



US 20240131066A1

(19) United States

(12) Patent Application Publication

Saha et al.

(10) Pub. No.: US 2024/0131066 A1

(43) Pub. Date: Apr. 25, 2024

(54) SENOLYTIC CRISPR CAR T CELLS
PRODUCED BY CRISPR-CAS9 GENOME
EDITING

CI2N 5/0783 (2006.01)
CI2N 9/22 (2006.01)
CI2N 15/11 (2006.01)

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(52) U.S. Cl.
CPC *A61K 35/17* (2013.01); *A61K 39/4611*
(2023.05); *A61K 39/4631* (2023.05); *A61K
39/464429* (2023.05); *C07K 14/005* (2013.01);
C07K 16/2896 (2013.01); *CI2N 5/0056*
(2013.01); *CI2N 5/0636* (2013.01); *CI2N
9/22* (2013.01); *CI2N 15/11* (2013.01); *A61K
2239/15* (2023.05); *A61K 2239/17* (2023.05);
A61K 2239/21 (2023.05); *A61K 2239/22*
(2023.05); *C07K 2317/622* (2013.01); *CI2N
2310/10* (2013.01)

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(21) Appl. No.: 18/295,036

(22) Filed: Apr. 2, 2023

Related U.S. Application Data

(60) Provisional application No. 63/327,189, filed on Apr.
4, 2022.

Publication Classification

(51) Int. Cl.

A61K 35/17 (2006.01)
A61K 39/00 (2006.01)
C07K 14/005 (2006.01)
C07K 16/28 (2006.01)
CI2N 5/00 (2006.01)

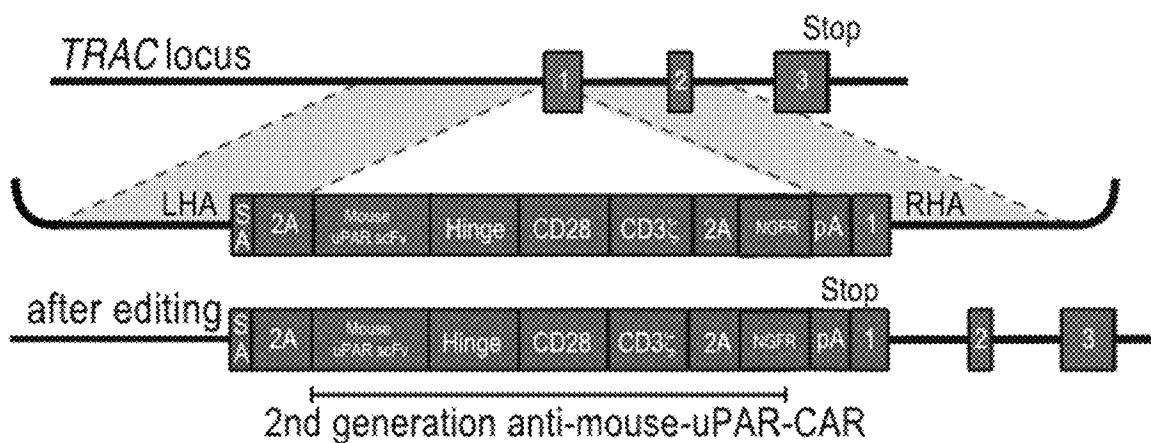
(57)

ABSTRACT

Described herein are methods using CRISPR-Cas9 and DNA templates that can generate chimeric antigen receptors (CARs) on T cells to target the cell surface protein urokinase Plasminogen Activator Receptor (uPAR) on senescent cells. Also described are methods of preparing CAR T cells, their use to treat neurodegenerative disease, stroke, craniocebral trauma and/or accident, or elderly individuals in need of treatment for aging.

Specification includes a Sequence Listing.

