

[0014] In some aspects, the first CDL target sequence, the second CDL target sequence, and the genomic target sequence comprise the same nucleic acid sequence.

[0015] In some aspects, the genomic target sequence comprises a safe-harbor nucleic acid sequence. In some aspects, the safe-harbor nucleic acid sequence comprises the nucleic acid sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2). In some aspects, one, both, or neither of the PAM sequences are encoded between the CDL target sequences and the HDR template.

[0016] In some aspects, the targetable nuclease and each of the gRNAs are in a molar ratio of between 1:10 and 2:1, respectively. In some aspects, the targetable nuclease and the donor template are in a molar ratio of between 10:1 and 1000:1, respectively.

[0017] In some aspects, the targetable nuclease protein and/or the second targetable nuclease protein comprises a transcription activator-like (TAL) effector DNA-binding protein and a nuclease.

[0018] In some aspects, the targetable nuclease protein and/or the second targetable nuclease protein comprises a zinc finger DNA-binding protein and a nuclease.

[0019] In some aspects, the targetable nuclease protein and/or the second targetable nuclease protein is fused to a nuclear localization signal (NLS) sequence.

[0020] In some aspects, the targetable nuclease protein and/or the second targetable nuclease protein is a Cas9 protein.

[0021] Also provided herein is a method for modifying the target nucleic acid in the cell, comprising introducing or having non-virally introduced into the cell any of the compositions described herein, wherein the HDR template is integrated into the target nucleic acid. In some aspects, the introducing comprises electroporation. In some aspects, the cell is a primary cell. In some aspects, the primary cell is a primary T cell.

[0022] Also provided for herein is a ribonucleoprotein complex for modifying a target nucleic acid, comprising any of the compositions described herein.

[0023] Also provided for herein is a ribonucleoprotein complex for modifying a target nucleic acid, (a) a CRISPR-CAS RNA-guided nuclease; and (b) a donor guide RNA (gRNA), wherein the donor gRNA comprises at least 17 nucleotides that are complementary to a co-delivery linearization (CDL) target sequence, and wherein the composition is formulated for non-viral delivery into a cell. In some aspects, the composition further comprises a plasmid

the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences wherein the donor gRNA comprises at least 17 nucleotides that are complementary to the genomic target sequence of the cell, and wherein the homology arms are complementary to nucleic acid sequences flanking the genomic target sequence of the cell, wherein the nucleic acid for insertion is configured for insertion into the genomic target sequence of the cell. In certain embodiments, the cell is a human cell. In certain embodiments, the cell is an immune cell. In certain embodiments, the immune cell is a T cell. In certain embodiments, the T cell is a primary T cell. In certain embodiments, the introducing the composition formulated for non-viral delivery comprises electroporation. In certain embodiments, the amount of donor template is at least about 80, 10-120, 10, 20, 30, 40, 50, 60, 70, 90, 100, 110, or 120 μ g. In certain embodiments, the number of cells for an individual electroporation reaction is at least about 5, 1-10, 1, 2, 3, 4, 6, 7, 8, 9, or 10 e7. In certain embodiments, the total number of cells provided is at least greater than 10 e7 and greater than one electroporation reaction is performed. In certain embodiments, the total volume of cell suspension is about 1 mL. In certain embodiments, the method results in increased template insertion in the genomic target sequence of the cells relative to an otherwise identical control composition but lacking the CDL target sequences, optionally wherein the template insertion increases by at least about 1-5, 1, 2, 3, 4, or 5 fold relative to the control.

[0028a] An in-vitro or ex-vivo method for modifying a target nucleic acid in a cell, comprising introducing or having non-virally introduced into the cell any of the compositions as described herein, wherein the HDR template is integrated into the target nucleic acid.

[0028b] In one aspect, provided herein is the use of any of the compositions as described herein in the manufacture of a medicament for modifying a target nucleic acid in a cell of a subject in need thereof, wherein the composition is to be introduced or non-virally introduced into the cell, and wherein the HDR template is to be integrated into the target nucleic acid.

