78.48
77.52
74.49
3.36
2.09
1.21
0.21
0.23
VSVG
CD19 scFv
Control scFv
Untreated
G
Target cells
Bystander cells
% cells
% target cells 2 6 14 53 92
% bystander cells 98 94 86 47 8
Target cells B2M-
Target cells B2M+
Bystander cells B2M-
Bystander cells B2M+

Figure 1 - Cell-specific genome editing with antibody-targeted Cas9-EDVs

(**A**) Schematic of an antibody-derived single-chain variable fragment (scFv) targeting molecule for Cas9-EDV display. The scFv is fused to the stalk and transmembrane domain (TMD) of human CD8α. IRES = internal ribosome entry site. WPRE = Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element. (**B**) Schematic of a Cas9-EDV decorated with a scFv targeting molecule (blue) and VSVGmut (orange). (**C**) Schematic of the lentiviral vector used for engineering 293T cells that express human CD19 and EGFP. (**D-F**) Assessment of antibody-targeted Cas9-EDV activity. 293T and CD19 EGFP 293T cells were mixed at an approximate ratio of 3:1 and treated with *B2M*-targeting Cas9-EDVs displaying various targeting molecule pseudotypes. Analysis was performed at 7 days post-treatment to assess B2M knockout in EGFP+ (on-target) and EGFP- (bystander) cells by flow cytometry (D, E) and amplicon sequencing (F). Cas9-EDVs were concentrated 10x and cells were treated with 50µl Cas9-EDVs (D, F) or in a dilution curve (E). (**G**) Antibody-targeted Cas9-EDVs mediate targeted genome editing regardless of target cell frequency. CD19 EGFP 293T cells were mixed with 293T cells to achieve target cell frequencies of ~2-92%. Heterogeneous cell mixtures were challenged with antibody-targeted Cas9-EDVs (100µl, 2.5x concentration) and B2M knockout was assessed in EGFP+ (on-target) and EGFP- (bystander) cells by flow cytometry. N = 3 technical replicates for all panels except for the 100µl dose of CD19-scFv in 1E (N = 2). Error bars represent the standard error of the mean.