A



A

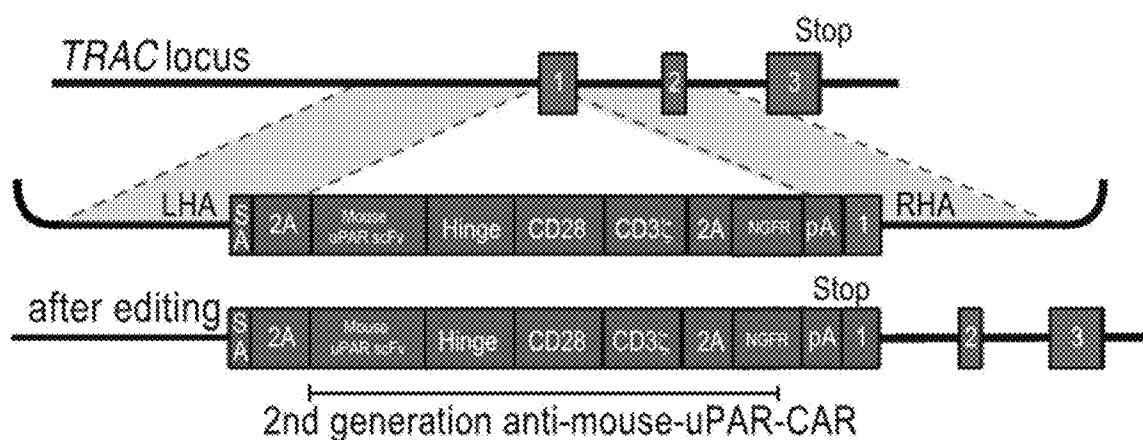


FIG. 1A

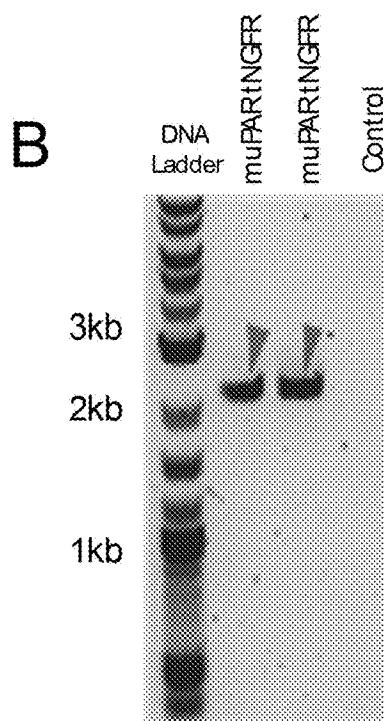


FIG. 1B

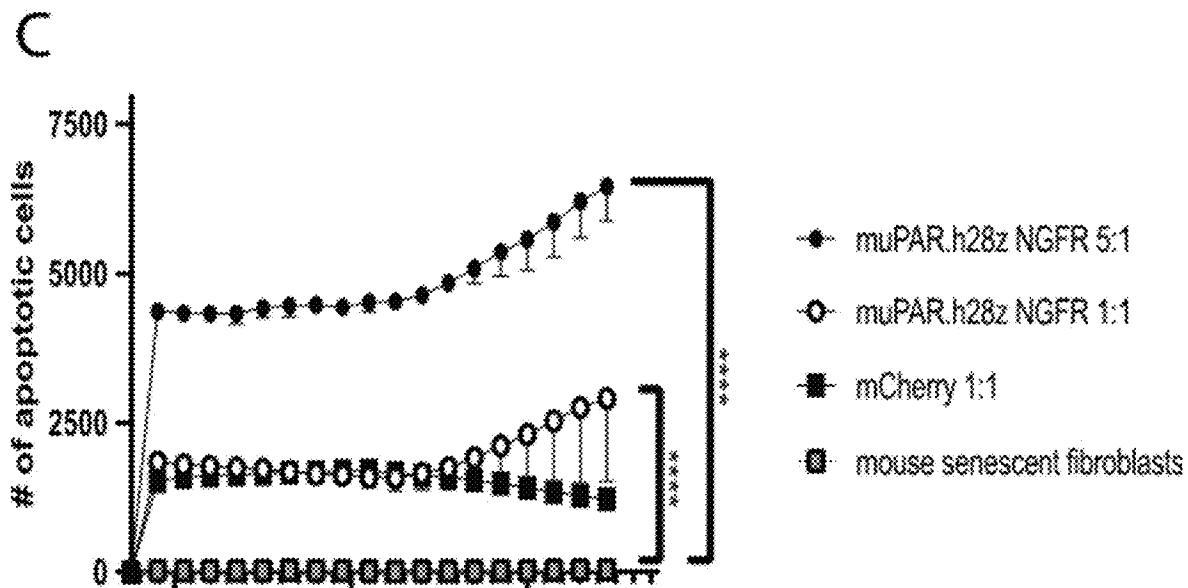


FIG. 1C

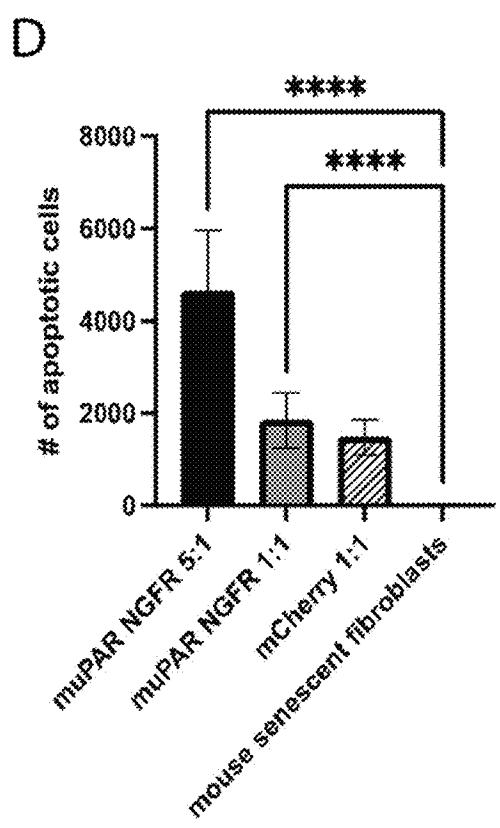


FIG. 1D

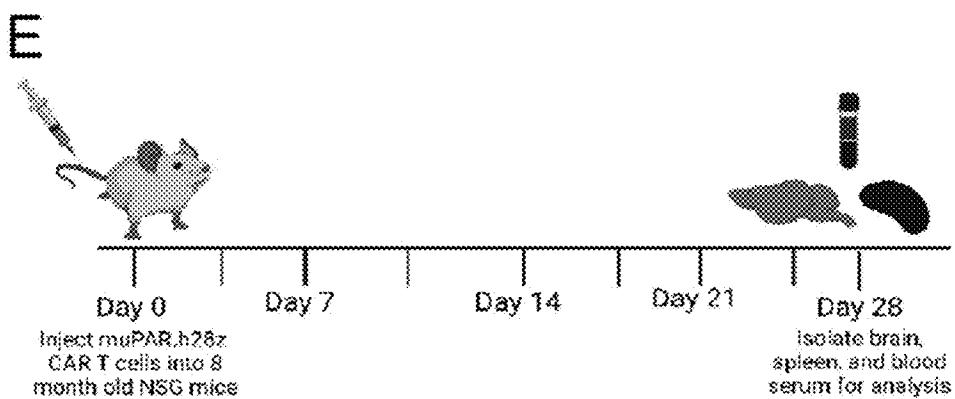


FIG. 1E

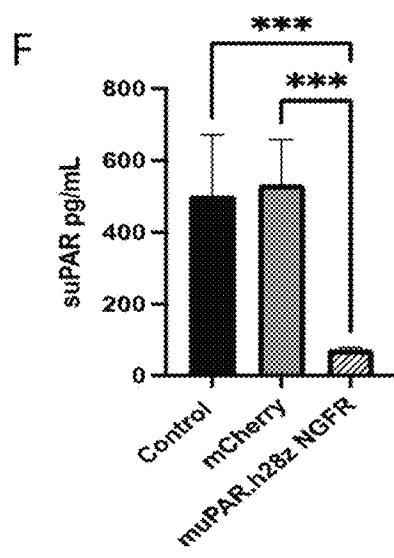


FIG. 1F

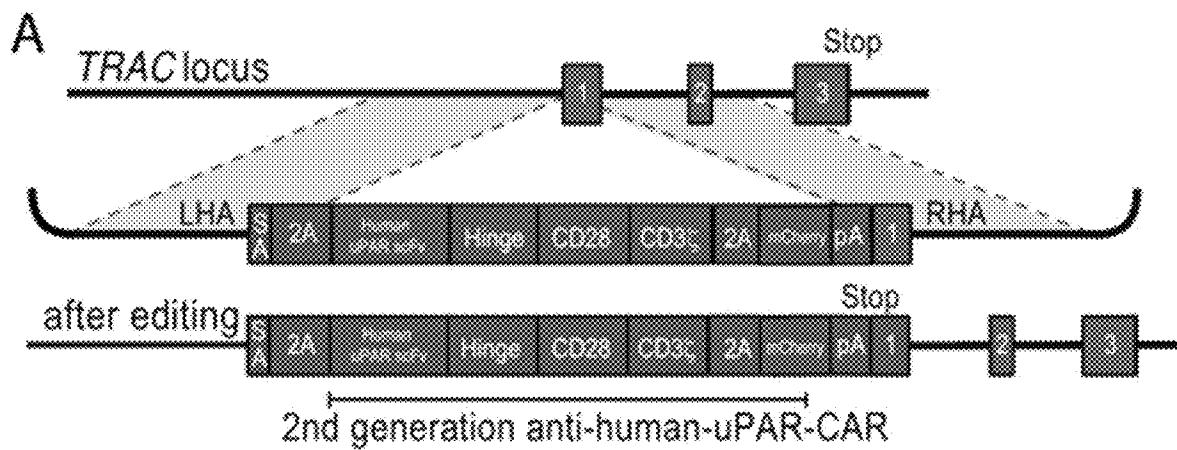


FIG. 2A

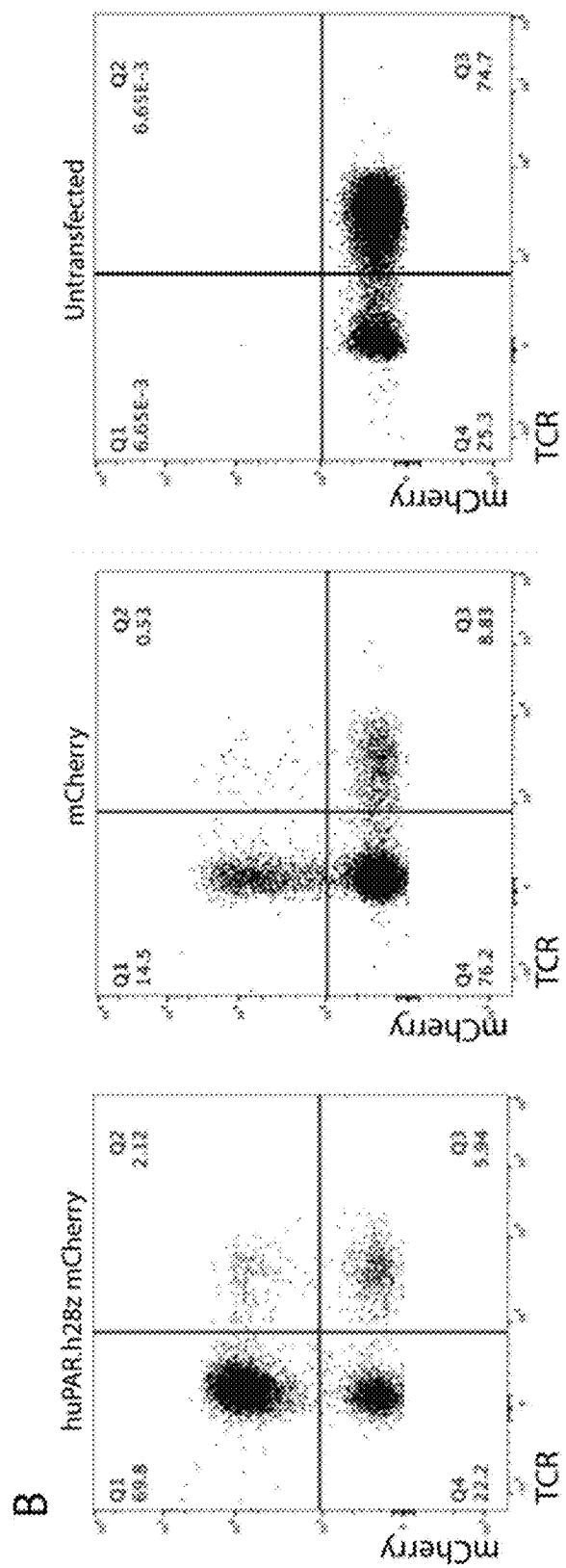


FIG. 2B

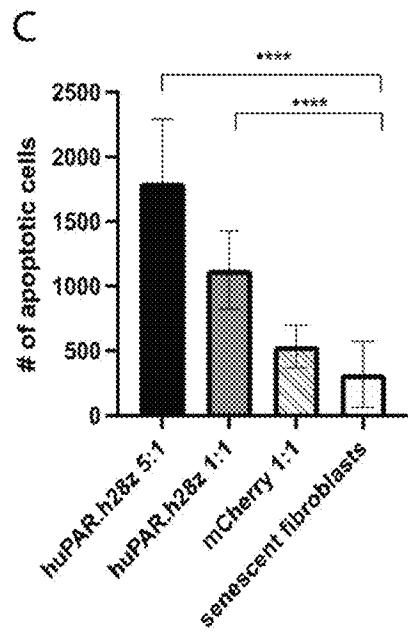


FIG. 2C

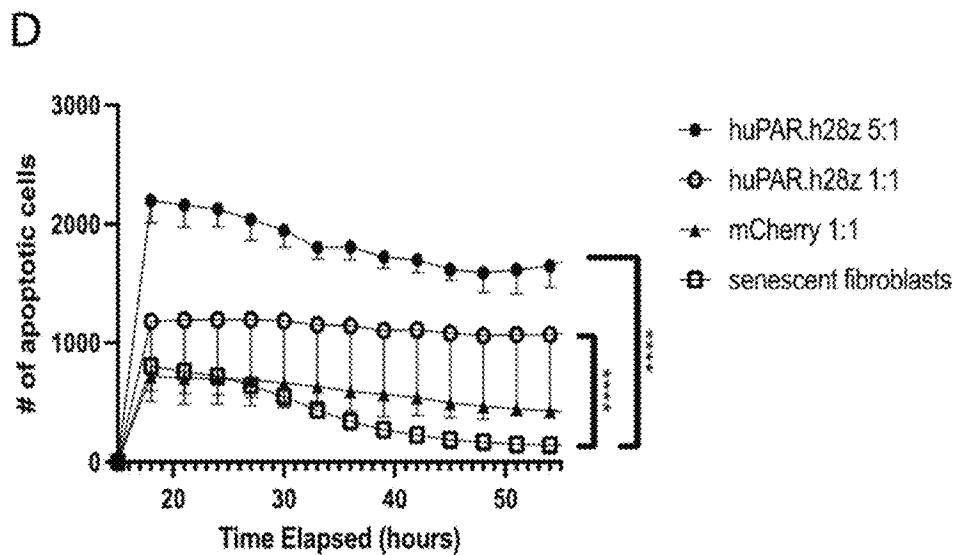


FIG. 2D

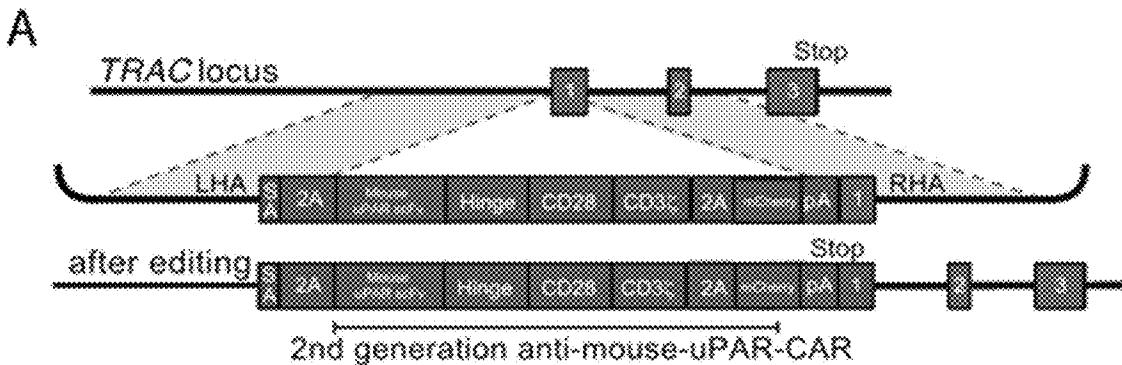


FIG. 3A

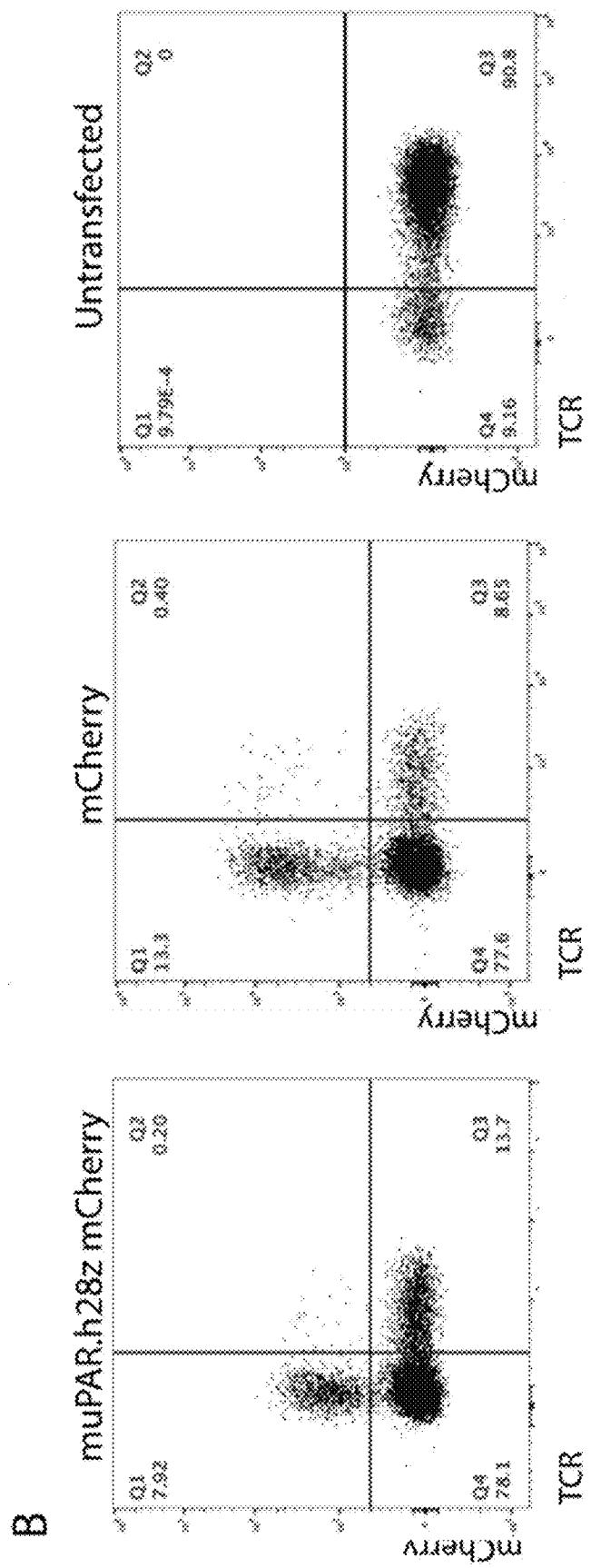


FIG. 3B

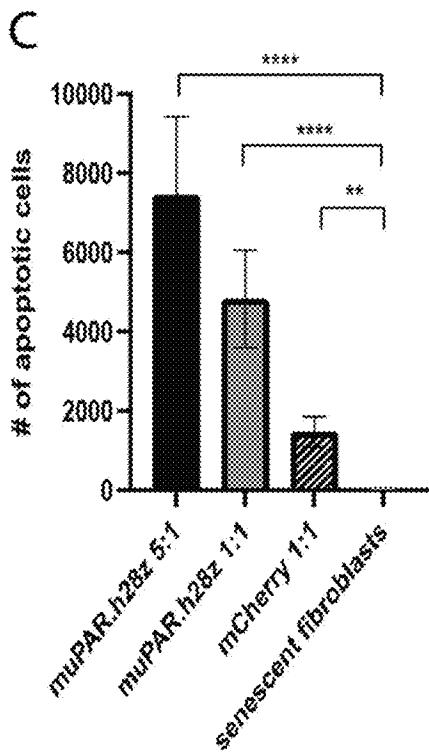


FIG. 3C

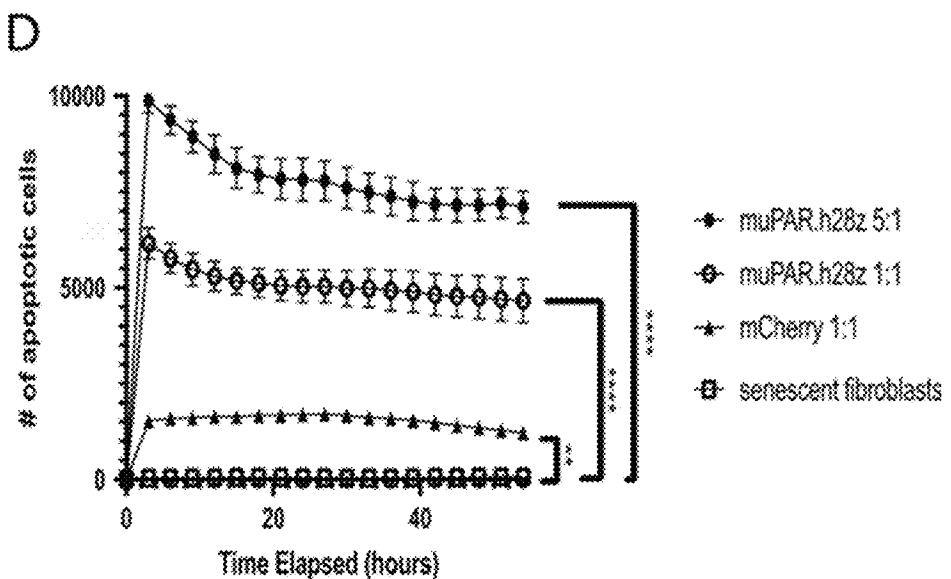


FIG. 3D

A

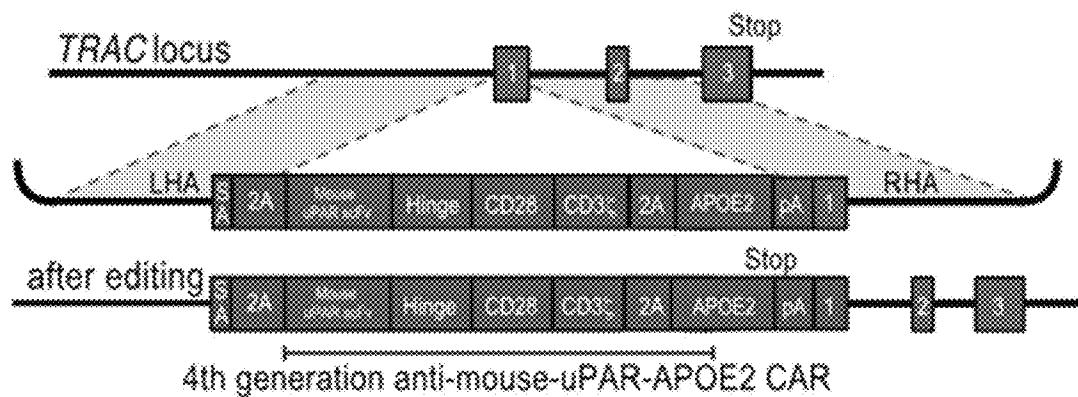


FIG. 4A

B

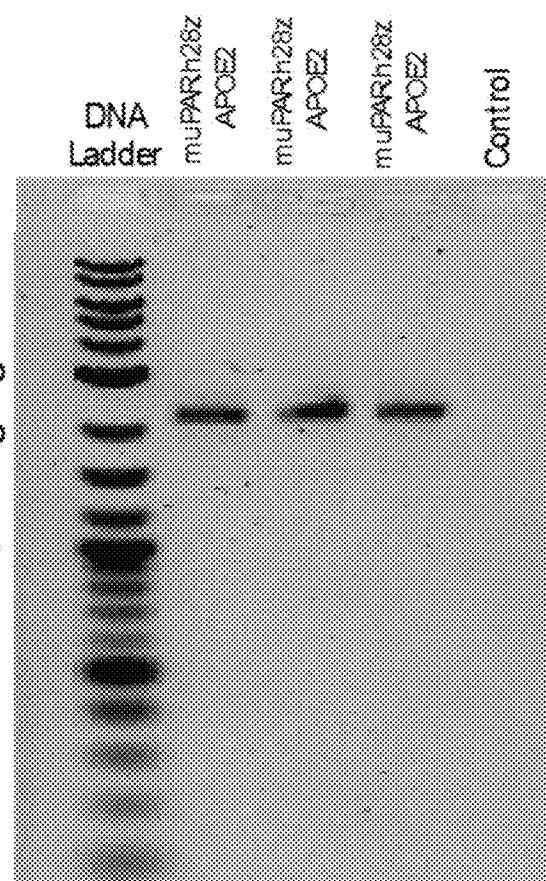


FIG. 4B

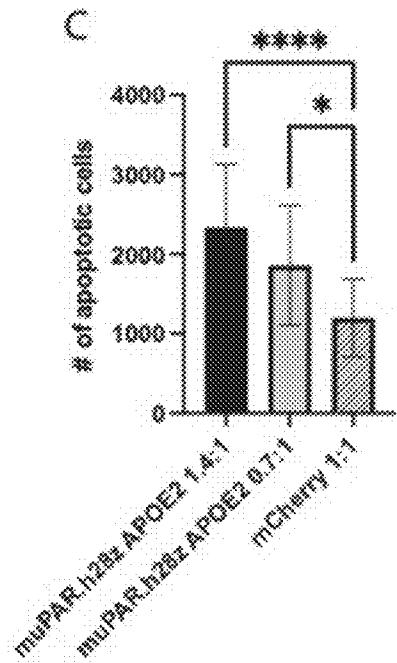


FIG. 4C

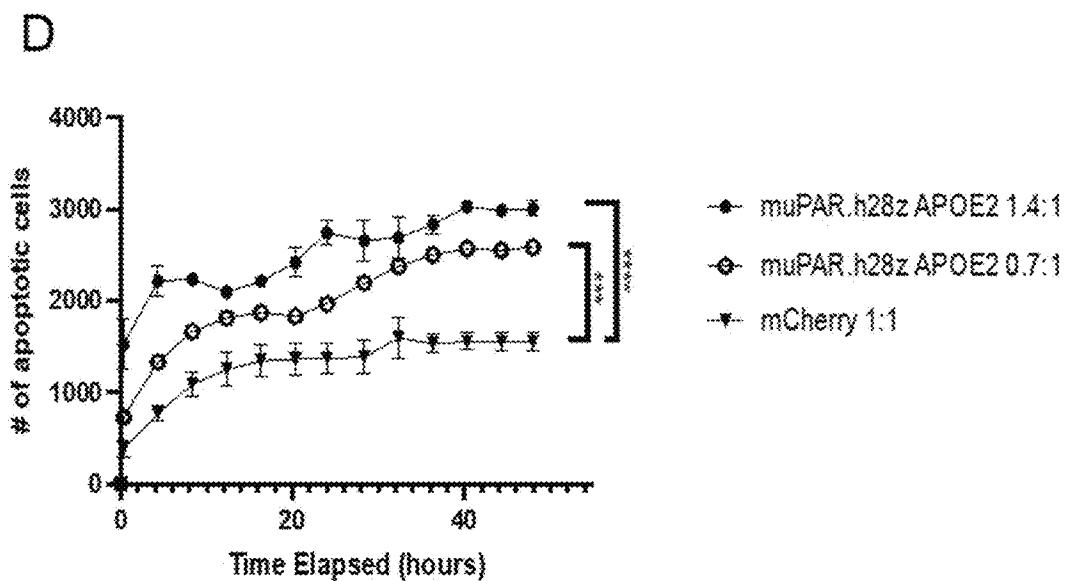


FIG. 4D

SENOLYTIC CRISPR CAR T CELLS PRODUCED BY CRISPR-CAS9 GENOME EDITING

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application 63/327,189 filed on Apr. 4, 2022, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH & DEVELOPMENT

[0002] This invention was made with government support under GM119644 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] The Instant Application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on Jul. 19, 2023 is named "WIS0068US2" and is 124,266 bytes in size. The Sequence Listing does not go beyond the disclosure in the application as filed.

BACKGROUND

[0004] Senescence is a multifaceted cellular response to endogenous and exogenous stress signals that involves the induction of cell cycle arrest to eliminate unwanted cells. A fundamental feature of cell senescence is the senescence-associated secretory phenotype (SASP), which involves the secretion of tissue specific inflammatory, oxidative, and matrix-degrading factors that can attract immune cells and promote matrix rearrangement to eliminate senescent cell populations. However, in persistently damaged or aged tissues, senescent cell clearance can be compromised due to a lack immune cell recruitment, ultimately resulting in tissue dysfunction. To overcome these challenges researchers have looked to eliminate accumulated senescent cell populations that evade immune cell responses by developing antisenescent therapies also known as "senolytic" treatments. While these therapies yield promising therapeutic potential, new approaches for eliminating senescence cells are critically needed for the further understanding and prevention of tissue dysfunction in senescence associated disease pathologies.

[0005] Chimeric Antigen Receptor (CAR) T cell therapies redirect T cell specificity and effector potential functions to attack a desired target in an MHC-1 independent manner, bypassing requirements for peptide presentation. In this way, T cells can be engineered to activate against cell surface antigens for several different pathologies such as cancer, HIV, and fibrosis. Amor and colleagues (Nature, 583(7814), pp. 127-132, 2020) recently demonstrated the ability to reprogram CAR T cell effector function to target senescence associated pathologies by targeting the cell surface antigen urokinase Plasminogen Activator Receptor (uPAR). These T cells were manufactured with γ -retroviruses to target uPAR+ cells to eliminate senescent cell in vivo to reduce inflammation in lung and liver fibrosis. These genomes of these cells were not edited by CRISPR-Cas9, which provides new opportunities to increase the potency, specificity, and persistence of T cell therapies.

[0006] What is needed are alternative CAR T cell therapies, incorporating CRISPR-Cas9 genome editing, as potent senolytic agents.

BRIEF SUMMARY

[0007] In an aspect, an DNA HDR template for a transgene comprising a chimeric antigen receptor (CAR) gene for inserting the transgene into a T cell expressed gene to generate CAR T cells having the composition:

[0008] (left HA)-(SA)-(first self-cleaving peptide polynucleotide or IRES)-(optional inducible control sequence)-(uPAR binding fragment polynucleotide)-(hinge domain polynucleotide)-(transmembrane domain polynucleotide)-(intracellular domain polynucleotide)-(second self-cleaving peptide polynucleotide or IRES)-(first secreted factor or first selection marker polynucleotide)-(optional third self-cleaving peptide polynucleotide or IRES)-(optional second secreted factor or second selection marker polynucleotide)-(polyA terminator)-(right HA);

[0009] or

[0010] (left HA)-(SA)-(first self-cleaving peptide polynucleotide or IRES) (uPAR binding fragment polynucleotide)-(hinge domain polynucleotide)-(transmembrane domain polynucleotide)-(intracellular domain polynucleotide)-(optional second self-cleaving peptide polynucleotide or IRES)-(optional inducible control sequence)-(optional first secreted factor or first selection marker polynucleotide)-(optional third self-cleaving peptide polynucleotide or IRES)-(optional second secreted factor or second selection marker polynucleotide)-(polyA terminator)-(right HA);

[0011] or

[0012] (left HA)-(SA)-(first self-cleaving peptide polynucleotide or IRES) (uPAR binding fragment polynucleotide)-(hinge domain polynucleotide)-(transmembrane domain polynucleotide)-(intracellular domain polynucleotide)-(optional second self-cleaving peptide polynucleotide or IRES)-(optional first secreted factor or first selection marker polynucleotide)-(optional third self-cleaving peptide polynucleotide or IRES)-(optional inducible control sequence)-(optional second secreted factor or second selection marker polynucleotide)-(polyA terminator)-(right HA);

[0013] wherein the left HA and the right HA are homology arms complementary to sequences on both sides of a cleavage site in the T cell expressed gene;

[0014] wherein SA is a splice acceptor site;

[0015] wherein the first, second and third self-cleaving peptide polynucleotide or IRES are polynucleotides encoding a first, second and third self-cleaving peptide or an internal ribosome entry site (IRES), respectively;

[0016] wherein the optional inducible control sequence is a regulatory sequence which provides control of protein expression in response to a small molecule inducer;

[0017] wherein the uPAR binding fragment polynucleotide is a polynucleotide encoding a polypeptide that specifically binds uPAR;

[0018] wherein the hinge domain polynucleotide encodes a CD28 or CD8 α hinge domain;

[0019] wherein the transmembrane domain polynucleotide encodes a transmembrane domain;

- [0020] wherein the intracellular domain polynucleotide encodes one or more intracellular domains;
- [0021] wherein the first and second secreted factor polynucleotides are coding sequences for a neurotrophic factor, growth factor, or cytokine;
- [0022] wherein the first and second selection marker polynucleotides are coding sequences for a detectable protein; and
- [0023] wherein the polyA terminator is a sequence-based element that defines the end of a transcriptional unit.
- [0024] In another aspect, included are plasmids comprising the HDR template described above.
- [0025] In another aspect, an ex vivo, virus-free method of site-specifically inserting a transgene containing a chimeric antigen receptor (CAR) gene into a T cell expressed gene to generate CAR T cells comprises
- [0026] preparing the homology-directed repair (HDR) template described above,
- [0027] introducing into a population of unmodified T cells a Cas9 ribonucleoprotein (RNP) and the HDR template to provide the CAR T cells,
- [0028] wherein the Cas9 RNP comprises a Cas9 protein and a guide RNA that directs double stranded DNA cleavage of a cleavage site in the T cell expressed gene, and
- [0029] wherein the transgene is specifically integrated into the cleavage site of the T cell expressed gene locus created by the Cas9 RNP in the cells, and
- [0030] culturing the CAR T cells in xeno-free medium to provide a cultured population of CAR T cells having the transgene specifically integrated in the T cell expressed gene,
- [0031] wherein, in the cultured population of CAR T cells, an endogenous promoter of the T cell expressed gene drives expression of the transgene, or wherein the transgene includes a promoter that drives expression of the transgene, and
- [0032] wherein the CAR gene encodes a fusion protein comprising the translated anti-uPAR binding motif, hinge domain, transmembrane domain, and intracellular domain.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] FIGS. 1A-F show generation, characterization, and potency of virus-free CRISPR (VFC) anti-muPAR-tNGFR T cells. (1A) Schematic of anti-muPAR-2A-tNGFR CAR construct targeting using the first encoding exon of the human TRAC gene (grey). SA: splice acceptor, T2A: self-cleaving peptide, mouse uPAR (muPAR) scFv: single chain variable fragment targeting murine uPAR, P2A: self-cleaving peptide, tNGFR: truncated nerve growth factor receptor, pA: rabbit β-globin polyA terminator. (1B) In-out PCR indicates proper on-target genomic integration of the CAR transgene in VFC-muPAR-tNGFR CAR cells. Control, untransfected donor-matched T cells. (1C) Incucyte Live-Cell Analysis system in vitro potency assay with murine fibroblasts at 5:1 and 1:1 effector:target ratio, averaged across two donors. The consistent increase in apoptotic cells after T cells were added at 0 hours indicates high potency of VFC-muPAR-tNGFR T cells. VFC-muPAR-tNGFR 5:1 (black circle) N=3; VFC-muPAR-tNGFR 1:1 (open circle) N=3; VFC-mCherry 1:1 (black square) N=3; mouse senescent fibroblast control (grey square) N=3. (1D) Summary of Incucyte Live-Cell Analysis over 48 hours. *p<0.05, one-way ANOVA. *p<0.01, one-way ANOVA.