Definitions

[0029] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[0030] As used herein, the "CRISPR-Cas" system refers to a class of bacterial systems for defense against foreign nucleic acid. CRISPR-Cas systems are found in a wide range of eubacterial and archaeal organisms. CRISPR-Cas systems include type I, II, and III sub-types. Wild-type type II CRISPR-Cas systems utilize an RNA-mediated nuclease, for example, Cas9 protein, in complex with guide and activating RNA (*e.g.*, single-guide RNA or sgRNA) to recognize and cleave foreign nucleic acids, *e.g.*, foreign nucleic acids including natural or modified nucleotides.

BRIEF DESCRIPTION OF THE DRAWINGS

[0044] The present application includes the following figures. The figures are intended to illustrate certain embodiments and/or features of the compositions and methods, and to supplement any description(s) of the compositions and methods. The figures do not limit the scope of the compositions and methods, unless the written description expressly indicates that such is the case.

[0045] FIG. 1 is a conceptual drawing illustrating an exemplary mechanism of the CDL (Co-Delivery Linearization) plasmid design for mediating genomic insertion of a cassette. As shown on the right, adding the CDL sequence (a Cas9 target sequence identical to the Cas9 genomic target for HDR integration) and a 3-base pair protospacer adjacent motif (PAM) outside of the homology arms flanking the cassette allows the Cas9-sgRNA ribonucleoprotein (RNP)-mediated endonuclease activity to release the cassette plus homology arms from the plasmid, in addition to the Cas9-sgRNA RNP-mediated cut in the genome. On the contrary, with standard plasmid, shown on the left, Cas9-sgRNA RNP only cuts the genomic locus but not the plasmid.

[0046] FIG. 2 are flow cytometry dot plots and graphs showing knock-in (KI) efficiency and KI cell yield of T-cells electroporated with a CRISPR RNP and homology-directed repair (HDR) templates of either standard plasmids or CDL plasmids. FIG. 2A shows a series of flow cytometry dot plots of T-cells electroporated with a CRISPR RNP and different doses (increasing from left to right, ranging between 0-100 mg/L) of homology-directed repair (HDR) templates of either standard plasmids (top panels) or CDL plasmids (bottom panels) encoding a MYC-tagged surface protein. FIG. 2B is a graph showing the KI% with varying doses of either standard plasmids or CDL plasmids. FIG. 2C is a graph showing the KI+ viable (live/dead stain; ThermoFisher) cell yield per 1 million starting cells with varying doses of either standard plasmids or CDL plasmids.

[0047] FIG. 3 is a graph showing the CD8/CD4 ratio with varying doses of either standard plasmids or CDL plasmids.

[0048] FIG. 4 is a graph showing transgene expression of T cells electroporated with 3 plasmids containing the CDL sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2) and 3 plasmids containing non-CDL sequences on day 6 post-electroporation.

10:1 and 400:1, between 10:1 and 300:1, between 10:1 and 200:1, between 10:1 and 100:1, or between 10:1 and 50:1), respectively.

[0056] A RNA-guided nuclease can also be fused with a localization peptide or protein. For example, the RNA-guided nuclease can be fused with one or more nuclear localization signal (NLS) sequences, which can direct the nuclease and the RNP complexes it forms to the nucleus to modify the target nucleic acid. Examples of NLS sequences are known in the art, *e.g.*, as described in Lange et al., *J Biol Chem.* 282(8):5101-5, 2007, and also include, but are not limited to, AVKRPAATKKAGQAKKKLD (SEQ ID NO: 3), MSRRRKANPTKLSENAKKLAKEVEN (SEQ ID NO: 4), PAAKRVKLD (SEQ ID NO: 5), KLKIKRPVK (SEQ ID NO: 6), and PKKKRKV (SEQ ID NO: 7). Examples of other peptide or proteins that can be used to a RNA-guided nuclease, such as cell-penetrating peptides and cell-targeting peptides are available in the art and described, *e.g.*, Vivès et al., *Biochim Biophys Acta.* 1786(2):126-38, 2008.