Figure 2

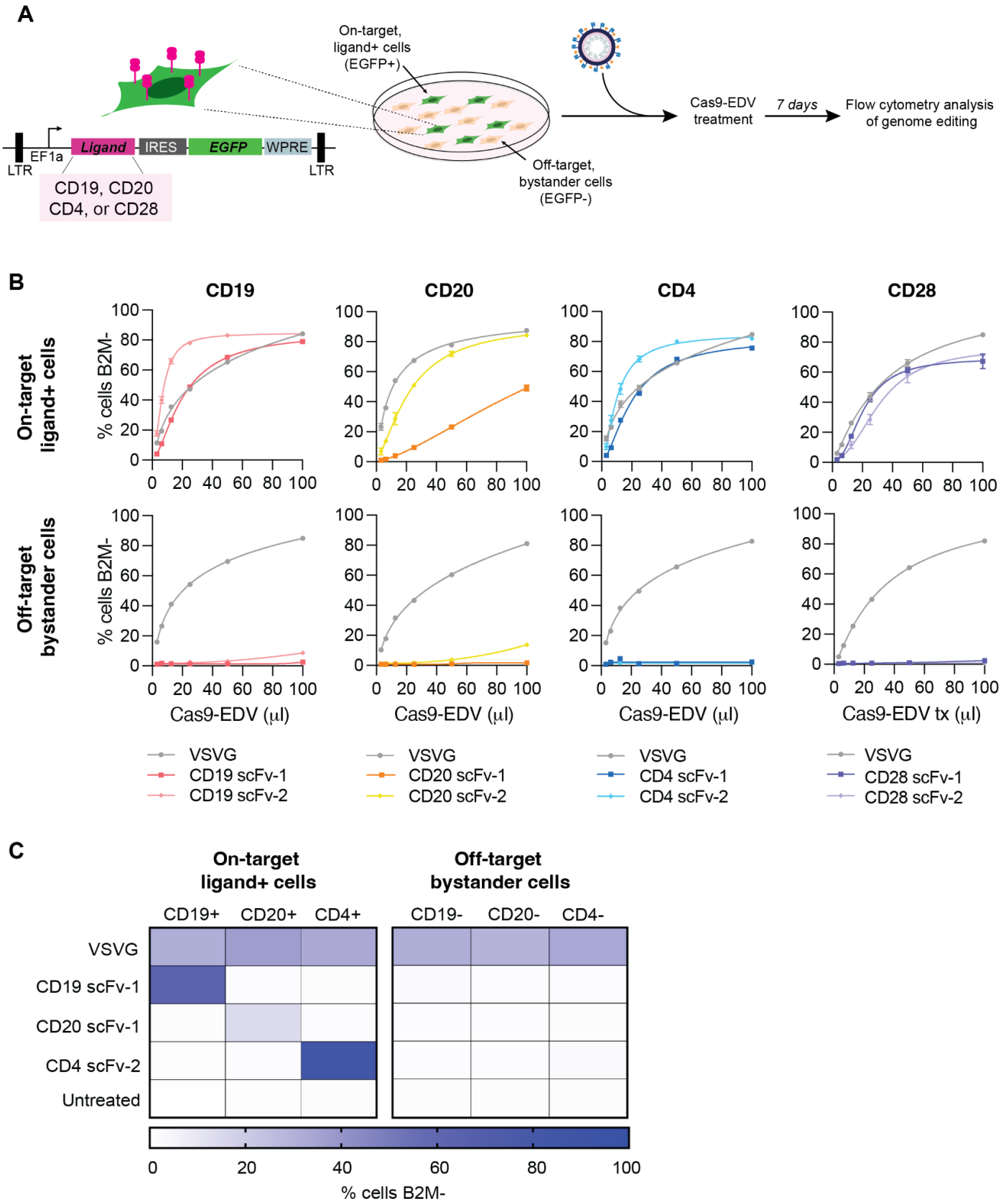
A
On-target, ligand+ cells (EGFP+)
Off-target, bystander cells (EGFP-)
Cas9-EDV treatment
7 days
Flow cytometry analysis of genome editing
LTR
EF1a
Ligand
IRES
EGFP
WPRE
LTR
CD19, CD20 CD4, or CD28
B
CD19
CD20
CD4
CD28
On-target ligand+ cells
Off-target bystander cells
% cells B2M-
Cas9-EDV (µl)
Cas9-EDV tx (µl)
VSVG
CD19 scFv-1
CD19 scFv-2
CD20 scFv-1
CD20 scFv-2
CD4 scFv-1
CD4 scFv-2
CD28 scFv-1
CD28 scFv-2
C
On-target ligand+ cells
Off-target bystander cells
CD19+
CD20+
CD4+
CD19-
CD20-
CD4-
VSVG
CD19 scFv-1
CD20 scFv-1
CD4 scFv-2
Untreated
% cells B2M-

Figure 2 - Antibody-targeted Cas9-EDVs are a programmable, modular strategy for mediating genome editing of specific cells

(**A**) Experimental outline and schematic of the lentiviral vector used for engineering 293T EGFP cells that express various ligands on the plasma membrane. To promote cellular engineering via single lentiviral integration events, engineered cell mixtures were generated via low MOI transduction to achieve <25% EGFP+ cells. Engineered cell mixtures were challenged with *B2M*-targeting Cas9-EDVs displaying various targeting molecule pseudotypes. (**B**) Various scFv targeting molecules were developed to target ligands expressed on human immune cells: CD19, CD20, CD4, and CD28. Cell-specific antibody-retargeted Cas9-EDVs activity was assessed for on-target, ligand+ cells (EGFP+) and off-target, bystander cells (EGFP-) 7 days post-treatment. (**C**) Antibody-targeted Cas9-EDV-mediated genome editing is highly specific for cells expressing the scFv cognate ligand. scFv-Cas9-EDVs were tested on engineered cells expressing the cognate and noncognate ligands and genome editing activity was measured via flow cytometry 3 days post-treatment. N = 3 technical replicates. All error bars represent standard error of the mean.