Cell Analysis over 48 hours. (1E) Schematic depicting in vivo mouse experiment timeline over a 28 day period. (1F) suPAR ELISA (R&D systems) assay results of blood serum collected from mice after 28 days post VFC-muPAR-tNGFR, VFC-mCherry, or no T cell infusion. Flow cytometry plots for transgene and TCR surface protein levels on the manufactured cell products. Y-axis shows mCherry levels and x-axis shows TCR levels on day 7 post-isolation. (1D) UTF, untransfected donor-matched T cells. (1E) DNA isolated from VFC-huPAR-mCh edited CAR T cells was subjected to “in-out” PCR and sequenced to evaluate TRAC locus integration. *p<0.05, one-way ANOVA. *p<0.01, one-way ANOVA.

[0034] FIGS. 2A-D show the generation, characterization, and potency of anti-huPAR-mCherry VFC-CART cells. (2A) Schematic of anti-huPAR-2A-mCherry CAR construct targeting using the first encoding exon of the human TRAC gene (grey). SA: splice acceptor, T2A: self-cleaving peptide, human uPAR (huPAR) scFv: single chain variable fragment targeting human uPAR, P2A: self-cleaving peptide, mCherry: fluorescent protein. pA: rabbit β-globin polyA terminator. (2B) Flow cytometry plots for transgene and TCR surface protein levels on the manufactured cell products. Y-axis shows mCherry levels and x-axis shows TCR levels on day 7 post-isolation. (2C) Incucyte Live-Cell Analysis system in vitro potency assay with human dermal fibroblasts (HDFa) at 5:1 and 1:1 effector:target ratio, averaged across two donors. The consistent increase in apoptotic cells after T cells were added at 0 hours indicates high potency of VFC-huPAR-mCherry T cells. VFC-huPAR-mCherry 5:1 (black circle) N=3; VFC-huPAR-mCherry 1:1 (open circle) N=3; VFC-mCherry 1:1 (black square) N=3; mouse senescent fibroblast control (grey square) N=3. (2D) Summary of Incucyte Live-Cell Analysis over 48 hours. *p<0.05, one-way ANOVA. *p<0.01, one-way ANOVA.

[0035] FIG. 3A-D show the generation, characterization, and potency of anti-muPAR-mCherry VFC-CART cells. (3A) Schematic of anti-muPAR-2A-mCherry CAR construct targeting using the first encoding exon of the human TRAC gene (grey). SA: splice acceptor, T2A: self-cleaving peptide, murine uPAR (huPAR) scFv: single chain variable fragment targeting murine uPAR, P2A: self-cleaving peptide, mCherry: fluorescent protein. pA: rabbit β-globin polyA terminator. (3B) Flow cytometry plots for transgene and TCR surface protein levels on the manufactured cell products. Y-axis shows mCherry levels and x-axis shows TCR levels on day 7 post-isolation. (3C) Incucyte Live-Cell Analysis system in vitro potency assay with murine senescent fibroblasts at 5:1 and 1:1 effector:target ratio, averaged across two donors. The consistent increase in apoptotic cells after T cells were added at 0 hours indicates high potency of VFC-muPAR-mCherry T cells. VFC-muPAR-mCherry 5:1 (black circle) N=3; VFC-muPAR-mCherry 1:1 (open circle) N=3; VFC-mCherry 1:1 (black square) N=3; mouse senescent fibroblast control (grey square) N=3. (3D) Summary of Incucyte Live-Cell Analysis over 48 hours. *p<0.05, one-way ANOVA. *p<0.01, one-way ANOVA.

[0036] FIGS. 4A-D show the generation, characterization, and potency of a fourth generation anti-muPAR-APOE2 VFC-CART cells. (4A) Schematic of anti-muPAR-2A-APOE2 CAR construct targeting using the first encoding exon of the human TRAC gene (grey). SA: splice acceptor, T2A: self-cleaving peptide, murine uPAR (huPAR) scFv: single chain variable fragment targeting murine uPAR, P2A:

self-cleaving peptide, APOE2: Apolipoprotein E 2 protein that forms lipoprotein particles and regulates lipid transport in both the central and peripheral nervous systems. pA: rabbit β-globin polyA terminator. (4B) In-out PCR indicates proper on-target genomic integration of the CAR transgene in VFC-muPAR-APOE2 CAR cells. Control, untransfected donor-matched T cells. (4C) Incucyte Live-Cell Analysis system in vitro potency assay with murine senescent fibroblasts at 5:1 and 1:1 effector:target ratio, averaged across two donors. The consistent increase in apoptotic cells after T cells were added at 0 hours indicates high potency of VFC-muPAR-APOE2 T cells. VFC-muPAR-APOE2 5:1 (black circle) N=3; VFC-muPAR-APOE2 1:1 (open circle) N=3; VFC-mCherry 1:1 (black square) N=3; mouse senescent fibroblast control (grey square) N=3. (4D) Summary of Incucyte Live-Cell Analysis over 48 hours. *p<0.05, one-way ANOVA. *p<0.01, one-way ANOVA.

[0037] The above-described and other features will be appreciated and understood by those skilled in the art from the following detailed description, drawings, and appended claims.

DETAILED DESCRIPTION

[0038] The present disclosure builds on the production of anti-senescence CAR T cell therapies and adapts this technology with CRISPR/Cas9 and homology directed repair (HDR) to integrate a 4.5 kb second-generation anti-uPAR CAR transgene at the human TRAC locus. We describe uPAR CAR T cell product, e.g., a completely virus-free product, featuring precise genomic integration of our CAR and elimination of senescent cells in vitro. Of particular note, there is an increased presence of senescent cells in neurodegenerative diseases and the CAR T therapies described herein are particularly useful to treat neurodegenerative diseases such as Alzheimer's Disease, Down Syndrome, and Parkinson's Disease.

[0039] In an aspect, a DNA HDR template for a transgene comprising a chimeric antigen receptor (CAR) gene for inserting the transgene into a T cell expressed gene to generate CAR T cells having the composition:

[0040] (left HA)-(SA)-(first self-cleaving peptide polynucleotide or IRES)-(optional inducible control sequence)-(uPAR binding fragment polynucleotide)-(hinge domain polynucleotide)-(transmembrane domain polynucleotide)-(intracellular domain polynucleotide)-(optional second self-cleaving peptide polynucleotide or IRES)-(optional first secreted factor or first selection marker polynucleotide)-(optional third self-cleaving peptide polynucleotide or IRES)-(optional second secreted factor or second selection marker polynucleotide)-(polyA terminator)-(right HA);

[0041] or

[0042] (left HA)-(SA)-(first self-cleaving peptide polynucleotide or IRES) (uPAR binding fragment polynucleotide)-(hinge domain polynucleotide)-(transmembrane domain polynucleotide)-(intracellular domain polynucleotide)-(optional second self-cleaving peptide polynucleotide or IRES)-(optional inducible control sequence)-(optional first secreted factor or first selection marker polynucleotide)-(optional third self-cleaving peptide polynucleotide or IRES)-(optional second secreted factor or second selection marker polynucleotide)-(polyA terminator)-(right HA);

[0043] or

[0044] (left HA)-(SA)-(first self-cleaving peptide polynucleotide or IRES) (uPAR binding fragment polynucleotide)-(hinge domain polynucleotide)-(transmembrane domain polynucleotide)-(intracellular domain polynucleotide)-(optional second self-cleaving peptide polynucleotide or IRES)-(optional first secreted factor or first selection marker polynucleotide)-(optional third self-cleaving peptide polynucleotide or IRES)-(optional inducible control sequence)-(optional second secreted factor or second selection marker polynucleotide)-(polyA terminator)-(right HA);

[0045] wherein the left HA and the right HA are homology arms complementary to sequences on both sides of a cleavage site in the T cell expressed gene;

[0046] wherein SA is a splice acceptor site;

[0047] wherein the first, second and third self-cleaving peptide polynucleotide or IRES are polynucleotides encoding a first, second and third self-cleaving peptide or an internal ribosome entry site (IRES), respectively;

[0048] wherein the optional inducible control sequence is a regulatory sequence which provides control of protein expression in response to a small molecule inducer;

[0049] wherein the uPAR binding fragment polynucleotide is a polynucleotide encoding a polypeptide that specifically binds uPAR;

[0050] wherein the hinge domain polynucleotide encodes a CD28 or CD8α hinge domain;

[0051] wherein the transmembrane domain polynucleotide encodes a transmembrane domain;

[0052] wherein the intracellular domain polynucleotide encodes one or more intracellular domain(s);

[0053] wherein the first and second secreted factor polynucleotides are coding sequences for a neurotrophic factor, growth factor, or cytokine;

[0054] wherein the first and second selection marker polynucleotides are coding sequences for a detectable protein; and

[0055] wherein the polyA terminator is a sequence-based element that defines the end of a transcriptional unit. In an aspect, the DNA HDR template is virus-free. In another aspect, the virus-free DNA HDR template is double-stranded.

HA

[0056] As used herein, homology arms (HA) are homology arms are complementary to sequences on both sides of the cleavage site in the T cell expressed gene. The homology arms guide insertion of a synthetic DNA sequence into the T cell expressed gene by endogenous DNA repair of the double-stranded DNA cleavage induced by Cas9 RNP. The homology arms are 50 to 3000 nucleotides in length and are complementary to sequences on either side of the cut site in the T cell expressed gene to facilitate incorporation of the synthetic DNA sequence into the genome of the T cell. Small sequence variations (<100 bases) from complementary sequences could be included to enable barcoding or tracking of various cell types or to increase efficiencies of insertion of the synthetic DNA sequence.

[0057] In an aspect, the length of the homology arms influences the efficiency of synthetic DNA sequence integration. In an aspect, the homology arms are 400 to 1000 base pairs, specifically 450 to 750 base pairs long.

[0058] In an aspect, the left homology arm includes 383 to 588 bp of the TRAC locus directly upstream of the cutsite, and the right homology arm includes 391 to 499 bp of the TRAC locus directly downstream of the cutsite.

Splice Acceptor

[0059] The splice acceptor site (SA) assists in the splicing of the synthetic DNA sequence into the transcript generated from the endogenous T cell expressed gene. The site at the 3' end of an intron typically contains an SA. Therefore, after homology directed repair, the SA in the integrated sequence before the synthetic CAR gene assists in splicing in the CAR and downstream sequences into the endogenous transcript driven by the T cell expressed gene promoter (e.g., TRAC promoter).

Self-Cleaving Peptides or Ires

[0060] A self-cleaving peptide sequence, e.g., T2A, assists in the separation or cleavage of the translated peptide of the protein product encoded by the synthetic DNA sequence from the protein product of the native T cell expressed gene. Exemplary self-cleaving peptides sequences include viral 2A peptides such as a porcine teschovirus-1 (P2A) peptide, a *Thosaea asigna* virus (T2A) peptide, an equine rhinitis A virus (E2A) peptide, or a foot-and-mouth disease virus (F2A) peptide.

[0061] An internal ribosome entry site (IRES) is a site that provides initiation of translation from an internal region of the mRNA. An IRES provides co-expression of two proteins from the same mRNA.

Inducible Control Sequence

[0062] As used herein an inducible control sequence is a regulatory sequence which takes advantage of alternative RNA splicing to provide control of protein expression in response to a small molecule inducer. An exemplary inducible control sequence is Xon which is described in Monteys et al., "Regulated control of gene therapies by drug-induced splicing", *Nature*, 596, pp. 291-95 (2021). By using the Xon element upstream of our CAR sequence, transcription and subsequent translation of the uPAR binding fragment can be controlled using an oral dosing of the inducer drug treatment LMI070.

Upar Binding Fragment

[0063] uPAR is the receptor for urokinase-type plasminogen activator (uPA), which promotes the degradation of the extracellular matrix components. uPAR expression is increased in many human cancers. As described in Amor et al., "Senolytic CAR T cells reverse senescence-associated pathologies", *Nature*, 583, pp. 127-132 (2020), uPAR is induced on the surface of senescent cells. Amor also described uPAR specific CAR T cells prepared using retroviral vectors for the treatment of senescence-associated diseases. These cells drove expression of uPAR by retroviral promoters and did not modify the TRAC gene, resulting in intact TCR protein on the surface and intact signaling by the receptor.

[0064] As used herein, a uPAR binding fragment is a polynucleotide encoding a polypeptide that specifically binds uPAR. WO 2020/0160518, incorporated by reference

herein for its description of uPAR binding fragments and polypeptides, describes uPAR antigen binding fragments (e.g., scFv).

[0065] In an embodiment, the uPAR binding fragment is an extracellular antigen-binding domain (e.g., human scFv) comprising a heavy chain variable (VH) region and a light chain variable (VL) region, optionally linked with a linker sequence, for example a linker peptide, between the heavy chain variable (VH) region and the light chain variable (VL) region. In certain embodiments, the extracellular antigen-binding domain is a human scFv-Fc fusion protein or full length human IgG with VH and VL regions.

[0066] In certain non-limiting embodiments, the uPAR binding fragment of the presently disclosed CAR can comprise a linker connecting the heavy chain variable (VH) region and light chain variable (VL) region of the extracellular antigen-binding domain.

[0067] In an aspect, the uPAR binding fragment comprises a VHCDR1 sequence, a VHCDR2 sequence, and a VHCDR3 sequence of GFTFSNY (SEQ ID NO: 27), STGGGN (SEQ ID NO: 28), and QGGGYSDSFDY (SEQ ID NO: 29); or GFSLSTSGM (SEQ ID NO: 30), WWDDD (SEQ ID NO: 31), and IGGSSGYMDY (SEQ ID NO: 32) respectively. Additionally or alternatively, in some embodiments, the uPAR binding fragment (e.g., scFv) comprises a VLCDRI sequence, a VLCDR2 sequence, and a VLCDR3 sequence of KASKSISKYLA (SEQ ID NO: 33), SGSTLQS (SEQ ID NO: 34), and QQHNEYPLT (SEQ ID NO: 35); RASEVDSYGNFSFMH (SEQ ID NO: 36), RASNLLKS (SEQ ID NO: 37), and QQSNEDPWT (SEQ ID NO: 38); or KASENVVTYVS (SEQ ID NO: 39), GASNRYT (SEQ ID NO: 40), and GQGYSYPYT (SEQ ID NO: 41), respectively.

[0068] Additionally or alternatively, in some embodiments, the amino acid sequence of the VH of the uPAR binding fragment (e.g., scFv) is:

(SEQ ID NO: 42)

EVQLVESGGGLVQPGRSLKLSCAASGTFNSNYAMAWVRQA
PTKGLEWVASISTGGG NT YYRD S VKGRFTISRDNAK
NTL YLQMD SLRSEDT AT YYCARQGGYSD SFD YW
G QGVMVTVSS,
or

(SEQ ID NO: 43)

Q VTLKE S GPGILQP SQTLSLTCFSGESLSTS GMG
V GWIRQP S GKGL E WLAHI WWDD DKRYNPALKSRL
TISKDPSSNQVFLKIASVDTADIATYYCVRIGGSSGYMDY
WGQGT SVTVSS.

[0069] Additionally or alternatively, in some embodiments, the amino acid sequence of the VL of the uPAR binding fragment (e.g., scFv) is:

(SEQ ID NO: 44)

DVQMTQSPSNLAASPGESVSINCKASKSISKYLAWYQQKP
GKANKLLIYSGSTLQSG TPSRFSGSGS GTDFTLTIRNL
EPEDF GL YY CQ QHNE YPLTF GS GTKLEIKR,

-continued

(SEQ ID NO: 45)

DI VLT Q SP ASL AV SLGQRATI S CRASE S VD

S Y GN SFMHW YQQKPGQPPKLLI YRASNLS KSGIP

ARFSGSGSGTDFLTINPVEADDVATYCCQQSNEPDWTFG

GGTKLEIKR,

or

(SEQ ID NO: 46)

NIVMT Q SPKSMSMS VGERVTLT CKASENVVTYV SW

Y QQKPEQ SPKLLIY GASNRYT GVPDRFTGSGSATDFT

LTISSVQAEDLADYHCGQGYSYPPYTFGGGTKEIKR.

[0070] Additionally or alternatively, in some embodiments, the uPAR binding fragment (e.g., scFv) comprises an amino acid sequence selected from the group consisting of:

(SEQ ID NO: 47)

EVQLVESGGGLVQPGRSLKLSCAASGFTFSNYAMA WVRQA

PTKGLEWVASISTGGG NT YYRD S VKGRFTISRDNAK

NTL YLQMD SLRSEDT AT YYCARQGGGYSDF QMTO PSNLAA S

G OGVMVTVSSGGGGGGGGGGSDVQMTQSPSNLAAS

PGEVS SINCKASKSISKYL AWYQQKPGK ANKLLIY S

GS TLQS GTP SRF S GS GS GTDFLTIRNLEPEDF

GL YY C QQH NE YPLTF GSGTKLEIKR;

(SEQ ID NO: 48)

Q VTLKE S GPGILQP SQTLSLTCFSFGFSLSTS GMG

V GWIRQP S GKGL E WLAHI WWDD DKRYNPALKSRL

TISKDPSSNQFLKIASVDTADIATYYCVRIGGSSGYMDY

WGQGT SVTVSSGGGGGGGGGGSDIVLTQSPASLAV

SLGQRATISCR ASESVDSYGNF MHWYQQKPGQPPKLL

IYRASNLSKGIPARFSGSGSGTDFLTINPVEADDVATYC

CQ Q SNEDP WTFGGGTKEIKR;
and

(SEQ ID NO: 49)

Q VTLKE S GPGILQP SQTLSLTCFSFGFSLSTS GMG

V GWIRQP S GKGL E WLAHI WWDD DKRYNP ALKSR

LTI SKDP S SN Q VFLKI AS VDT ADI AT YY C

VRIGGS S GYMD YWGQGT S VT V S S GGGGS GG

GGS GGGGSNI VMT QSPKSMSMS VGERVTLT CK AS

ENVVT YY S W YQQKPEQSPKLLIY GASNRYT GVPDRF

GTGSGSATDFLTITISSVQAEDLADYHCGQGY S YP YT F

GGGTKEIKR.