IV. Single-Guide RNAs

[0057] A Cas protein may be guided to its respective cognate DNA to be cleaved (*e.g.*, a CDL target sequence and/or genomic target sequence) by a single-guide RNA (sgRNA). An sgRNA is a version of the naturally occurring two-piece guide RNA (crRNA and tracrRNA) engineered into a single, continuous sequence. An sgRNA may contain a guide sequence (*e.g.*, the crRNA equivalent portion of the sgRNA) that targets the Cas protein to the cognate nucleic acid sequence and a scaffold sequence that interacts with the Cas protein (*e.g.*, the tracrRNAs equivalent portion of the sgRNA). An sgRNA may be selected using a software. As a non-limiting example, considerations for selecting an sgRNA can include, *e.g.*, the PAM sequence for the Cas9 protein to be used, and strategies for minimizing off-target modifications. Tools, such as NUPACK® and the CRISPR Design Tool, can provide sequences for preparing the sgRNA, for assessing target modification efficiency, and/or assessing cleavage at off-target sites.

Guide Sequence

[0058] The guide sequence in the sgRNA may be complementary to a specific sequence within a cognate nucleic acid sequence (*e.g.*, a CDL target sequence and/or genomic target sequence). The 3' end of a cognate nucleic acid sequence can be followed by a PAM sequence. A guide sequence is generally complementary to approximately 20 nucleotides upstream of the PAM sequence. In general, a Cas9 protein or a variant thereof cleaves about three nucleotides

endonuclease or the catalytic domain of FokI. Detailed descriptions of TALENs and their uses for gene editing are found, *e.g.*, in U.S. Patent Nos. 8,440,431; 8,440,432; 8,450,471; 8,586,363; and 8,697,853; Scharenberg *et al.*, *Curr Gene Ther*, 2013, 13(4):291-303; Gaj *et al.*, *Nat Methods*, 2012, 9(8):805-7; Beurdeley *et al.*, *Nat Commun*, 2013, 4:1762; and Joung and Sander, *Nat Rev Mol Cell Biol*, 2013, 14(1):49-55. Examples of a zinc finger DNA-binding protein fused to a protein that can cleave the target nucleic acid are described in the art and include, but are not limited to, those described in Urnov *et al.*, *Nature Reviews Genetics*, 2010, 11:636-646; Gaj *et al.*, *Nat Methods*, 2012, 9(8):805-7; U.S. Patent Nos. 6,534,261; 6,607,882; 6,746,838; 6,794,136; 6,824,978; 6,866,997; 6,933,113; 6,979,539; 7,013,219; 7,030,215; 7,220,719; 7,241,573; 7,241,574; 7,585,849; 7,595,376; 6,903,185; 6,479,626; and U.S. Application Publication Nos. 2003/0232410 and 2009/0203140.

[0088] In some embodiments, a targetable nuclease does not have nuclease activity. For example, the targetable nuclease (*e.g.*, a targetable nuclease without any nuclease activity) can regulate the expression of a cognate nucleic acid sequence. In some embodiments, the targetable nuclease can be a fusion protein that includes a protein that can bind to cognate nucleic acid sequence, such as a Cas protein variant without any cleavage activity (*e.g.*, a dCas9), a TAL effector DNA-binding protein, and a zinc finger DNA-binding protein as described above, and a protein that can modify the cognate nucleic acid sequence, such as a transcription activator or repressor.

[0089] A targetable nuclease can also be fused with a localization peptide or protein. For example, a targetable nuclease can be fused with one or more nuclear localization signal (NLS) sequences, which can direct a targetable nuclease, and a RNP complexes it forms, to the nucleus to modify a cognate nucleic acid sequence. Examples of NLS sequences are known in the art, *e.g.*, as described in Lange *et al.*, *J Biol Chem*. 282(8):5101-5, 2007, and also include, but are not limited to, AVKRPAATKKAGQAKKKKLD (SEQ ID NO: 3), MSRRRKANPTKLSENAKKLAKEVEN (SEQ ID NO: 4), PAAKRVKLD (SEQ ID NO: 5), KLKIKRPVK (SEQ ID NO: 6), and PKKKRKV (SEQ ID NO: 7). Examples of other peptide or proteins that can be used to a targetable nuclease, such as cell-penetrating peptides and cell-targeting peptides are available in the art and described, *e.g.*, Vivès *et al.*, *Biochim Biophys Acta*. 1786(2):126-38, 2008.