[0071] In an aspect, the uPAR binding fragment (e.g., scFv) is encoded by a nucleic acid sequence such as:

(SEQ ID NO: 50)

GAAGTCCA ACTCGTTGAAAGCGGCCGGTGGCTTGTCAGC

CAGGCAGATCACTG AAAC TGT CATGC GCC CGC AGT GGC

TCACTTTCTCCA ATTAC GCA ATGGCGT GGG TT AGAC A

GGCCCC AC GA AAGG CT TG GAGT GGGT CGC ATC AAT

CAGT AC AGGAG GT GGAAAC ACTT ACT ATCGCGA

T AGT GTTAAGGGGAGATTC ACGATTAGCCGG AC A

ACCGC AAAAAC AC GTTGTATCTGC AGATGGACTC ACT

T AGATCGAGGAC A C AGC GACTT ACT ACT GTGCG

AGGC AGGGCGGAGGGT AT AGT GAT AGCTTT GATT

ACTGGGCCAGGGCGTAATGGTA ACT GTTAGTCTGGTGG

AGGTGGATCAGGTG GAGGTGGATCTGGTGGAGGTGGATC

TGATGTGAGATGACACAGAGTCCTCAAATTGGCCGCT

TCACCCGGAGAATCAGTAAGTATCAACTGTAAAGCGTCCA

AGTCC ATTTC AAAGT ATTTGGC ATGGTAT C AAC A

GAAGCCGGAAAGGCGAAC AAAC TCTGATTATAGCGG

GAGTACCTTGCA GTCCGGCACCC TAGTAGATTTTCAGGC

TCCGGTTCTGGGACCGACTTC ACTTTGACGATTGCA ATT

TGGAACCAGAGGATT TGGCTGTACTATTGTCAGCAGCA

CAACGAATACCCGTTGACTTTGGTAGTGGTACAAAGCTG

GAAATCAAGAGAGCGGCC;

(SEQ ID NO: 51)

CAGGTGACCTGAGAGTCCGGCCGGCAT CCTGCAGC

CCAGCCAGACCTGAGCCTGACCTGCTCCTCAGCGGCTT

CTCCCTGTCCACCTCCGGCATGGCGTGGCTGGATCAGA

CAGCCCAGCGGAAGGGCTGGAGTGGCTGGCCACATCT

GGT GGGACGATGACAAGAGATA CAACCCGCTCTGAAGA

GCCGGCTGACATCAGCAAGGACCC TAGCAGTAACCAAGGT

GTT CCTGAAGATCGCTCCGTGGACACAGCAGACATCGCA

ACATACTATTGCGTGC GGATCCGGAAAGCAGTGGATACA

TGGACTACTGGGAC AGGGAAAC AGCGTGACCGT GAG

CAGT GGT GGAGGT GGAT CAGGTGGAGGTGGATCTGG

TGGAGGTGGATCTGACATCGTGTGACCCAGAGCCAGCT

AGCTTGGCAGT GAGCCTGGGACAGAGGGCTACCATCAGCT

GCAGAGCTTCAGAGAGCGTGGACAGCTACGGAAACAGCTT

CATGCACTGGTACCCAGCAGAAAGCCAGGACAGCC ACCT A

AGCT GCTGATCT ACCGGGCT AGC AACCT GAAGTCC

GGAAATCCCTGCTCGTTAGCGGAAGCGGTAGCGGCACCG

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ACTTCACCCCTGACAATCAACCCAGTGGAGGCCGACGATGT
GGC AACCT ACTGCTGT C AGC AGAGC AACGAGGA
CCC AT GGACCTTCGGCGGT GGAACC AAACT GGAGA
T CAAGAGA;
and

(SEQ ID NO: 52)
CAGGTGACCCCTGAAGGAGTCGGCCCCGGCATCCTGCAGC
CCAGGCCAGACCCCTGAGCTGACCTGCTCCTTCAGCGGCTT
CTCCCTGTCACCTCCGGCATGGCGTGGCTGGATCAGA
CAGCCCCAGCGCAAGGGCCTGGAGTGGCTGGCCACATCT
GGTGGGACGATGACAAGAGATAAACCCGCTCTGAAGAG
CCGGCTGACAATCAGCAAGGACCCTAGCAGTAACCAGGTG
TTCCTGAAGAGATCGCTTCCGTGGACACAGCAGACATCGCAA
CATACTATTGCGTGCAGGATCGGGAGCAGTGAGTGGATACAT
GGACTACTGGGACAGGGAACCGCGTGACCGTGAGCAGT
GGTGGAGGTGGATCAGGTGGAGGTGGATCTGGTGGAGGTG
GATCTAACATCGTGATGACCCAGTCCCCATAAGAGCATGAG
CATGAGCGTGGCGAGAGAGTGACCCCTGACCTGCAAGCC
TCCGAGAACGTGGTGACCTACGTGAGCTGGTACCGAGA
AGCCTGAGCAGAGCCCTAACGCTGCTGATCTACGGCGCTTC
CAACAGATACACCGGAGTGCCTGACAGATTACCCGGCAGC
GGAAGCGCAACCGACTTCACCTTGACCATCAGCAGCGTGC
AGGCTGAGGACCTGGCCGACTACCACTGCGCCAGGGCTA
CAGCTACCCCTACACCTTCGGTGGAGGCACCAAGCTGGAG
ATCAAGCGG.

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[0072] Additionally or alternatively, in some embodiments, the uPAR binding fragment (e.g., scFv) is encoded by a nucleic acid sequence that has at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to any one of SEQ ID NOS: 36-38. In some embodiments, the uPAR binding fragment (e.g., scFv) is encoded by a nucleic acid that is about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOS: 50-52.

[0073] In some embodiments, the chimeric antigen receptor comprises a uPAR binding fragment (e.g., a uPA fragment) comprising the amino acid sequence:

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(SEQ ID NO: 53)
MRALL ARLLLC VLVV SD SKGSNELHQ VP SN CDC
LN GGT C V SNKYFSNIHW CN CPKKFGQHCEIDKS
KTCYEGNNGHFYRGKASTDTMGRPCLPWNSATVLQQTYAH
RSDA LQLGLGKHNY CRNPDNRRRP W C YV Q V GL
KPL V QECMVHDCADGKKP;

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-continued

or

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(SEQ ID NO: 54)
MRALL ARLLLC VLVV SD SKGSNELHQ VP SN CDC
LN GGT C V SNKYFSNIHW CN CPKKFGQHCEIDKS
KTCYEGNNGHFYRGKASTDTMGRPCLPWNSATVLQQTYAH
RSDA LQLGLGKHNY CRNPDNRRRP W.

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[0074] Additionally or alternatively, in some embodiments, the uPAR binding fragment (e.g., uPa fragment) comprises an amino acid sequence that has at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 53 or SEQ ID NO: 54. In some embodiments, the uPAR binding fragment (e.g., uPa fragment) comprises an amino acid sequence that is about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 53 or SEQ ID NO: 54.

[0075] Additionally or alternatively, in some embodiments, the uPAR binding fragment (e.g., a uPAR fragment) is encoded by a nucleic acid sequence:

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(SEQ ID NO: 55)
ATGAGAGCCCTGCTGGCGCGCTGCTTCTCTGCGTCTGG
TCGTGAGCGACTCCA AAGGC AGC AAT GAACTTC AT
C AAGTTCC ATCGAACTGT GACTGTCTAAATGGAGGAA
CATGTGTGTCACAAAGTACTTCTCCAACATTCACTGGTG
CAACTGCCAAA GAAATTCGAGGGC AGC ACTGT GA
AAT AG AT AAGTCAAAACCTGCT ATGAGGGGAATGG
TCACCTTACCGAGGAAAGGCCAGCACTGACACCATGGGC
CGGCCCTGCCTGCCCTGGAACTCTGCCACTGTCCCTCAGC
AACCGTACCATGCCACAGATCT GAT GCTCTTC AGCT
GGCCTGGGAAAC AT AATT ACTGC AGGAACCC AG
AC AAC CGGAGGCGACCCCTGGTGCTAT GT GC AGGT
GGGCCT AAAGCGCTTGCC AAGAG T GC AT GGT
GC ATGACTGCC AGAT GAAAAAAAGCCC;
or

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(SEQ ID NO: 56)
ATGAGAGCCCTGCTGGCGCGCTGCTTCTCTGCGTCTGG
TCGTGAGCGACTCCA AAGGC AGC AAT GAACTTC AT
C AAGTTCC ATCGAACTGT GACTGTCTAAATGGAGGAA
CATGTGTGTCACAAAGTACTTCTCCAACATTCACTGGTG
CAACTGCCAAA GAAATTCGAGGGC AGC ACTGT GA
AAT AG AT AAGTCAAAACCTGCT AT GAGGGGAATGG
TCACCTTACCGAGGAAAGGCCAGCACTGACACCATGGGC
CGGCCCTGCCTGCCCTGGAACTCTGCCACTGTCCCTCAGC
AACCGTACCATGCCACAGATCT GAT GCTCTTC AGCT
GGCCTGGGAAAC AT AATT ACTGC AGGAACCC A
GAC AAC CGGAGGCGACCCCTGG

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[0076] Additionally or alternatively, in some embodiments, the uPAR binding fragment is encoded by a nucleic acid sequence that has at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to any one of SEQ ID NOs: 55 or 56. In some embodiments, the uPAR binding fragment is encoded by a nucleic acid that is about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOs: 55 or 56.

[0077] In an aspect, the uPAR binding fragment is an antibody fragment. As used herein, the term “single-chain variable fragment” ~ or “scFv” is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of an immunoglobulin (e.g., mouse or human) covalently linked to form a VH:VL heterodimer. The heavy (VH) and light chains (VL) are either joined directly or joined by a peptide-encoded linker (e.g., about 10, 15, 20, 25 amino acids), which connects the N-terminus of the VH with the C-terminus of the VL, or the C-terminus of the VH with the N-terminus of the VL. The linker is may be rich in glycine for flexibility, as well as serine or threonine for solubility. The linker can link the heavy chain variable region and the light chain variable region of the extracellular antigen binding domain. In certain embodiments, the linker comprises amino acids having the sequence GGGGS GGGGS GGGGS (SEQ ID NO: 57).

[0078] A specific uPAR binding fragment includes a heavy chain variable fragment and a light chain variable fragment, optionally connected by a linker (SEQ IDs 40-41).

Car Domains

[0079] Typically, the antigen-specific extracellular domain (uPAR binding fragment) is linked to the intracellular domain of the CAR by a transmembrane domain, e.g., derived from a CD4, CD8 α , CD28, IgG and/or or CD3zeta transmembrane domain. The transmembrane domain traverses the cell membrane, anchors the CAR to the T cell surface, and connects the extracellular domain to the intracellular signaling domain, thus impacting expression of the CAR on the T cell surface. The uPAR binding fragment is linked to the intracellular domain by a hinge domain such as a CD28 or CD8 α hinge domain. The hinge domain provides flexibility to the uPAR binding fragment and improves efficacy. CARs may also further comprise one or more costimulatory domain and/or one or more spacer. A costimulatory domain is derived from the intracellular signaling domains of costimulatory proteins that enhance cytokine production, proliferation, cytotoxicity, and/or persistence in vivo. A spacer or hinge connects (i) the antigen-specific extracellular domain to the transmembrane domain, (ii) the transmembrane domain to a costimulatory domain, (Hi) a costimulatory domain to the intracellular domain, and/or (iv) the transmembrane domain to the intracellular domain. For example, inclusion of a spacer domain (e.g., IgG1, IgG2, IgG4, CD28, CD8) between the antigen-specific extracellular domain and the transmembrane domain may affect flexibility of the antigen-binding domain and thereby CAR function. Transmembrane domains, costimulatory domains, and spacers are known in the art. Exemplary costimulatory domains include OX40, 41BB, ICOS, CD27, CD40, CD40L or a TLR.

Secreted Factors

[0080] The first and second secreted factors are a coding sequence for a neurotrophic factor or cytokine. Secreted

factors can include neuroprotective, pro-regenerative secreted factors such as APOE2, sAPPa; pro-memory secreted factors such as IL-4, IL-10; growth factors like BDNF, NGF; factors that attract pro-regenerative immune cells such as IL-1, IL-6, TNF-alpha, IFN-gamma; and the like. Exemplary secreted factors include a pro-regenerative secreted factor, a pro-memory secreted factor, growth factor, or a factor that attracts pro-regenerative immune cells

SELECTION MARKERS

[0081] In an aspect, the synthetic DNA sequence comprises a coding sequence for a selection marker which can be selectable cell surface receptors such as truncated NGFR (tNGFR) or a fluorescent protein such as mCherry, mKate, GFP, BFP, RFP, CFP, YFP, mCyan, mOrange, tdTomato, mBanana, mPlum, mRaspberry, mStrawberry, and mTangerine.

PolyA Terminator

[0082] The polyadenylation (polyA) terminator is a sequence-based element that defines the end of a transcriptional unit within the synthetic DNA sequence and initiate the process of releasing the newly synthesized RNA from the transcription machinery. Exemplary polyA terminators are rabbit beta-globin polyA and a bovine growth hormone polyA.

[0083] FIG. 1A is a schematic of an anti-huPAR-CAR-2A-mCh targeting strategy. FIG. 3 is a schematic of an anti-muPAR-CAR-2A-NGFR targeting strategy. muPAR is the scFv VH and VL of mouse anti-uPAR binding fragment with a CD8 linker. The CD28 hinge and transmembrane domain were used. The zeta chain is a CD3 zeta chain. NGFR is a truncated nerve growth factor affinity receptor. mCh is the mCherry fluorescent protein.

[0084] Exemplary sequences of the present disclosure include the following:

- [0085]** SEQ ID NO: 1—DNA: muPAR.h28z tNGFR
- [0086]** SEQ ID NO: 2—protein: muPAR.h28z tNGFR
- [0087]** SEQ ID NO: 3—DNA: huPAR.h28z tNGFR
- [0088]** SEQ ID NO: 4—protein: huPAR.h28z tNGFR
- [0089]** SEQ ID NO: 5 DNA: huPAR.h28z mCherry
- [0090]** SEQ ID NO: 6 protein: huPAR.h28z mCherry
- [0091]** SEQ ID NO: 7 DNA: muPAR.m28z tNGFR
- [0092]** SEQ ID NO: 8 protein: muPAR.m28z tNGFR
- [0093]** SEQ ID NO: 9 DNA: muPAR.h28z mCherry
- [0094]** SEQ ID NO: 10 protein muPAR.h28z mCherry
- [0095]** SEQ ID NO: 11 DNA: muPAR.m28z mCherry
- [0096]** SEQ ID NO: 12 protein: muPAR.m28z mCherry
- [0097]** SEQ ID NO: 13 DNA: muPAR.m28z APOE2
- [0098]** SEQ ID NO: 14 protein: muPAR.m28z APOE2
- [0099]** SEQ ID NO: 15 DNA: huPAR.h28z APOE2
- [0100]** SEQ ID NO: 16 protein: huPAR.h28z APOE2
- [0101]** SEQ ID NO: 17 DNA: miniXon huPAR.h28z mCherry
- [0102]** SEQ ID NO: 18 protein: miniXon huPAR.h28z mCherry
- [0103]** SEQ ID NO: 19 DNA: miniXon muPAR.m28z mCherry
- [0104]** SEQ ID NO: 20 protein: miniXon muPAR.m28z mCherry
- [0105]** SEQ ID NO. 21 DNA: miniXon muPAR.m28z APOE2
- [0106]** SEQ ID NO. 22 protein: miniXon muPAR.m28z APOE2

[0107] SEQ ID NO: 23 DNA: miniXon huPAR.h28z
APOE2

[0108] SEQ ID NO: 24 protein: miniXon huPAR.h28z
APOE2

[0109] SEQ ID NO: 25 DNA: muPAR.h28z APOE2

[0110] SEQ ID NO: 26 protein: muPAR.h28z APOE2

Plasmids

[0111] Also included herein is a plasmid comprising the virus-free double-stranded HDR template described herein. Exemplary plasmids are non-viral expression vectors such as pUC57 and pUC57-Mini.

Genomic Integration of the Car Expressing the Upar Binding Fragment Polynucleotide

[0112] In a gene editing method, guide RNAs direct Cas9 nuclease to create a double stranded DNA break at the target locus. DNA repair involving the DNA template containing the synthetic CAR sequence then allows the integration of the CAR described herein into T cells to provide genome-edited T-cells.

[0113] In an aspect, an ex vivo, virus-free method of site-specifically inserting a transgene containing a chimeric antigen receptor (CAR) gene into a T cell expressed gene to generate CAR T cells comprises

[0114] preparing the virus-free homology-directed repair (HDR) template described above,

[0115] introducing into a population of unmodified T cells a Cas9 ribonucleoprotein (RNP) and the HDR template to provide the CAR T cells,

[0116] wherein the Cas9 RNP comprises a Cas9 protein and a guide RNA that directs double stranded DNA cleavage of a cleavage site in the T cell expressed gene, and

[0117] wherein the transgene is specifically integrated into the cleavage site of the T cell expressed gene locus created by the Cas9 RNP in the cells, and

[0118] culturing the CAR T cells in xeno-free medium to provide a cultured population of CAR T cells having the transgene specifically integrated in the T cell expressed gene,

[0119] wherein, in the cultured population of CAR T cells, an endogenous promoter of the T cell expressed gene drives expression of the transgene, or wherein the transgene includes a promoter that drives expression of the transgene, and

[0120] wherein the CAR gene encodes a fusion protein comprising the translated anti-uPAR binding motif, hinge, transmembrane domain, and intracellular domain.

[0121] As used herein, "introducing" means refers to the translocation of the Cas9 ribonucleoprotein and a DNA template from outside a cell to inside the cell, such as inside the nucleus of the cell. Introducing can include transfection, electroporation, contact with nanowires or nanotubes, receptor mediated internalization, translocation via cell penetrating peptides, liposome mediated translocation, transduction with putative non-integrating viruses (e.g., adeno-associated virus, AAV), viral-like particles (VLPs), and the like.

[0122] Unmodified T cells include autologous T cells that are collected from a patient, such as a cancer patient, by peripheral blood draw or leukapheresis. Unmodified T cells can also include T cells from allogeneic healthy donors or

induced pluripotent stem cells which can be used to produce universal T cells for administration to a patient. T cells are generally modified ex vivo, that is outside of the patient, and then the modified T cells such as CAR T cells are returned to the patient, such as by intravenous infusion, subcutaneous, intratumoral, intraperitoneal or intravenous or intracerebroventricular infusion or intracerebral injection.

[0123] Genome editing of the T cells as described herein uses a CRISPR system, or Cas9 ribonucleoprotein. CRISPR refers to the Clustered Regularly Interspaced Short Palindromic Repeats type II system used by bacteria and archaea for adaptive defense. This system enables bacteria and archaea to detect and silence foreign nucleic acids, e.g., from viruses or plasmids, in a sequence-specific manner. In type II systems, guide RNA interacts with Cas9 and directs the nuclease activity of Cas9 to target DNA sequences complementary to those present in the guide RNA. Guide RNA base pairs with complementary sequences in target DNA. Cas9 nuclease activity then generates a double-stranded break in the target DNA.

[0124] CRISPR/Cas9 is a ribonucleoprotein (RNP) complex. CRISPR RNA (crRNA) includes a 20 base protospacer element that is complementary to a genomic DNA sequence as well as additional elements that are complementary to the transactivating RNA (tracrRNA). The tracrRNA hybridizes to the crRNA and binds to the Cas9 protein, to provide an active RNP complex. Thus, in nature, the CRISPR/Cas9 complex contains two RNA species.

[0125] Guide RNA, or gRNA, can be in the form of a crRNA/tracrRNA two guide system, or an sgRNA single guide RNA. The guide RNA is capable of directing Cas9-mediated cleavage of target DNA. A guide RNA thus contains the sequences necessary for Cas9 binding and nuclease activity and a target sequence complementary to a target DNA of interest (protospacer sequence).

[0126] As used herein, a guide RNA protospacer sequence refers to the nucleotide sequence of a guide RNA that binds to a target genomic DNA sequence and directs Cas9 nuclease activity to a target DNA locus in the genome of the T cell such the TRAC gene, a T cell receptor beta subunit constant gene (TRBC), AAVS1 (i.e., PPP1R12C), TET2, FAS, BID, CTLA4, PDCD1, CBLB, PTPN6, CIITA and B2M genes. In some embodiments, the guide RNA protospacer sequence is complementary to the target DNA sequence. "Complementary" or "complementarity" refers to specific base pairing between nucleotides or nucleic acids. Base pairing between a guide RNA and a target region in exon 1 of the TRAC gene can be via a DNA targeting sequence that is perfectly complementary or substantially complementary to the guide RNA. As described herein, the protospacer sequence of a single guide RNA may be customized, allowing the targeting of Cas9 activity to a target DNA of interest.

[0127] Any desired target DNA sequence of interest may be targeted by a guide RNA target sequence. Any length of target sequence that permits CRISPR-Cas9 specific nuclease activity may be used in a guide RNA. In some embodiments, a guide RNA contains a 20 nucleotide protospacer sequence.

[0128] In addition to the protospacer sequence, the targeted sequence includes a protospacer adjacent motif (PAM) adjacent to the protospacer region which is a sequence recognized by the CRISPR RNP as a cutting site. Without wishing to be bound to theory, it is thought that the only requirement for a target DNA sequence is the presence of a protospacer-adjacent motif (PAM) adjacent to the sequence

complementary to the guide RNA target sequence. Different Cas9 complexes are known to have different PAM motifs. For example, Cas9 from *Streptococcus pyogenes* has a NGG trinucleotide PAM motif; the PAM motif of *N. meningitidis* Cas9 is NNNNGATT; the PAM motif of *S. thermophilus* Cas9 is NNAGAAW; and the PAM motif of *T. denticola* Cas9 is NAAAAC.

[0129] A “Cas9” polypeptide is a polypeptide that functions as a nuclease when complexed to a guide RNA, e.g., an sgRNA or modified sgRNA. That is, Cas9 is an RNA-mediated nuclease. The Cas9 (CRISPR-associated 9, also known as Csn1) family of polypeptides, for example, when bound to a crRNA:tracrRNA guide or single guide RNA, are able to cleave target DNA at a sequence complementary to the sgRNA target sequence and adjacent to a PAM motif as described above. Cas9 polypeptides are characteristic of type II CRISPR-Cas systems. The broad term “Cas9” Cas9 polypeptides include natural sequences as well as engineered Cas9 functioning polypeptides. The term “Cas9 polypeptide” also includes the analogous Clustered Regularly Interspaced Short Palindromic Repeats from *Prevotella* and *Francisella* 1 or CRISPR/Cpf1 which is a DNA-editing technology analogous to the CRISPR/Cas9 system. Cpf1 is an RNA-guided endonuclease of a class II CRISPR/Cas system. This acquired immune mechanism is found in *Prevotella* and *Francisella* bacteria. Additional Class I Cas proteins include Cas3, Cas8a, Cas5, Cas8b, Cas8c, Cas 10d, Case1, Cse 2, Csy 1, Csy 2, Csy 3, GSU0054, Cas 10, Csm 2, Cmr 5, Cas10, Csx11, Csx10, and Csf 1. Additional Class 2 Cas9 polypeptides include Csn 2, Cas4, C2c1, C2c3 and Cas13a.

[0130] Exemplary Cas9 polypeptides include Cas9 polypeptide derived from *Streptococcus pyogenes*, e.g., a polypeptide having the sequence of the Swiss-Prot accession Q99ZW2 (SEQ ID NO: 58); Cas9 polypeptide derived from *Streptococcus thermophilus*, e.g., a polypeptide having the sequence of the Swiss-Prot accession G3ECR1 (SEQ ID NO: 59); a Cas9 polypeptide derived from a bacterial species within the genus *Streptococcus*; a Cas9 polypeptide derived from a bacterial species in the genus *Neisseria meningitidis* (e.g., GenBank accession number YP_003082577; WP_015815286.1 (SEQ ID NO: 60)); a Cas9 polypeptide derived from a bacterial species within the genus *Treponema denticola* (e.g., GenBank accession number EMB41078 (SEQ ID NO: 61)); and a polypeptide with Cas9 activity derived from a bacterial or archaeal species. Methods of identifying a Cas9 protein are known in the art. For example, a putative Cas9 protein may be complexed with crRNA and tracrRNA or sgRNA and incubated with DNA bearing a target DNA sequence and a PAM motif.

[0131] The term “Cas9” or “Cas9 nuclease” refers to an RNA-guided nuclease comprising a Cas9 protein, or a fragment thereof (e.g., a protein comprising an active, inactive, or partially active DNA cleavage domain of Cas9, and/or the gRNA binding domain of Cas9). In some embodiments, a Cas9 nuclease has an inactive (e.g., an inactivated) DNA cleavage domain, that is, the Cas9 is a nickase. Other embodiments of Cas9, both DNA cleavage domains are inactivated. This is referred to as catalytically-inactive Cas9, dead Cas9, or dCas9.

[0132] Functional Cas9 mutants are described, for example, in US20170081650 and US20170152508, incorporated herein by reference for its disclosure of Cas9 mutants.

[0133] As used herein, the term editing refers to a change in the sequence of the genome at a targeted genomic location. Editing can include inducing either a double stranded break or a pair of single stranded breaks in the genome, such as in a T cell expressed gene. Editing can also include inserting a synthetic DNA sequence into the genome of the T cell at the site of the break(s).

[0134] As used herein, a Cas9 RNP that targets a T cell expressed gene comprises a Cas9 protein and a guide RNA that directs double stranded DNA cleavage of the T cell expressed gene. The guide RNA thus includes a crRNA comprising a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide, wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide. The single-stranded protospacer region of the guide RNA hybridizes to a sequence in the T cell expressed gene, directing cleavage of the T-cell expressed gene to a specific locus of the T cell expressed gene.

[0135] Exemplary T cell expressed genes which can be cleaved by the methods described herein include the AAVS1 (i.e., PPP1R12C), TET2, FAS, BID, CTLA4, PDCD1, CBLB, PTPN6, CIITA, B2M, TRAC and TRBC genes, specifically TRAC. The T cell expressed gene-targeted by Cas9 ribonucleoprotein may result in a reduction or elimination of expression of functional TRAC gene product (e.g., knockout of expression of functional TRAC gene product).

[0136] In an aspect, the T cell expressed gene is TRAC and wherein the guide RNA targets the 5' end of the first exon of TRAC. An exemplary guide RNA useful to target the first encoding exon of TRAC comprises SEQ ID NO: 62; CAGGGTTCTGGATATCTGT or SEQ ID NO: 63; GGGAGTCAAAGTCGGTGAAC

[0137] In addition to the Cas9 RNP, the virus-free double-stranded HDR template comprising the synthetic DNA sequence is introduced into the T cells.

[0138] The genome-edited T cells are then cultured in in xeno-free medium to provide a cultured population of T cells having the synthetic DNA sequence specifically integrated in the T-cell expressed gene locus. The term “xeno” comes from the Greek “*xenos*” meaning strange. Xeno-free (or xenogeneic-free) therefore means free from “strange” components, or components from a “strange” species (strange being relative to the native species you’re working with). In terms of cell culture, this would mean human cell lines can be cultured using human-derived components (like human serum), and it is considered xeno-free, since there is no difference between species.

[0139] As used herein culturing the genome-edited T cells in xeno-free medium can include recovery from integration of the synthetic DNA sequence and/or expansion of the edited T cell population.

[0140] In an aspect, the CAR T cells produced by the methods described herein have activity against a neurodegenerative disease, stroke, craniocerebral trauma and/or accident, or an elderly patient in need of treatment for aging, for example. Thus, the methods further comprise administering the cultured population of CAR T cells to a patient in need of treatment for a neurodegenerative disease, stroke, craniocerebral trauma and/or accident, or an elderly patient in need of treatment for aging. Exemplary neurodegenera-

tive diseases include Alzheimer's disease, dementia, Parkinson's disease, Lewy body disease, ataxia, Huntington's disease, amyotrophic lateral sclerosis, Down syndrome, and spinal muscular atrophy.

[0141] In an aspect, administering the CAR T cells is by intravenous or intracerebroventricular infusion of intracerebral injection.

[0142] The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Methods

[0143] Cell lines: Primary Human Dermal Fibroblasts adult (HDFa) were purchased from ATCC and maintained in Dulbecco's Modified Eagle Medium high glucose (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% penicillin-streptomycin. For drug-induced senescence experiments, trametinib (S2673) and palbociclib (S1116) were purchased from Selleck Chemicals and dissolved in DMSO to yield 10 mM stock solutions, which were stored at -80° C. Cells were treated with MEK inhibitor (25 nM) and CDK4/6 inhibitor (500 nM). The cells were induced for 48 hours, the growth medium was then changed every two days. Cortical Glutamatergic GFP+ Neurons were purchased from BrainXell. These cells were maintained in 50% Dulbecco's Modified Eagle Medium Nutrient Fixture F-12 (Gibco) and 50% Neurobasal Medium (Gibco) supplemented with 2% B27 Supplement (Thermofischer), 1% N2 Supplement (Thermofischer), 0.5 mM Glutamax™ (Gibco), BDNF 10 ng/mL (Peprotech), 10 ng/mL GDNF (Peprotech), 1 ng/mL TGF-β1 (Peprotech), Geltrex® 15 µg/mL (Thermofischer), Neuron Seeding Supplement Day 1 1× (BrainXell), Supplement K 1× (BrainXell). For drug-induced senescence experiments 300 µM Hydrogen Peroxide (Sigma Aldrich) was added to the neuron cultures for 2 hours to induce oxidative stress. After incubation, media was taken off of the cells and replaced with normal glutamatergic neuron culture. Cell lines were maintained in culture at 37° C. in 5% CO₂ and tested negative for *mycoplasma*.

[0144] Isolation of primary T cells from healthy donors: This study was approved by the Institutional Review Board of the University of Wisconsin-Madison (#2018-0103), and informed consent was obtained from all donors. Peripheral blood was drawn from healthy donors into sterile syringes containing heparin and transferred to sterile 50 mL conical tubes. Primary human T cells were isolated using RosetteSep™ Human T Cell Enrichment Cocktail (STEMCELL Technologies). T cells were counted using a Countess™ II FL Automated Cell Counter (Thermo Fisher Scientific) with 0.4% Trypan Blue viability stain (Thermo Fisher Scientific) at a 1:1 dilution. T cells were cultured at a final density of 1 million cells/mL in ImmunoCult™—XF T cell Expansion Medium (STEMCELL) supplemented with 200 U/mL IL-2 (Peprotech) and stimulated with ImmunoCult™ Human CD3/CD28/CD2 T cell Activator (STEMCELL) immediately after isolation, per the manufacturer's instructions.

[0145] T cell culture: T cells were cultured in ImmunoCult™—XF T cell Expansion Medium at a density of 1 million cells/mL and stimulated with ImmunoCult™ Human CD3/CD28/CD2 T cell Activator (STEMCELL) for 48 hours prior to electroporation. After 24 hours post-electroporation, VFC T cells were transferred without centrifugation to 1 mL of fresh culture medium with 500 U/mL IL-2. T cells

were passaged, counted, and adjusted to 1 million/mL in fresh medium+IL-2 on days 5, 7, 9, 11, and 14 after isolation.

[0146] Double-stranded DNA HDR template production: Plasmids were generated by Genscript by inserting CAR constructs into a pUC57 vector. VFC-huPAR.28z-2A-mCherry (also termed VFC-huPAR-mCh) and VFC-mCherry (also termed VFC-mCh) plasmids were transformed in 5-alpha competent *E. coli* (NEB) and purified using the PureYield™ MiniPrep system (Promega). PCR amplicons were generated from plasmid templates using Q5® Hot Start Polymerase (NEB) and pooled into 600 µl reactions for Solid Phase Reversible Immobilization (SPRI) cleanup (6×) using AMPure XP beads according to the manufacturer's instructions (Beckman Coulter). Each of the 600 µl starting products was eluted into 30 µl of water. Bead incubation and separation times were increased to 5 minutes, and elution time was increased to 15 minutes at 37° C. to improve overall yield. PCR products from round 1 cleanup were pooled and subjected to an ethanol precipitation to increase total concentration. Template concentration and purity was quantified using a IMPLEN NanoPhotometer® N50. Concentrated template products were diluted in Ultra-Pure H2O at a concentration of 2.5 µg/µl according to Nanodrop™ measurements.

[0147] SpCas9 RNP preparation: RNPs were produced by complexing a two-component gRNA to SpCas9. In brief, tracrRNA and crRNA were ordered from IDT, suspended in nuclease-free duplex buffer at 100 µM, and stored in single-use aliquots at -80° C. tracrRNA and crRNA were thawed, and 4.15 µl of each component was mixed 1:1 by volume and annealed by incubation at 37° C. for 30 minutes to form a 50 µM gRNA solution in individual aliquots for each electroporation replicate. Recombinant sNLS-SpCas9-sNLS Cas9 (Aldevron, 10 mg/ml, total 3.33 µl) was added to the complexed gRNA at a 1.2:1 molar ratio and incubated for 15 minutes at 37° C. to form an RNP. Individual aliquots of RNPs were incubated for at least 30 seconds at room temperature with HDR templates for each sample prior to electroporation.

[0148] T cell nucleofection: Following guidance from the protocols in the art, RNPs and HDR templates were electroporated 2 days after T cell isolation and stimulation. During crRNA and tracrRNA incubation, T cells were centrifuged for 3 minutes at 200 g and counted using a Countess™ II FL Automated Cell Counter with 0.4% Trypan Blue viability stain (Thermo Fisher). 4.13 million T cells were aliquoted and centrifuged for 10 min at 90 g. During cell spin, 8.33 µl of HDR template (total 16.66 µg) per condition were aliquoted to PCR tubes, followed by RNPs (11.66 µl per well) and were incubated for at least 5 minutes. After cell centrifugation, supernatants were removed by vacuum, and cells were resuspended in 80 µl P3 buffer (Lonza), then transferred to PCR tubes containing RNPs and HDR templates, bringing the total volume per sample to 100 µl. Each sample was transferred directly to a 100 µL Nucleocuvette™ Vessel. T cells were electroporated with a Lonza 4D Nucleofector™ with X Unit using pulse code EH115. Immediately after nucleofection, 100 µl of pre-warmed recovery medium with 500 U/mL IL-2 and 25 µl/mL ImmunoCult™ CD3/CD28/CD2 activator was added to each cuvette. Cuvettes were rested at 37° C. in the cell culture incubator for 15 minutes. After 15 minutes, cells

were moved to 200 μ l total volume of recovery media and equally distributed to 4 wells round bottom 96 well plate. [0149] Flow cytometry Analysis: T cells were stained and analyzed on day 7 of manufacture for mCherry and TCR expression. Ghost DyeTM Red780 was used as a live dead stain to access cell viability. TCR a/b antibody clone IP26 was used to detect TCR knockout in BD Brilliant Stain Buffer (BD Biosciences). All stained samples were run on an AttuneTM NxT Flow cytometer (Thermo Fisher Scientific). T cells were stained and analyzed on day 7 of manufacture for mCherry and TCR expression, and day 10 of manufacture for the full Aurora immunophenotyping panel, using fresh cells. Downstream analyses of all spectral cytometry data were performed in FCS Express 7 Software.

[0150] In-out PCR: Following guidance from the art, genomic DNA was extracted from 100,000 cells per condition using DNA QuickExtractTM (Lucigen), and incubated at 65° C. for 15 min, 68° C. for 15 min, and 98° C. for 10 min. Genomic integration of the CAR was confirmed by in-out PCR using a forward primer upstream of the TRAC left homology arm, and a reverse primer binding within the CAR sequence. (ATCTTGTGCGCATGTGAGGGGC (SEQ ID NO: 64) and GCAAGCCAGGACTCCACCAACC (SEQ ID NO: 65)). PCR was performed according to the manufacturer's instructions using Q5TM Hot Start Polymerase (NEB) using the following program: 98° C. (30 s), 35 cycles of 98° C. (10 s), 67° C. (20 s), 72° C. (2 min), and a final extension at 72° C. (2 min).

[0151] In Vitro Cytotoxicity Assays: For FIG. 2: 10,000 HDFa fibroblasts at varying passage numbers cells were seeded in triplicate per condition in a CytoView-Z 96 Well plate (Axion Biosystems) and maintained in the Maestro Z (Axion Biosystems) stored at 37° C., 5% CO₂. Cell viability and impedance was tracked continuously for 24 hours and then treated with CDK4/6 and MEK media additives for 48 hours. VFC T cells were added to each well with varying effector: target ratios based to reach 100%, 50%, 25% CAR positivity. Cytotoxicity was measured every hour for 48 hrs and data output was imported and analyzed with AXIS software.

[0152] SA- β -Gal Staining: SA- β -gal staining was performed using CHEMICON[®] Cellular Senescence Assay Kit (cat. KAA002 Millipore Sigma) at a pH 6.0 for human cells. Adherent cells plated in a 12 well plate and fixed with 500 μ l Fixing solution (Millipore Sigma) and incubated at room temperature for 10 minutes, washed twice with 1xPBS and stained with freshly prepared 1xSA- β -gal Detection Solution (Millipore Sigma) at 37° C., without CO₂ and protected from the light and left overnight. The SA- β -gal Detection Solution was removed and the cells were washed with twice with 1xPBS. Blue stained cells were imaged on a Leica light microscope and three high power fields per well were counted and averaged to quantify the percentage of SA- β -gal+ cells per population.