[0090] A targetable nuclease that modifies a target nucleic acid (*e.g.*, a genomic target sequence, such as in a T cell genome) can be the same as a targetable nuclease capable of cleaving a CDL target sequence. In an illustrative non-limiting example, a targetable nuclease

pulsed in a Lonza HT Nucleofector System with code EH-115. Cells were allowed to rest for 15 minutes at room temperature before transfer to 96-well plates (Sarstedt) in TexMACS medium supplemented with 12.5 ng/ml human IL-7 and IL-15 (Miltenyi premium grade).

Flow Cytometry Analysis

[0119] Transgene expression (gene of interest with a Myc epitope-tag) was detected by staining with anti-Myc antibody (Cell Signaling Technology clone 9B11) and analysis on an Attune NxT Flow Cytometer. Other antibodies used were TCRalpha/beta antibody (BioLegend clone IP26), CD4 antibody (BioLegend clone RPA-T4), CD8 antibody (BioLegend clone SK1).

Sequences

[0120] CRISPR protospacer (*e.g.*, CDL target sequence): GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2)

[0121] Full CRISPR site, protospacer and exemplary PAM:

GAGCCATGCTTGGCTTACGAGGG (SEQ ID NO: 8)

[0122] Standard plasmid donor backbone sequence used (X indicates where our transgene insertion of length 5696 bp was; Standard uppercase indicates homology arms):

CGACCAACCCATCAAACCTCCCCGCCCCCAGCACTTTTATTTCTCCTCTTTAGGAA
GTACACTTCAGTATCTTTGGCACAGTGCATGAGCACGACTAAAGTAAAACATCGC
AGAAAACATAGCTTTAGTCTACCCTTCGTGTCCTAAAAGGAAAACCAGTAGCTTC
CCAGGCCACCGGAAGGGCAACACATGTCCTCTGCAGTTTCTGCACACGGGAAGG
TAAAGACAGAGAGAGGACCTACTCCTCAACACAGAAACATTTCAAAATCTTTCC
TCGCCTGCAACCCAAGCTGAAGTCATTCTCCCCAGAAATAACAAAAGTTGGAAG
AGAAGCCGGAGACAGGATAGGTGCAGGAAGCCCACACTTTGAGGGCAGCACTC
AGACACCCTCTCCTGTGTGCAGGACGTGCCGAATGTTTCAGGTGCAATGAGAATG
AGCCATGCTTGGCTTA (SEQ ID NO: 9)-X-

CGAGGGCAATCTGGCCCATCAAGTGGCCTTCGCCTCTGGGAGTAACAAAAATGC
ACTTCAAAATAGCTTCTGTAATCAAGCTGCATGGGTGGAGTACTCCCCAGCTGAC
TCCAGGAAGTTCTCTATCCAAAGCTATTCATTAGGCCAGAGCTGTGCAAATAATT
AGTCACCCACTTGCTCCATAACCCTCCATGACAGCCCAGGCATTGAGTCCAGGTG
GGACCATCAAGCCATGCTCTGGTGGCTCATGCATTATCATAGAAATGGGAGGCTT
TATTTATTTTACTAAAAAGAACAAAAACAACAGACTGCTGTCCTTTAGACAATAG
GATCACGTCATCTGAGCCCTCTGTGCCCCAGGTGACAAGCCCAGCCCCAAGTTCT
CTTTCCTCAGCCTCCCCACACATGTTCTGGAGGAGATGGGCCCAGCAGGCTGCTC