Example 1: Vfc-Hupar-Mcherry T Cells Eliminate Senescent Cell Populations in In Vitro Coculture Assay

[0153] To avoid the use of viral vectors in our manufacturing process we began by cloning a second generation huPAR CAR sequence with an appended mCherry fluorescent protein with homology arms at the desired cut site for the start of the first encoding exon, exon 6, of the TRAC locus (FIG. 2A). We next generated double-stranded DNA

(dsDNA) HDR templates via PCR amplification and performed a two-step purification process first with a Solid Phase Reversible Immobilization (SPRI) with AMPureXP beads followed by an ethanol precipitation to purify and concentrate the templates.

[0154] Primary human T cells from healthy donors were electroporated with the purified HDR templates and Spy-Cas9 ribonucleoproteins (RNPs) targeting the human TRAC locus. Cells were recovered for 24 hours at a 1 million/mL density in round-bottom 96-well plates and were expanded in ImmunocultTM xeno-free human T cell expansion medium. The cell viability and proliferation of VFC-huPAR-mCh was monitored over 9 days throughout the manufacturing process. Cells were then assayed on day 7 post-isolation to confirm the integration of the VFC-huPAR-mCh CAR T cell products as well as a virus-free CRISPR mCherry only control (VFC-mCh), in place of the hupAR-mCherry CAR sequence. We achieved consistently high genome editing with the dsDNA templates across 2 donors and demonstrated up to 70% knock-in efficiency, with an average of 20% uPAR+ and >90% total TCR-cells, as measured by flow cytometry (FIG. 2B).

[0155] To evaluate the efficiency of the uPAR-mCh CAR T cells in eliminating uPAR+ cells we measured the in vitro potency against senescent induced fibroblasts. Human dermal fibroblasts (HDFa) were plated at 30% confluence and allowed to adhere for 24 hrs, after incubation cells were induced with CDK4/6 and MEK inhibitors. The cells were then stained with SA- β -galactosidase to access for the presence of the senescence associated secretory phenotype (SASP). We performed an impedance assay measuring loss of resistance from induced and non-induced fibroblast populations over a 48 hour period. We observed potent killing 5:1 effector:target ratios. These results demonstrate potent target cell killing of uPAR+ senescent cells through multiple stimuli (FIG. 2C-D).

Example 2: Vfc-Mupar-Ngfr T Cells Eliminate Murine Senescent Cell Populations in In Vitro Coculture Assay

[0156] To avoid the use of viral vectors in our manufacturing process we began by cloning a second generation muPAR CAR sequence with an appended a tNGFR selectable marker with homology arms at the desired cut site for the start of the first encoding exon, exon 6, of the TRAC locus (FIG. 1A). We next generated double-stranded DNA (dsDNA) HDR templates via PCR amplification and performed a two-step purification process first with a Solid Phase Reversible Immobilization (SPRI) with AMPureXP beads followed by an ethanol precipitation to purify and concentrate the templates.

[0157] Primary human T cells from healthy donors were electroporated with the purified HDR templates and Spy-Cas9 ribonucleoproteins (RNPs) targeting the human TRAC locus. Cells were recovered for 24 hours at a 1 million/mL density in round-bottom 96-well plates and were expanded in ImmunocultTM xeno-free human T cell expansion medium. Cells were then assayed on day 7 post-isolation to confirm the integration of the VFC-muPAR-NGFR CAR T cell products. Genomic integration of muPAR-NGFR CAR was confirmed via "in-out" PCR amplification assay on genomic DNA extracted from 100,000 cells from both VFC-muPAR-NGFR and untransfected control cells with primers specific to the TRAC locus and CAR transgene

(FIG. 1B). The cell viability and proliferation of VFC-muPAR-NGFR was monitored over 9 days throughout the manufacturing process.

[0158] To evaluate the efficiency of the muPAR-NGFR CAR T cells in eliminating uPAR+ cells we measured the in vitro potency against mouse senescent induced fibroblasts. Mouse dermal fibroblasts from Ail4 transgenic mice were plated at 30% confluence and allowed to adhere for 24 h, after incubation cells were induced with CDK4/6 and MEK inhibitors. The cells were then stained with SA- β -galactosidase to access for the presence of the senescence associated secretory phenotype (SASP). We observed potent killing of senescent cells at 5:1 effector:target ratio (FIG. 1C-D). These results demonstrate potent target cell killing of uPAR+ murine senescent cells through multiple stimuli.

[0159] Exemplary templates include:

- [0160] SEQ ID NO: 1-DNA: muPAR.h28z tNGFR
- [0161] SEQ ID NO: 2-protein: muPAR.h28z tNGFR
- [0162] SEQ ID NO: 3-DNA: huPAR.h28z tNGFR
- [0163] SEQ ID NO: 4-protein: huPAR.h28z tNGFR
- [0164] SEQ ID NO: 5 DNA: huPAR.h28z mCherry
- [0165] SEQ ID NO: 6 protein: huPAR.h28z mCherry
- [0166] SEQ ID NO: 7 DNA: muPAR.m28z tNGFR
- [0167] SEQ ID NO: 8 protein: muPAR.m28z tNGFR
- [0168] SEQ ID NO: 9 DNA: muPAR.h28z mCherry
- [0169] SEQ ID NO: 10 protein: muPAR.h28z mCherry
- [0170] SEQ ID NO: 11 DNA: muPAR.m28z mCherry
- [0171] SEQ ID NO: 12 protein: muPAR.m28z mCherry
- [0172] SEQ ID NO: 13 DNA: muPAR.m28z APOE2
- [0173] SEQ ID NO: 14 protein: muPAR.m28z APOE2
- [0174] SEQ ID NO: 15 DNA: huPAR.h28z APOE2
- [0175] SEQ ID NO: 16 protein: huPAR.h28z APOE2
- [0176] SEQ ID NO: 17 DNA: miniXon huPAR.h28z mCherry
- [0177] SEQ ID NO: 18 protein: miniXon huPAR.h28z mCherry
- [0178] SEQ ID NO: 19 DNA: miniXon muPAR.m28z mCherry
- [0179] SEQ ID NO: 20 protein: miniXon muPAR.m28z mCherry
- [0180] SEQ ID NO: 21 DNA: miniXon muPAR.m28z APOE2
- [0181] SEQ ID NO: 22 protein: miniXon muPAR.m28z APOE2
- [0182] SEQ ID NO: 23 DNA: miniXon huPAR.h28z APOE2

[0183] SEQ ID NO: 24 protein: miniXon huPAR.h28z APOE2

[0184] SEQ ID NO: 25 DNA: muPAR.h28z APOE2

[0185] SEQ ID NO: 26 protein: muPAR.h28z APOE2

[0186] The use of the terms "a" and "an" and "the" and similar referents (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms first, second etc. as used herein are not meant to denote any particular ordering, but simply for convenience to denote a plurality of, for example, layers. The terms "comprising", "having", "including", and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to") unless otherwise noted. Recitation of ranges of values are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. The endpoints of all ranges are included within the range and independently combinable. All methods described herein can be performed in a suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as"), is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention as used herein.

[0187] While the invention has been described with reference to an exemplary embodiment, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the scope of the invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from the essential scope thereof. Therefore, it is intended that the invention not be limited to the particular embodiment disclosed as the best mode contemplated for carrying out this invention, but that the invention will include all embodiments falling within the scope of the appended claims. Any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

SEQUENCE LISTING

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aggagcgcag	acgccccccgc	gtaccagcag	ggccagaacc	agctctataa	cgagctcaat	1200
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tcgcgcgg	ocaaccacgt	ggaccctgt	ctgcccgtca	ccgtgcgc	ggacaccgg	2040
cgccagctcc	gcccgtgcac	acgtggcc	gacgcgcgt	cgaggagat	ccctggccgt	2100
tggattacac	ggtccacacc	cccagaggc	tcggacagca	cagccccag	caccaggag	2160
cctgaggcac	ctccagaaca	agacccata	gcccgcac	ttggcgggt	ggtgaccaca	2220
gtgtatgggc	gtccggcc	cgtgtgtacc	cgaggccac	ccgacaac	catccctgtc	2280
tattgttcca	tcctggctgc	tgtgttgt	ggtcttgc	cttacatagc	cttcaagagg	2340
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SEQ ID NO: 4	moltype = AA	length = 791
FEATURE	Location/Qualifiers	
source	1..791	
	mol_type = protein	
	note = huPAR.h28z tNGFR	
	organism = synthetic construct	
SEQUENCE: 4		
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RQPSGKGLEW LAHIWWDDDK RYNPALKSRL TISKDPSSNQ VFLKIASVDT ADIATYYCVR	120	
IGGSSGYMDY WGQGTTSVTVS SGGGSSGGGG SGGGGSDIVL TQSPLASLAVS LGQRATISCR	180	
ASESVDSYGN SFMHWYQQKP QOPPKLIIYR ASNLSKSGIPA RFSGSGSGTD FTLTINPVEA	240	
DDVATYCCQQ SNEDPWTFGG GTKLEIKRIE VMYPPPYLDN EKSNTGIIHV KGKHLCPSP	300	
FPGPSKGLEW YSLLVGGVLAC YSLLVGGVLA IFWVRSKRSL LLHSDYMMNT PRRPGPTRKH	360	
YQPYAPPDRF AAYRSRVKFS RSADAPPAQYQ GQNQLYNELN LGRREEYDVL DKRRGRDPEM	420	
GKPKPRRNPNQ EGLYNELQKD KMAEAYSEIG MKGERRRKGK HDGLYQGLST ATKDTYDALH	480	
MQALPPRATN FSLLKQAGDV EENPGPMGAG ATGRAMDGP RLLL LLLLGVS LGGAKEACPT	540	
GLYTHSGEKC KACNLGEVGA QPCGANQTV CEPCLDSVTFS DVVSATEPCK PCTECVGLQS	600	
MSAPCVAEADD AVRCRAYYY QDETTGRCEA CRVCEAGSGL VFSCQDKQNT VCEECPDGTY	660	
SDEANHVDPC LPCTVCEDETR RQLRECTRWA DABEEIPGR WITRSTPPEG SDSTAPSTQE	720	
PEAPPEQDLI ASTVAGVVTT VMGSSQPVVT RGTTDNLIPV YCSILA AVVV GLVAYIAFKR	780	
WNSRAKRSGS G	791	

SEQ ID NO: 5	moltype = DNA	length = 2625			
FEATURE	Location/Qualifiers				
source	1..2625				
	mol_type = other DNA				
	note = huPAR.h28z mCherry				
	organism = synthetic construct				
SEQUENCE: 5					
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gtgttctctg	agatcgcttc	gtggacaca	cgacatcgat	caacactata	360
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agtgggtgg	gtggatcagg	tggaggtgg	tctgggtgg	gtggatctga	480
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gacgtatgtt	caacactactg	ctgtcagc	agcaacgg	acccatggac	780
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tatagctgc	tagtaacagt	ggccttatt	atttctgg	tgaggtagtaa	1020
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taccagccct	atgccccacc	acgcgacttc	gcagcctatc	gctccagagt	1140
aggagcgcag	acgccccccgc	gtaccagc	ggccagaacc	agctctataa	1200
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aacttcgagg	acggccggct	ggtgacccgt	acccaggact	cctacgttgc	ggacggccgag	2280
tttcatctaca	aggtaa	gtcgccac	aacttccct	ccggaggccc	ctgtatcgac	2340
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aaggccgaga	tcaagcagag	gctgttgt	aggacggcg	gcacacta	cgtgtgggtc	2460
aagggcacct	acaaggccat	gaaggccc	cgtgtcc	ggccatcaca	cgtcaacatc	2520
aatgtggaca	tcacccctcca	caacggagg	tacacatcg	ttaaacaggta	cgaacgcgc	2580
ggggccgc	actccaccc	cgccatggac	gagctgtaca	agtaa		2625

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SEQ ID NO: 6 moltype = AA length = 874
FEATURE Location/Qualifiers
source 1..874
mol_type = protein
note = huPAR.h28z mCherry
organism = synthetic construct
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SEQUENCE : 6	60
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RQPSGKGLEW LAHIWWDDDK RYNPALKSRL TISKDPSSNQ VFLKIASVDT ADIATYYCVR	180
IGGSSGYMDY WGQGTSTVTS SGGGSSGGG SGGGGSIDLQ TQSPASLAWS LGQRATISCR	240
ASESVDSYGN SFMHWYQQKP QGPPKLLIYR ASNLKSGIPA RFSGSGSGTD FTLTINPVEA	300
DDVATYCCQQ SNEDPWTFFG GTKLEIKRIME VMPYPPYLDN EKSNGTIIH VKGKHLCPSPSPL	360
FPGPSPKFVW LVVVGGVLAC YSSLVTVAIFI FWRSRKRSL LLHSDYMNMT PRRPGPTRK	420
YQPYAPPDRD AAYRSRUVFKS RSDAPADAYQO GQNQLYNLIN LGRREYEDVL DKRRRDPPEM	480
GGKPRRKNPQ EGLYNELQKD KMAEAYSEIG MKGERRRGKG HDGLYQGLST ATKDTYDALH	540
MQALPFRATN FSLLKQAGDV EENPGPMPEP SKSAPAKGG SKKAITQAEG KDGKKRKSRE	600
KESYSIVYVK VLKVQHPDTD ISSKAMGIM SFVNDFIERT AGEASRLAHY NKRSTSRSR	660
IQTAVRLLLP GELAKHAXVE GTKAVTKYTS SKDPVPAATMV SKGEEDNMAI IKEFMRPFKVH	720
MEGSVNGHEF EIEGEGEGRP YEGTQTAKLK VTKGGPLPFA WDILSPQFMY GSKAYVKHPA	780
DIPDYLKLSE PEGFKWERMV NFEDGVVTVT TDQSSLDQGE FIYKVLRGT NFPSDGPVMQ	840
KKTMGWEASS ERMYPEDGAL KGEIKQLKL DGDDGHDAEV KTTYAKKPKV QLPGAYNVNI	874
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SEQ ID NO: 7 moltype = DNA length = 2382
FEATURE Location/Qualifiers
source 1..2382
mol_type = other DNA
note = muPAR.m28z tNGFR
organism = synthetic construct
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SEQUENCE: 7
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ggcccgacca aaggcctgga atgggtggcg agcattagca cccggccggg caaacacctat 240
tatcgcgata gcgtgaaagg ccgtttaacc attagcccgat ataacgcgaa aaaacacctgg 300
tatctgcgaa tggatagctc ggcgcggcggaa gataccgcga ccttattatgg cgcgcggcc 360
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atccctgtct	attgtccat	cctggctgt	gtgggtgtgg	gtctgtggc	ctacatagcc	2340
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SEQ ID NO: 8 moltype = AA length = 794

FEATURE Location/Qualifiers
source 1..794
mol_type = protein
note = muPAR.m28z tNGFR
organism = synthetic construct

SEQUENCE: 8

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APTKGLEWVA	SISTGGNTY	YRDSVKGRFT	ISRDNAKN	TYL	QMDSLRSE	DTATYCARQ	120
GGGYSDSF	DYWGQGVMTVS	SGGGGSGGGG	SGGGGSDVQ	TM	SPSNLAAS	PGEVSINCK	180
ASKSISKLYA	WYQQKPGKAN	KLLIYSGSTL	QS	GTPSRFSG	SGSGTDF	TLT IRNLEPEDFG	240
LYYCQHQHNEY	PLTFGSGTKL	EIKREQKLIS	EEDLIEFMYP	PPY	YLNDERSN	GTIIHIKEKH	300
LCHTQSSPKL	FWALVVVAGV	LFCYGLLTV	ALCVIWTNSR	RNRNLLQSDYM	NMTPRRPGLT	360	
RKPYQPYAPA	RDFAAYRPRV	KFSRSAEPPA	YQQGQNQLYN	ELNLGRREEY	DVL	DKRGRD	420
PEMGGKPRK	NPQEGLYNEL	QDKMAEAE	EIGMKGEERR	GKGHDGLYQG	LSTATKD	TYD	480
ALHMQALPPR	ATNFSLLKQ	GDVEENPGPM	GAGATGRAMD	GPRLLL	GVSLGGAK	EA	540
CPTGLYTHSG	ECCKACNLGE	GAQVPCGANQ	TVC	CEPCLLD	SVATE	PCKPCTECVG	600
LQSMSAPC	VE ADDAVRCAY	GYYQDETTGR	CEACRVCEAG	SGLVFSCQDK	QNTVCEEC	CPD	660
GTYSDEAHNV	DPCLPCTVCE	DTERQLRECT	RWADAECEE	PGRWIR	STP PEGSD	STAPS	720
TOEPEAPPEQ	DLIASTVAGV	VTTVMGSSQP	VVTRGTTDNL	IPVYCSILAA	VV	VLVAYIA	780
FKRWNNSRAKR	SGSG						794

SEQ ID NO: 9 moltype = DNA length = 2613

FEATURE Location/Qualifiers
source 1..2613
mol_type = other DNA
note = muPAR.h28z mCherry
organism = synthetic construct

SEQUENCE: 9

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gcccgcacca	aaaggcgttga	atgggtggcg	agcattagca	ccggccgg	caacacccat	240
tatcgcata	gctgttac	attagcccg	ataacgcgaa	aaacaccc	tg	300
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acctcccaaca	acggaggacta	caccatgtg	gaacagtacg	aacgcgcgcg	2580
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SEQ ID NO: 10 moltype = AA length = 870

FEATURE Location/Qualifiers
source 1..870
 mol_type = protein
 note = muPAR.h28z mCherry
 organism = synthetic construct

SEQUENCE: 10

MALPVTALL	PLALLHAAR	PEVQLVESGG	GLVQPGRSLK	LSCAASGFTF	SNYAMAWVRQ	60
APTKGLEWVA	SISTGGGNTY	YRDSVKGRPT	ISRDNAKNTL	YLQMDSLRSE	DTATYVCARQ	120
GGGYSDFSDY	WGQGVMTVS	SGGGSGGGG	SGGGSDVQM	TQSPSNLAAS	PGESVSIINCK	180
ASKSISKYLA	WYQQKPGKAN	KLLIYSGSTL	QSGETPSRFSG	SGSGTDFTLT	IRNLPEPDGF	240
LYYCQQHNEY	PLTFCGSGTKL	EIKRIEVMP	PPYLDNEKSN	GTIIHVKGKH	LCPSPLFPGP	300
SKPFWVLVVV	GGVLACYSLL	VTVAIFIWPV	RSKRSRLLHS	DYMNMTPRRP	GPTRKHYQPY	360
APPFRDFAAYR	SRVKFSRSAD	APAYQQGQNQ	LYNELNLGR	EYEDVLDKRR	GRDPEMGGKP	420
RRKNPQEGLY	NELQDKMMAE	AYSEIGMKGE	RRRGKGDGL	YQLGSTATKD	TYDALHMQL	480
PPRATNFSLL	KQAGDVENE	GPMPEPSKSA	PAPKKGSKKA	ITKAQKKDGK	KRKRSRKEFY	540
SIIVYKVLKQ	VHPDTGISSK	AMGIMNSFVN	DIFERIAGEA	SRLAHYNKRS	TITSREIQT	600
VRLLLPGELA	KHAVSEGTKA	VTKYTSSKDP	PVATMVSKE	EDNMAIIKEF	MRFKVHMEGS	660
VNGHEFIEG	EGEGRPYEGT	QTAALKVTKG	GPLPFWADIL	SPQFMYGSKA	YVKHPADIPD	720
YLKLSFPEGF	KWERVMNFED	GGVVTVTQDS	SLQDGEFIYK	VKLRTGNFPS	DGPVMQKTM	780
GWEASSERMY	PEDGALKGEI	KQRLKLKDGG	HYDAEVKTTY	KAKKPVQLPG	AYNVNIKLDI	840
TSHNEDYTIV	EQYERAEGRH	STGGMDELYK				870

SEQ ID NO: 11 moltype = DNA length = 2238

FEATURE Location/Qualifiers
source 1..2238
 mol_type = other DNA
 note = muPAR.m28z mCherry
 organism = synthetic construct

SEQUENCE: 11

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SEQ ID NO: 12 moltype = AA length = 745

FEATURE
source
1..745
mol_type = protein
note = muPAR.m28z mCherry
organism = synthetic construct

SEQUENCE: 12

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GCGYSDSFVDY	WGQGMVTVS	SGGGGGGGG	SGGGGSVDQM	TQSPSNLAAS	PGESVSINCK	180
ASKSISKYLA	WYQQPKPGKAN	KLLIYSGSTL	QSGTPSRFSG	SGSGTDFLT	IRNLEPEDFG	240
LYYCQQHNEY	PLTFGSGTKL	EIKREQKLIS	EEDLIEFMYP	PPYLDNERSN	GTIIHIKEKH	300
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RKPYQPYAPPA	RDFAAYRPRA	KF SRS AETAA	NLQDPNQLYN	ELN LGRREY	DVLEKKRARD	420
PEMGGKQQR	RNPQEGVYNA	LQKDKM A EAY	SEIGTKGER	RGKGDHGLYQ	GLSTATKDTY	480
DALHMOTLAP	RATNFSLLKQ	AGDVEENGP	VSKGEEDNMA	IIKEFMRFKV	HMEGSVNNGHE	540
FEIEGEGEGR	P YEGTQAKL	KVTKGGLP	FW DILSPQMF	YGSKAVVHP	ADIPDYLKLS	600
FPEGF KWERV	MNFEDGGVVT	WTQDSSLQDG	E FIYKVKL RG	TNFPSDG PVM	QKKTMGWEAS	660
SERMPEDGA	LKGEIKQRLK	LKDGGHYDAE	VKTTYKAKKP	VQLPGAYNVN	IKLDITSHNE	720
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SEQ ID NO: 13 moltype = DNA length = 2484

FEATURE
source
1..2484
mol_type = other DNA
note = muPAR.m28z APOE2
organism = synthetic construct

SEQUENCE: 13

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gcccgcacca	aaggcttga	atgggtggcg	agcattagca	ccggccgggg	caacacctat	240
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FEATURE		Location/Qualifiers				
source		1..827				
		mol_type = protein				
		note = muPAR.m28z APOE2				
		organism = synthetic construct				
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AYKSELEEQL	TPVAEETRAR	LSKELOQAAQA	RLGADMEDVC	GRLVQYRGEV	QAMLQSTEE	660
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SEQ ID NO: 15		moltype = DNA	length = 2502			
FEATURE		Location/Qualifiers				
source		1..2502				
		mol_type = other DNA				
		note = muPAR.h28z APOE2				
		organism = synthetic construct				
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SEQ ID NO: 18 moltype = AA length = 1077
 FEATURE Location/Qualifiers
 source 1..1077
 mol_type = protein
 note = miniXon huPAR.h28z mCherry
 organism = synthetic construct

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 SNQVFLKIAS VDTADIATYY CVRIGGSVY MDYWQGQTSV TVSSGGGGSG GGGSGGGGSD 360
 IVLTQSAPSL AVSLGQRATI SCRASESVDS YGNFSFMHWYQ QKPGQPCKL IYRASNLKSG 420
 IPARFSGSGS GTDFLTINP VEADDVATYC CQQSNEPDWT FGGGTKEIK RIEVMYPY 480
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SEQ ID NO: 19 moltype = DNA length = 2889
 FEATURE Location/Qualifiers
 source 1..2889
 mol_type = other DNA
 note = miniXon muPAR.m28z mCherry
 organism = synthetic construct

SEQUENCE: 19
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organism = synthetic construct
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mol_type = other DNA
note = miniXon muPAR.m28z APOE2
organism = synthetic construct

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	note = miniXon muPAR.m28z APOE2					
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	organism = synthetic construct					
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	note = miniXon huPAR.h28z APOE2					
	organism = synthetic construct					
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                        organism = synthetic construct
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SEQUENCE:	26	Organism:	2'-Deoxyribonucleic acid			
MALPVTLALL	PLALLLHAAR	PEVQLVESGG	GLVQPGRSLK	LSCAASGFTF	SNYAMAWVRQ	60
APTKGLEWAA	SISTGGGNTY	YRDSVKGRFT	ISRDNAKNTL	YLQMDSLRSE	DTATYYCARQ	120
GGGGYSDSFDY	WGQQGVMVTVS	SGGGGSGGGG	SGGGGSDVQM	TQSPSNNLAES	PGEVSINCK	180
ASKSKISYKLW	WYQQKEPGKAN	YIILYSGSTL	QSGTSPSFSGS	SGSGTDFDTLT	IRNLEPELDFG	240
LYYYCQQHNEY	PLTFGSGTKL	EIKRIEVMPY	PPYLDNEKSN	GTIIHVKGKH	LCPSPLFPGP	300
SKPFWVLVVV	GGVLACYSLL	VTVAFIIFWV	RSKRSRLLHS	DYMNMTPRRP	GPTRKHYQPY	360
APPDRFAAYR	SRVKFSSRSD	APAYQQGQNG	LYNELNLGRR	EELYDVLKDPR	GRDPMEGKGP	420
RRKNPQEGLY	NELQDKDMAE	AESEIGMKGE	RRRGKGHDGL	YGLSTATKD	TYDALHMQL	480
PRRATNFSSL	QOAGDVEENP	GPDYKDDDDK	MKVLAWLALL	TFLAGCOAKV	EQAVETEEPEP	540
ELRQQTEWQS	GQRWELALGR	FWDYLRWVQT	LSEQVQEELL	SSQVTQELRA	LMDETMKELK	600
AYKSELEEQL	TPVAAEETRAR	LSEKELQAAQA	RLGADMEDVC	GRLVQYRGEV	QAMLQGQSTEE	660
LRVRLASHLKR	KLRKQLLRLRDA	DDLQKCLAVY	QAGAREGAER	GLSAIRERLQ	PLVEQGRVRA	720
ATVGSLAGQP	LQERAQAWGE	RLLRAMEEMG	SRTDRLDEV	KEQVAEVRAK	LEEQAAQQIRL	780
QAEAFQARLK	SWFEPLVEDM	QROWAGLVEK	QVAAVGTSSA	PVPVPSDH		827

SEQUENCE: 27
GFTFSNY
organism = synthetic construct

-continued

SEQ ID NO: 29 FEATURE source	moltype = AA length = 11 Location/Qualifiers 1..11 mol_type = protein note = VHCDR3 sequence organism = synthetic construct	
SEQUENCE: 29 QGGGYSDSFD Y		11
SEQ ID NO: 30 FEATURE source	moltype = AA length = 9 Location/Qualifiers 1..9 mol_type = protein note = VHCDR1 sequence organism = synthetic construct	
SEQUENCE: 30 GFSLSTSGM		9
SEQ ID NO: 31 FEATURE source	moltype = AA length = 5 Location/Qualifiers 1..5 mol_type = protein note = VHCDR2 sequence organism = synthetic construct	
SEQUENCE: 31 WWDDDD		5
SEQ ID NO: 32 FEATURE source	moltype = AA length = 10 Location/Qualifiers 1..10 mol_type = protein note = VHCDR3 sequence organism = synthetic construct	
SEQUENCE: 32 IGGSSGYMDY		10
SEQ ID NO: 33 FEATURE source	moltype = AA length = 11 Location/Qualifiers 1..11 mol_type = protein note = VHCDR1 sequence organism = synthetic construct	
SEQUENCE: 33 KASKSISKYL A		11
SEQ ID NO: 34 FEATURE source	moltype = AA length = 7 Location/Qualifiers 1..7 mol_type = protein note = VHCDR2 sequence organism = synthetic construct	
SEQUENCE: 34 SGSTLQS		7
SEQ ID NO: 35 FEATURE source	moltype = AA length = 9 Location/Qualifiers 1..9 mol_type = protein note = VHCDR3 sequence organism = synthetic construct	
SEQUENCE: 35 QQHNEYPLT		9
SEQ ID NO: 36 FEATURE source	moltype = AA length = 15 Location/Qualifiers 1..15 mol_type = protein note = VHCDR1 sequence organism = synthetic construct	
SEQUENCE: 36 RASESVDSYG NSFMH		15
SEQ ID NO: 37 FEATURE source	moltype = AA length = 7 Location/Qualifiers 1..7	

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mol_type = protein
note = VHCDR2 sequence
organism = synthetic construct

SEQUENCE: 37
RASNLKS                                                 7

SEQ ID NO: 38      moltype = AA length = 9
FEATURE          Location/Qualifiers
source           1..9
mol_type = protein
note = VHCDR3 sequence
organism = synthetic construct

SEQUENCE: 38
QOSNEDPWT                                              9

SEQ ID NO: 39      moltype = AA length = 11
FEATURE          Location/Qualifiers
source           1..11
mol_type = protein
note = VHCDR1 sequence
organism = synthetic construct

SEQUENCE: 39
KASENVVTYV S                                             11

SEQ ID NO: 40      moltype = AA length = 7
FEATURE          Location/Qualifiers
source           1..7
mol_type = protein
note = VHCDR2 sequence
organism = synthetic construct

SEQUENCE: 40
GASNRYT                                                 7

SEQ ID NO: 41      moltype = AA length = 9
FEATURE          Location/Qualifiers
source           1..9
mol_type = protein
note = VHCDR3 sequence
organism = synthetic construct

SEQUENCE: 41
GQGYSYPYT                                              9

SEQ ID NO: 42      moltype = AA length = 120
FEATURE          Location/Qualifiers
source          1..120
mol_type = protein
note = VH of the uPAR binding fragment
organism = synthetic construct

SEQUENCE: 42
EVQLVESGGG LVQPGRLSLK SCAASGFTFS NYAMAWVRQA PTKGLEWVAS ISTGGGNNTYY 60
RDSVKGRFTI SRDNNAKNTLY LQMDSLRSED TATYYCARQG GGYSDSFDYW GQGVMVTVSS 120

SEQ ID NO: 43      moltype = AA length = 120
FEATURE          Location/Qualifiers
source          1..120
mol_type = protein
note = VH of the uPAR binding fragment
organism = synthetic construct

SEQUENCE: 43
QVTLKESGPG ILQPSQTLSL TCSFSGFSL S TSGMGVGWIR QPSGKGLEWL AHIWWDDDKR 60
YNPALKSRLT ISKDPSSNQV FLKIASVDTA DIATYYCVRI GGSSGYMDYW GQGTSVTVSS 120

SEQ ID NO: 44      moltype = AA length = 108
FEATURE          Location/Qualifiers
source          1..108
mol_type = protein
note = VL of the uPAR binding fragment
organism = synthetic construct

SEQUENCE: 44
DVQMTQSPSN LAASPGESVS INCKASKSIS KYLAWYQQKP GKANKLLIYS GSTLQSGTPS 60
RFSGSGSGTDF TLTIRNLEP EDFGLYYCQQ HNEYPLTFGS GTKLEIKR               108

SEQ ID NO: 45      moltype = AA length = 112
FEATURE          Location/Qualifiers
source          1..112
mol_type = protein

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note = VL of the uPAR binding fragment
organism = synthetic construct

SEQUENCE: 45
DIVLTQSPAS LAVSLGQRAT ISCRASESVD SYGNNSFMHWY QQKPGQPPKL LIYRASNLS 60
GIPARFSGSG SGTDFTLTIN PVEADDVATY CCQGSNEDPW TFGGGTKEI KR 112

SEQ ID NO: 46      moltype = AA length = 108
FEATURE           Location/Qualifiers
source            1..108
mol_type = protein
note = VL of the uPAR binding fragment
organism = synthetic construct

SEQUENCE: 46
NIVMTQSPKS MSMSVGERVT LTCKASENVV TYVSWYQQKP EQSPKLLIYG ASNRYTGVPD 60
RFTGSGSATD FTLTISSVQA EDLADYHCGQ GYSYPYTFGG GTKLEIKR 108

SEQ ID NO: 47      moltype = AA length = 243
FEATURE           Location/Qualifiers
source            1..243
mol_type = protein
note = uPAR binding fragment
organism = synthetic construct

SEQUENCE: 47
EVOLVESGGG LVQPGRLSLK SCAASGFTFS NYAMAWVRQA PTKGLEWVAS ISTGGNTYY 60
RDSVKGRFTI SRDNAKNTLY LQMDSLRSED TATYYCARQG GGYSDSFDYW GQGVMVTVSS 120
GGGGSGGGGS GGGGSDVQMT QSPSNLAASP GESVSINCKA SKSISKYLAW YQQKPGKANK 180
LLIYSGSTLQ SGTPSRFSGS GSGTDFTLTI RNLEPEDFGL YYCQQHNEYP LTFGSGTKLE 240
IKR 243

SEQ ID NO: 48      moltype = AA length = 247
FEATURE           Location/Qualifiers
source            1..247
mol_type = protein
note = uPAR binding fragment
organism = synthetic construct

SEQUENCE: 48
QVTLKESPG ILQPSQTLSL TCSFSGFSL TSGMGVGWIR QPSGKGLEWL AHIIWWDDKR 60
YNPALSKRLT ISKDPSNSQV FLKIASVDTA DIATYYCVRI GGSSGYMDYW GQGTSVTVSS 120
GGGGSGGGGS GGGGSDIVLT QSPASLAVAL GQRATISCRA SESVDSYGNS FMHWYQQKPG 180
QPPKLLIYRA SNLKGIPAR FSGSGSGTDF TLTINPVEAD DVATYCCQQS NEDPWTFGGG 240
TKLEIKR 247

SEQ ID NO: 49      moltype = AA length = 243
FEATURE           Location/Qualifiers
source            1..243
mol_type = protein
note = uPAR binding fragment
organism = synthetic construct

SEQUENCE: 49
QVTLKESPG ILQPSQTLSL TCSFSGFSL TSGMGVGWIR QPSGKGLEWL AHIIWWDDKR 60
YNPALSKRLT ISKDPSNSQV FLKIASVDTA DIATYYCVRI GGSSGYMDYW GQGTSVTVSS 120
GGGGSGGGGS GGGGSNIVMT QSPKSMMSMV GERVTLCKA SENVVTVSW YQQKPEQSPK 180
LLIYGAASNRY TGVPDRFTGS GSATDFTLTI SSVQAEDLAD YHCGQGYSYP YTFGGGTKE 240
IKR 243

SEQ ID NO: 50      moltype = DNA length = 735
FEATURE           Location/Qualifiers
source            1..735
mol_type = other DNA
note = encoding sequence of uPAR binding fragment
organism = synthetic construct

SEQUENCE: 50
gaagtccaaac tcgttgaaag cggcggttgtt ctgttccaggc caggcagatc actgaaactg 60
tcatgcggcc cgagtggctt cactttctcc aattacgcaaa tggcggttgtt tagacaggcc 120
cccacgaaag ggtcgcatca atcagtagcggat gaggtggaaa cacttactat 180
cgcgcataatgtt ttaaggggatc attacgatt agccccggaca acgcgcaaaa cacgttgtat 240
ctgcagatgg actcaatgg atcccgaggac acagcgactt actactgtgc gaggcaggcc 300
ggagggtata gtgatagctt tgattactgg ggccaggccg taatggtaac tgtagttct 360
ggtggaggtt gatcaggatctt ggtggagggtt gatctgtatgtt gcagatgaca 420
cagagtccctt caaatggc cgcttcaccgg gagaatcatcg taatgtatcaa ctgtaaagcg 480
tccaagtccaa ttccaaatgtt tttggcatgg tatcaacaga agccggaaa ggcgaacaaa 540
ctccctgttattt atagcggggatc taccttgcag tccggcacgc ctatgtatgtt ttcaggctcc 600
ggttctggaa ccgacttccac ttgacgatt cgcaattttgg aaccagagga ttttggctg 660
tactattgtc agcagcacaa cgaatacccg ttgacttttg gtatgtgtac aaagctggaa 720
atcaagagag cggcc 735

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SEQ ID NO: 51      moltype = DNA length = 741
FEATURE           Location/Qualifiers
source            1..741
mol_type = other DNA
note = encoding sequence of uPAR binding fragment
organism = synthetic construct

SEQUENCE: 51
caggtgaccc tgaaggagtc cggecccgcc atcctgcagc ccagccagac cctgagctg 60
actctgtctt tcagcggtt ctccccgtcc acctccggca tggcgctgg ctggatcaga 120
cagcccacgg gcaaggccct ggagtggctg gcccacatct ggtgggacga tgacaagaga 180
tacaaccccg ctctgaagag ccgggtgaca atcagcaagg accctagcag taaccagg 240
ttcctgaaga tcgcttcgt ggacacagca gacatcgca catactattt cgtggatc 300
ggcggaaagca gtggatacat ggactactgg ggacaggaa ccagcgtgac cgtgac 360
gttggaggtg gatcagggtt aggtggatct gttggaggtt gatctgacat cgtgtgacc 420
cagagcccgat ctatgttgcg agtggacgtt ggacagaggg ctaccatcag ctgcagac 480
tcagagacgc tggacagcta cggaaacacg ttcatgacat ggttaccacca gaagccaa 540
cagccaccta agctgtgtat ctaccgggtt agcaacctga agtccggat ccctgctgg 600
tttagcggaa gcggtagcgg caccgactt accctgacaa tcaacccagt ggaggccac 660
gatgtggcaa octactgttgc tcagcagac aacgaggacc catggacccctt cggcggtg 720
accaaactgg agatcaagag a 741

SEQ ID NO: 52      moltype = DNA length = 729
FEATURE           Location/Qualifiers
source            1..729
mol_type = other DNA
note = encoding sequence of uPAR binding fragment
organism = synthetic construct