TGAGGCCTGGCAtcccaatggcgcgccgagcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcac
 aattccacacaacatacagagccggaagcataaagtgtaaagcctgggggtgcctaatagtgagtaactcacattaattgcgttgcgct
 cactgcccgtttccagtcgggaaacctgtcgtgccagctgcattaatgaatcgccaacgcgcggggagaggcggtttgcgtattgg
 gcgctgttccgcttctcgtcactgactcgtcgcctcggctgttcggctgcggcgagcggtatcagctcactcaaaggcggtataac
 gggtatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccg
 cgttgcgtggcggttttccataggctccgccccctgacgagcatcacaaaaatcgacgtcaagtgcagaggtggcgaaacccgacag
 gactataaagataccaggcggttccccctggaagctccctcgtgcgtctcctgttccgacctgccgcttaccggatacctgtccgcct
 ttctcccttcgggaagcgtggcgcttttctcatagctcacgctgtaggtatctcagttcgggtgtaggtcgttcgtccaagctgggctgtgtg
 cacgaacccccgttcagcccagccgctgcgccttatccggttaactatcgtcttgagccaacccggtgaagacacgacttatccgcat
 ggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttctgaagtgggtggcctaactacggcta
 cactagaagaacagtatttggatatcgcgtctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaaaaac
 caccgctggtagcggtgggttttttggcaagcagcagattacgcgcagaaaaaaggatctcaagaagatcctttgatcttttctacg
 gggctgcagctcagtggaacgaaaactcacgttaagggatttgggtcatgagattatcaaaaaggatcttcacctagatccttttaatta
 aaaatgaagttttaatacatctaaagtatatatgagtaaaactggctgcagttaccaatgcttaatcagtgaggcacctatctcagcgat
 ctgtctatttcgttcatccatagttgcctgactccccgtcgtgtagataactacgatacgggagggccttaccatctggccccagtgctgca
 atgataccgcgagaaccacgctcaccggctccagatttatcagcaataaaccagccagccggaaggggcgcagcgagaagtggctc
 ctgcaactttatccgctccatccagctctattaattgttgcgggaagctagagtaagtagttccaggttaatagtttgcgaacggtgttg
 ccattgctacaggcatcgtggtgtcacgctcgtcgtttgggtatggcttcattcagctccggttcccaacgatcaaggcgagttacatgatc
 ccccatgttggtgcaaaaaagcggttagctccttcggctcctcgatcgttgcagaagtaagttggccgcagtggtatcactcatggttatg
 gcagcactgcataattcttactgtcatgccatccgtaagatgcttttctgtactgggtgagtactcaaccaagtcattctgagaatagtg
 atcgggcgaccgagttgctcttgcggcgctcaatacgggataataccgcgccacatagcagaactttaaaagtgtcatcattggaaa
 acgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaaccactcgtgcaccaactgatcttcagc
 atcttttactttcaccagcgtttctgggtgagcaaaaaacaggaaggcaaaatgccgcaaaaaagggaataaaggcgacacggaaatgt
 tgaatactcatactcttcttttcaatattattgaagcatttatcaggggtattgtctcatgagcggatacatatttgaatgtatttagaaaaata
 aacaaataggggttcgcgcacattccccgaaaagtccacctgacgtctaagaaaccattattatcatgacattaacctataaaaaata
 ggcgtatcacgaggcccttttctcgcgcgtttcgggtgatgacgggtgaaaacctctgacacatgcagctcccgagactgtcacagct
 tgtctgtaagcggatgccgggagcagacaagcccgctcagggcgctcagcgggtgttggcggtgtcggggctggcttaactatgc
 ggcatcagagcagattgtactgagagtgaccatatgcgggtgtgaaataccgcacagatgcgtaaggagaaaaataccgcatcaggcg
 ccattgccattcaggctgcgcaactgttgggaaggcgatcgggtgcgggcctcttcgctattacgccagctggcgaaagggggatg
 tgtgcaaggcgattaaagtgggtaacgccaggggtttccagtcacgacgttgtaaacgacggccagtgtaattgacgcgtattggga
 t (SEQ ID NO: 10).

[0123] CDL plasmid donor backbone sequence used (X indicates where our transgene insertion of length 5696 bp was; Bold indicates protospacer (CDL target sequence); Lowercase italic indicates PAM; Standard uppercase indicates homology arms):

GAGCCATGCTTGGCTTACGA*ggg*CGACCAACCCATCAAACCTCCCCGCCCCCAGC
 ACTTTTATTTCTCCTCTTTAGGAAGTACACTTCAGTATCTTTGGCACAGTGCATGA
 GCACGACTAAAGTAAAACATCGCAGAAAACATAGCTTTAGTCTACCCTTCGTGTC
 CTAAGAGGAAAACCAGTAGCTTCCCAGGCCACCGGAAGGGCAACACATGTCCTC
 TGCAGTTTCTGCACACGGGAAGGTAAAGACAGAGAGAGGACCTACTCCTCAACA
 CAGAAACATTTCAAATCTTTCCTCGCCTGCAACCCAAGCTGAAGTCATTCTCCC
 CAGAAATAACAAAAGTTGGAAGAGAAGCCGGAGACAGGATAGGTGCAGGAAGC
 CCACACTTTGAGGGCAGCACTCAGACACCCTCTCCTGTGTGCAGGACGTGCCGAA
 TGTTCAAGGTGCAATGAGAATGAGCCATGCTTGGCTTA (SEQ ID NO: 11)-X-
 CGAGGGCAATCTGGCCCATCAAGTGGCCTTCGCCTCTGGGAGTAACAAAAATGC
 ACTTCAAATAGCTTCTGTAATCAAGCTGCATGGGTGGAGTACTCCCCAGCTGAC
 TCCAGGAAGTTCTCTATCCAAAGCTATTCATTAGGCCAGAGCTGTGCAAATAATT
 AGTCACCCACTTGCTCCATAACCCTCCATGACAGCCCAGGCATTGAGTCCAGGTG
 GGACCATCAAGCCATGCTCTGGTGGCTCATGCATTATCATAGAAATGGGAGGCTT
 TATTTATTTTACTAAAAAGAACAAAAACAACAGACTGCTGTCTTTAGACAATAG
 GATCACGTCATCTGAGCCCTCTGTGCCCCAGGTGACAAGCCCAGCCCCAAGTTCT
 CTTTCCTCAGCCTCCCCACACATGTTCTGGAGGAGATGGGCCCAGCAGGCTGCTC
 TGAGGCCTGGC*ccc***TCGTAAGCCAAGCATGGCTC***atccaatggcgcgccgagcttggcgtaatcat*
ggtcatagctgtttcgtgtgaaattgtatccgctcacaaatccacacaacatacagagccggaagcataaagtgtaaagcctggggtg
cctaatagtgagctaaactacattaattgcgttgcgctcactgcccgtttccagtcgggaaacctgtcgtgccagctgcattaatgaat
cggccaacgcgcggggagagggcggttgcgtattgggcgctgtccgcttcctcgtcactgactcgtcgcgtcggctcgttcggctg
cggcgagcgggtatcagctcactcaaaggcggtatccaggtatccacagaatcaggggataacgcaggaaagaacatgtgagcaaa
aggccagcaaaaggccaggaaccgtaaaaaggccgctgtggcggttttccataggctccgccccctgacgagcatcacaaaa
atcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataaccaggcggttccccctggaagctccctcgtgcgctctc
ctgttccgacctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctc
agttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaacccccgttcagcccagccgtgcgccttatccgtaactatcg
tcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcgg
tgctacagagttctgaagtgggtggcctaactacggctacactagaagaacagtatttggtatctgcgctctgctgaagccagttacctc
ggaaaaagagttggtagctcttgatccggcaacaaaccaccgctggtagcggtggtttttgtttgcaagcagcagattacgcgcag
aaaaaaaggatctcaagaagatcctttgatctttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatg

agattatcaaaaaggatcttcacctagatccttttaataaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgaca
gttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcacccatagttgcctgactccccgtcgtgtagataacta
cgatacgggagggccttaccatctggccccagtgctgcaatgataccgcgagaaccacgctcacgggtccagatttatcagcaataaa
ccagccagccggaagggccgagcgcagaagtggctcgaactttatccgctccatccagctctattaattgttgcgggaagctaga
gtaagtagttcgccagttaatagtttgcgcaacggttggccattgctacaggcatcgtggtgtcacgctcgtcgtttggtatggcttcattc
agctccggttcccaacgatcaaggcgagttacatgatccccatggttgcaaaaaagcggtagctccttcggctcctccgatcgttgct
agaagtaagttggccgagtggtatcactcatggttatggcagcactgcataattcttactgtcatgccatccgtaagatgctttctgtg
actggtgagtactcaaccaagtcattctgagaatagtgatgcggcgaccgagttgctcttgcggcgctcaatacgggataataccgc
gccacatagcagaactttaaaagtgtcatcattggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccag
ttgatgtaacccactcgtgcacccaactgatcttcagcatctttactttcaccagcgtttctgggtgagcaaaaaacaggaaggcaaat
gccgcaaaaaagggaataagggcgacacggaaatgtgaatactcactcttcttttcaatattattgaagcatttatcagggttattgt
ctcatgagcggatacatattgaatgtatttagaaaaataaacaataggggttcgcgcacatttccccgaaaagtgccacctgacgtct
aagaacattattatcatgacattaacctataaaaaataggcgatcacgaggccctttgtctcgcgcgttccggtgatgacggtgaaaa
cctctgacacatgcagctcccgagactgtcacagcttgtctgtaagcggatgccgggagcagacaagcccgtcagggcgcgctcag
cgggtgttggcgggtgtcggggctggcttaactatcgggcatcagagcagattgtactgagagtgcacatcgcggtgtgaaatacc
gcacagatgcgtaaggagaaaaataccgcatcaggcgccattcgccattcaggctgcgcaactgttgggaagggcgatcggtgcggg
cctcttcgctattacgccagctggcgaaaagggggatgtgctgcaaggcgattaagttgggtaacgccagggtttccagtcacgacgt
tgtaaacgacggccagtgaattgacgcgtattgggat (SEQ ID NO: 12)

Example 2 – Co-delivery Linearization (CDL) Designs for CRISPR-mediated Gene Editing of T Cells

[0124] Genetic engineering of T cells using RNP electroporation was explored comparing a plasmid donor templates with (1) a homology-directed repair template cassette (HDRT) featuring a standard HDRT design of a transgene simply flanked by homology arms (FIG. 1 – left panel) or (2) a co-delivery linearization (CDL) design where the HDRT was additionally flanked with CDL target sequences (CRISPR target sites with a match to the protospacer of the CRISPR gRNA used to target the T cell genome) and protospacer-adjacent motif (PAM) (FIG. 1 – right panel).

[0125] As shown in Figure 2, T cells were electroporated with RNP complexes with varying amounts of either standard or CDL plasmid donor templates then assessed for transgene integration by flow cytometry (FIG. 2A, quantified in Table 1). The CDL design demonstrated greater KI% at lower doses of HDRT DNA than standard HDRT plasmids without the CDL sequence modifications (FIG. 2B, quantified in Table 2). Furthermore, overall

performed on the ThermoFisher Attune cytometer with results analyzed using FlowJo software.

Results

[0132] Performance on a clinical scale platform. The effect of modifying the nucleic acid sequence of a plasmid containing a homology directed repair template to include the CDL sequence immediately upstream of the 5' HDR arm and downstream of the 3' HDR arm was assessed in the context of the previously described CRISPR-Cas9 system on a platform and scale relevant to clinical manufacturing of a cell therapy product. The purpose of this study was to demonstrate the utility of the CDL sequence in a clinical context and highlight the applicability of the technology to manufacturing cell therapy products.

[0133] Inclusion of the CDL sequence in the HDR cassette increases the percentage of cells with transgene integration following clinical scale transfection. T cells electroporated with plasmids containing the CDL sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2) and a sequence encoding a transgene or control plasmids comprising sequences encoding a transgene without the CDL sequence were assessed for both cell number and transgene expression on day 6 post-electroporation. The length of the polynucleotide sequence encoding the transgene was: 6533 base pairs in length (for plasmid pS3798), 7042 base pairs in length (for plasmid pS3631), and 6236 base pairs in length (for plasmid pS3797). The results shown in Figure 4 indicate a significant ($p < 0.05$) increase in transgene expression in the conditions receiving the plasmid containing the CDL sequence compared to the conditions receiving the control, non-CDL site containing plasmids. The difference is evident in the percentage of cells harboring an integrated transgene. On average, transgene expression as assessed by flow cytometry increased from 3.35% for the non-CDL conditions to 9.76% for the CDL conditions at 20 ug DNA. Overall, these data demonstrate the enhanced effect the inclusion of the CDL sequence has on the gene editing process.

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