SEQUENCE: 52
caggtgaccc tgaaggagtc cggecccgcc atcctgcagc ccagccagac cctgagctg 60
actctgtctt tcagcggtt ctccccgtcc acctccggca tggcgctgg ctggatcaga 120
cagcccacgg gcaaggccct ggagtggctg gcccacatct ggtgggacga tgacaagaga 180
tacaaccccg ctctgaagag ccgggtgaca atcagcaagg accctagcag taaccagg 240
ttcctgaaga tcgcttcgt ggacacagca gacatcgca catactattt cgtggatc 300
ggcggaaagca gtggatacat ggactactgg ggacaggaa ccagcgtgac cgtgac 360
gttggaggtg gatcagggtt aggtggatct gttggaggtt gatctaaat cgtgtgacc 420
cagtccttca agagcatggat catggcgtg ggcgagagag tgaccctgac ctgcaagac 480
tcggagaaacg tgggtgacca cttgtggatgg taccagcaga agcctgacca gagccctaa 540
ctgctgtatct acggcgcttc caacagatac accggagtgc ctgacagatt caccggcagc 600
ggaaaggccaa cccgacttac ctttgaccatc agcagcgtgc aggctgagga cctgcccac 660
taccactgcg cccagggtca cagctaccct tacaccctcg gtggaggcac caagctgg 720
atcaagcgg 729

SEQ ID NO: 53      moltype = AA length = 157
FEATURE           Location/Qualifiers
source            1..157
mol_type = protein
note = uPAR binding fragment
organism = synthetic construct

SEQUENCE: 53
MRALLARLLL CVLVVSDSKG SNELHQVPSN CDCLNGGTVC SNKYFSNIHW CNCPKKFQQ 60
HCEIDDKSRTC YEGNGHFYRG KASTDTMGRP CLPWNSATVL QQTYYHAHRSD ALQLGLGKH 120
YCRNPDNRRR PWCVVQVGLK PLVQECMVHD CADGKKP 157

SEQ ID NO: 54      moltype = AA length = 132
FEATURE           Location/Qualifiers
source            1..132
mol_type = protein
note = uPAR binding fragment
organism = synthetic construct

SEQUENCE: 54
MRALLARLLL CVLVVSDSKG SNELHQVPSN CDCLNGGTVC SNKYFSNIHW CNCPKKFQQ 60
HCEIDDKSRTC YEGNGHFYRG KASTDTMGRP CLPWNSATVL QQTYYHAHRSD ALQLGLGKH 120
YCRNPDNRRR PW 132

SEQ ID NO: 55      moltype = DNA length = 471
FEATURE           Location/Qualifiers
source            1..471
mol_type = other DNA
note = uPAR binding fragment coding sequence
organism = synthetic construct

SEQUENCE: 55
atgagagccc tgctggcgcg cctgtttctc tgcgttctgg tgcgtgacca ctccaaagg 60
agcaatgaac ttcatcaagt tccatcgaa tgcgtactgtc taaaatgggg aacatgtgt 120
tccaaacaatg acttctccaa cattcactgg tgcgtactgtc caaagaatt cggaggcag 180
cactgtgaaa tagataagtc aaaaacctgc tatgaggggg atggtcactt ttaccgg 240
aaggccagca ctgacacccat gggccggccc tgcctgcctt ggaactctgc cactgtc 300

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cagcaaacgt accatgcccc cagatctgtat gctttcagc tggccctgg gaaacataat 360
tactgcaggaa accccagacaa ccggaggcgca cccgggtgc atgtgcagggt gggcttaag 420
ccgcttgcc aagagtgcatt ggtgcattgac tgccatgat gaaaaaaagcc c 471

SEQ ID NO: 56 moltype = DNA length = 396
FEATURE Location/Qualifiers
source 1..396
mol_type = other DNA
note = uPAR binding fragment coding sequence
organism = synthetic construct

SEQUENCE: 56
atggagagccc tgctggcgcg cctgttttc tgcgttctgg tcgtgagcga ctccaaaggc 60
agcaatgaac ttcatcaagt tccatcgaa tgcgtactgtc taaaatgggg aacatgtgt 120
tccaacaatg actttccaa cattcgatcg tcgaactgc caaagaattt cggaggcgag 180
cactgtggaa tagataatgc aaaaacctgt tatggggggg atgggttcatt ttaccgggg 240
aaggccacga ctgacacccat gggccggccc tgctgcctt ggaacttcgc cactgtcctt 300
cagcaaacgt accatgcccc cagatctgtat gctttcagc tggccctgg gaaacataat 360
tactgcaggaa accccagacaa ccggaggcgca ccctgg 396

SEQ ID NO: 57 moltype = AA length = 15
FEATURE Location/Qualifiers
source 1..15
mol_type = protein
note = linker
organism = synthetic construct

SEQUENCE: 57
GGGGSGGGGS GGGGS 15

SEQ ID NO: 58 moltype = AA length = 1368
FEATURE Location/Qualifiers
source 1..1368
mol_type = protein
organism = Streptococcus pyogenes

SEQUENCE: 58
MDKKYSIGLD IGTNSVGWAV ITDEYKVPSK KFKVLGNTR HSIKKNLIGA LLFDSGETA 60
ATRLKRTARR RYTRRKNRIC YLQBIFSNEM AKVDDSFPHR LEESFLVEED KKHERHPIFG 120
NIVDEVAYHE KYPTIYHLRK KLVSTDKA DRLIYLALAH MIKFRGHFL EGDLNPNSD 180
VDKLFIFIQLVQ TYNQLFEEPN INASGVDAKA ILSARLSKS RLENLTIAQLP GEKKNGLFGN 240
LIALSGLTLP NFKSNFDLAE DAKLQLSKSD QIDQYADLF LAAKNLSDAI 300
LLSDILRVNT EITKAPLAS MTKRYDEHHQ DLTLKALVR QQLPEKYKEI FFDQSKNGYA 360
GYIDGGASQE EFYKPKIPIL EKMDGTEELL VKLNRDPLL KQRTFDNGSI PHQIHGLELH 420
AILRRQEDFY PFLKDNRREKI EKILTFRIPY YVGPLARNS RPAWMTRKSE ETITPWNEE 480
VVDKGASAQS FIERMTNFQD NLPNEKVLFPK HSLLYEYFTV YNELTKVKYV TEGMRKP AFL 540
SGEQKKAIQD LDFKTNRKVT VKQLKFDSVIE KIECFDSDVIE SGVEDRFNAS LGTYHDLK 600
IKDKDFLDNE ENEDILEDIV LTLLTFEDRE MIBERLKTYA LHFDDKVMQ LKRRRTGNG 660
RLSRKLINGI RDQKSGKTIL DFLKSDGFAN RNMQLIHDH SLTFKEDIQK AQVSGQGDSDL 720
HEHIANLAGS PAIKKGILQT KVVKVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER 780
MKRIEEGIKE LGSQILKEP VENTOLQNEK LYLYYLQNQR DMYVDQELDI NRLSDYDVH 840
IVPQSFLKDD SIDNKVLTRS DKNRGKSDMV PSBEVVKKM PNYWRQLLNAK LITQRKF DNL 900
TKAERGLLSE LDKAGFIKRQ LVETRQITKH VAQILDLSRMN TKYDENDKLI REVKVITLKS 960
KLVSDFKDQF QFYKVERINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK 1020
MIAKSEFEIG KATAKYFFYS NMIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF 1080
ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDQLI ARKKDWDPKK YGGFDSPVTA 1140
YSVLTVVAKVE KGKSKKLKSV KELLGITIME RSSFEKNPID FLEAKGYKEV KKDLIILKPK 1200
YSLFEELENGR KRMLASAGEL QKGKNEALPS KVNFLYLAS HYEKLGSPPE DNEQQLFVE 1260
QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA 1320
PAAFKYFDTT IDRKYRTSTK EVLDATLHQ SITGLYETRI DLSQLGGD 1368

SEQ ID NO: 59 moltype = AA length = 1409
FEATURE Location/Qualifiers
source 1..1409
mol_type = protein
organism = Streptococcus thermophilus

SEQUENCE: 59
MLFNKCIIS INLDFSNKEK CMTPYPSIGL DIGTNNSVGWA VITDNYKVPS KKMVKVLGNTS 60
KKYIKKNLLG VLLFDGSITA EGRRLKRTAR RRYTRRRRNRI LYLQEIFSTE MATLDAFFQ 120
RLDDDFLVPD DKRDKSYPIF GNLVEEKVYH DEFPTIYHLR KYLADSTKKA DLRLVYLALA 180
HMIKYRGHFL IEGEFNSKNN DIQKNFQDPL DTYNAIFESD LSLENSQLE EIVDKKISK 240
EKKDRILKLF PGEKNSGIFS EFLKLIVGNO ADFRKCFNLD EKASLHFSKE SYDEDLETL 300
GYIGDDYSDV FLKAKKLYDA ILLSGFLTVT DNTEAPLSS AMIKRYNEHK EDLALLKEYI 360
RNISLKTYYNA VFKDDTKNGY AGYIDGKTMQ EDFYVYLNLA LAAEFGADYI LEKIDREDFL 420
RKQRTFDNGS IPYQIHLQEM RAILDKQAKF YPFLAKNKER IEKILTFRIP YYVGPLARGN 480
SDFAWSIRKR NEKITPWNFE DVIDKESSAE AFINRMTSFD LYLPEEKVLP KHSLLYETFN 540
VYNELTKVRF IAESMRDYQF LDSKQKKDIV RLYFKDKRKV TDKDIIIEYLH AIYGYDIEL 600
KGIEKQFNSS LSTYHDLNI INDKEFLDDS SNEAIIIEEI HTLTIFEDRE MIKQRLSKFE 660
NIFDKSVLKK LSRRHYTGNG KLSAKLINGI RDEKSGNTIL DYLIDDGISN RNFQMQLIHDD 720

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ALSFKKKIQK	AQIIGDEDKG	NIKEVVKS LP	GSPAIIKKGIL	QSIKIVDELV	KVMGGRK PES	780
IVVEMARENQ	YTNQGKSNSQ	QRLKRLEKSL	KELGSKILKE	NIPAKLSKID	NNALQNDRLY	840
LYYLQNGKDM	YTGDDLDIDR	LSNYDIDHII	PQAFLKD NSI	DNKVLVSSAS	NRGKSDDFPS	900
LEVVKRRKTF	WYQLLKS KL	SQRKF DNLT K	AERGGLL PED	KAGFIQRQLV	ETRQITKHVA	960
RLLDEKFNNK	KDENNRAV RT	VKIITLKST L	VSQFRKDFEL	YKVREINDFH	HAHDAYLNAV	1020
IASALLKKYP	KLEPEFVYGD	YPKYNSFR ER	KSATEKVYFF	SNIMNIFK KS	ISLADGRVIE	1080
RPLIEVNEET	GESVN KESD	LATVRRVLS Y	PQNVNVK VVE	EQNHL DRGK	PKGLF NANS	1140
SKPKPNSNEM	LVGAK EYLDP	KKYGGYAGIS	NSPAVLVKG T	IEKGAKKKIT	NVLEF QG I S	1200
LDRIN YRKDK	LNFLLEKG YK	DIELIIELPK	YSLFELS DGS	RRMLASILST	NNKRGEIHKG	1260
NQIFLSQKFV	KLLYHAKRIS	NTINENH RKY	VENHKKEFEE	LFYYI LEF N	NYVGA KKG NG	1320
LLNSAQFSWQ	NHSIDELC S	FIGPTGSRK	GLFELTS RG S	AADFE FLG V	IPRYRDYTPS	1380
SLLKDATLH	QSVT GLYETR	IDLAKLGE G				1409

SEQ ID NO: 60 moltype = AA length = 1082
 FEATURE Location/Qualifiers
 source 1..1082
 mol_type = protein
 organism = Neisseria meningitidis

SEQUENCE: 60
 MAAFKPNPIN YILGLDIGIA SVGWAMV EID EEE NPIRLID LGVRV FERA E VPKTGDS LAM 60
 VRRLARS VRR LTRRRAH RLL RARR LLKREG VLQAADF DEN GLIKSLP NT P WQLRAA LDR 120
 KLT PLESAV LH LHK HRG Y LSQRK NEGET ADKELGALL K GVADNAHAL Q TGDFRT PAEL 180
 ALNKFEKESG HIRNQ RGDYS LSQRK NEGET ADKELGALL K GVADNAHAL Q TGDFRT PAEL 240
 TQP ALP GS DA VQ KML GH CTF EPABPKA AKN TYTAERF IWL TKL NN RL R QGS ERPL TD T 300
 ERATLM DEPY RK SKL TYA QA RK LLL GLEDTA FF KG L RY GKD NAE AS TLM MEM KAY HAIS RAL 360
 EKE GLK DKK S PLN LSP ELD EIGT AFS L FK T DED IT GR LK DRI QPE ILEA LL KH IS FD KF 420
 VQ ISL KAL R IVPLM EQ GK YDEACAEI YG DHY GKK NTE KI YL PIP AD EIR NPV V LRA 480
 LSQ ARK VING V VRR YG SPAR I HI ETAREVG KSP KDR KEIE K RQE ENR KDR EKA AAK F REY 540
 FPF NVG EP K S KDI LKL RLY E QHG KCL YSG KE IN LGR LNE KGY VEID HAL PFS RTW DDS F 600
 NN KV VL LG SE NQ NKG NQ TP Y EY FNG KDN SR E WO EF KAR VE TS RF PRS K KQ RILL QKF DED 660
 GF KERN LND T RY VN RFL CQ F VAD RM R GLK RY GKD NAE AS TLM MEM KAY HAIS RAL 720
 RH HAL DAVV AC STV AM QK Q EY FNG KDN SR E WO EF KAR VE TS RF PRS K KQ RILL QKF DED 780
 QEV MIR VFG K PDG KPE FEE A DT PEK LRL TLL AE KL SS RPE A VHEY V TPL FV SR APN R K MSG 840
 QGH MET V KSA KRL D EGV S VSL R VPL T Q LK D L E K M V N R E R E P K LY E A L K A R L E A H K D D P A 900
 KAFAE P Y K D KAG N R T Q Q V KAV R G E V Q K Y TG V W V R N H N G IAD N A T M V R V DV F E K G D K Y Y 960
 LPV I Y SW QVA KG IL P D R A V V I D D S F N K F S L H P N D L V E V I T K K A R M F G Y F 1020
 ASCH RGT GNI N IRI HLD H K I G N G I L E G I G V K T A L S F Q K Y Q I D E L G K E I R P C R L K K R P P 1080
 VR

SEQ ID NO: 61 moltype = AA length = 1395
 FEATURE Location/Qualifiers
 source 1..1395
 mol_type = protein
 organism = Treponema denticola

SEQUENCE: 61
 MKKEIKDYFL GLD VGT GSVG WAV TDY KL LK ANR KDL W G MRC FETA E TA EV RRL H R G A R 60
 RRI E R R K K R I K L L Q E L F S Q E I A K T D E G F F Q R M K E S P F Y A E D K T I Q E N T L F N D K D F A D K T 120
 YHKAYPTINH LIKA WIEN KV KPD P R L L Y LA CHN I K K R G H F L F E G D F D S E N Q F D T S I Q A L 180
 FEYLREDMEV DID A DS Q K V E I L K D S S L L K N S E K Q S R L N K I L G L K P S D K Q K K A I T N L I S G N 240
 KINFADLYDN PDL KDAE KNS I S F S K D D F D A L S D D L A S I L G D S F E L L L K A K A V Y N C S V L S K 300
 VIG D E Q Y L S E A K V K I Y E K H K T D L T K L K N V I K K H F P K D Y K K V F G Y N K N E K U N N N Y S G Y V G V 360
 CKTKSKKL N N S V N Q E D F Y K F L K T I L S A K S E I K E V N D I L T E I E T G T F L P K Q I S K S N A E I 420
 PYQLRK MEL K I L S N A E K H K S F L K Q K D E K G L S H S E K I I M L L T F K I P Y Y I G P I N D N H K K F F 480
 P DRC W V V K K E K S P S G K T T P W N F F D H I D K E K T A E A F I T S R T N F C T Y L V G E S V L P K S S L L Y S 540
 E Y T V L N E I N N L Q I I D G K N I C D I K L K Q K Y E D L F K K Y K K I T Q K Q I S T F I K H E G I C N K T D E 600
 V I I L G I D K E C T S S L K S Y I E L K N I F G K Q V D E I S T K N M L E E I T Q W A T Y D E G E G K T I L K T K I 660
 K A E Y G K Y C S E Q I K K I L N L K F S G W G R L S R K F L E T V T S E M P I G F S E P V N I I T A M R E T Q N N L M 720
 E L L S S E F T F T E N I K K I N S G F E D A E K Q F S Y D G L V K P L F L S P V K K M L W Q T L K L V K E I S H I T 780
 Q A P P K K I F I E M A K G A E L E P A R T K T R L K I L Q D L Y N N C K N D A D A F S S E I K D L S G K I E N E D N L 840
 R L R S D K L Y L Y T Q L G K C M Y C G K P I E G H V F D T S N Y D I D H I Y P Q S K I K D S I S N R V L V C S S 900
 C C N K N K E D K Y P L K S E I Q S K Q R G F W N F L Q R O N N F I S L E K L N R L T R A T P I S D D E T A K F I A R Q L V 960
 E T R Q A T K V A A K V L E K M F P E T K I V Y S K A E T V S M F R N K F D I V K C R E I N D F H H A H D A Y L N I V V 1020
 G N V Y N T K F T H N P W N I K E K P D N P K I A D T Y N Y Y K V F D Y D V K R N N I T A W E K G K T I I T V K D M L 1080
 K R N T P I Y T R Q A A C K K G E L F N Q T I M K K G L Q Q H P L K K E G P F S N I S K Y G G Y N K V S A A Y T T L I E 1140
 Y E E K G N K I R S L E T I P L Y L V K D I Q K D Q D V L K S Y L T D L L G K K E F K I L V P K I K I N S L L K I N G F 1200
 P C H I T G K T N D S F L L R P A V Q F C C S N N E V L Y F K K I I R F S E I R S Q R E K I G K T I S P Y E D L S F R S 1260
 Y I K E N L W K K T K N D E I G E K E F Y D L L Q K K N L E I Y D M L L T K H K D T I Y K K R P N S A T I D I L V K G K 1320
 E K F K S L I I E N Q F E V I L E I L K L F S A T R N V S D L Q H I G G S K Y S G V A K I G N K I S S L D N C I L I Y Q 1380
 S I T G I F E K R I D L L K V

SEQ ID NO: 62 moltype = DNA length = 19
 FEATURE Location/Qualifiers
 source 1..19
 mol_type = other DNA
 note = guide sequence
 organism = synthetic construct

-continued

SEQUENCE: 62 cagggttctg gatatctgt	19
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moltype = DNA length = 20 Location/Qualifiers 1..20 mol_type = other DNA note = guide sequence organism = synthetic construct	
SEQUENCE: 63 gggagtcaaa gtcgggtgaac	20
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moltype = DNA length = 22 Location/Qualifiers 1..22 mol_type = other DNA note = primer organism = synthetic construct	
SEQUENCE: 65 gcaaggccagg actccaccaa cc	22

1. A DNA HDR template for a transgene comprising a chimeric antigen receptor (CAR) gene for inserting the transgene into a T cell expressed gene to generate CAR T cells having the composition:

(left HA)-(SA)-(first self-cleaving peptide polynucleotide or IRES)-(optional inducible control sequence)-(uPAR binding fragment polynucleotide)-(hinge domain polynucleotide)-(transmembrane domain polynucleotide)-(intracellular domain polynucleotide)-(optional second self-cleaving peptide polynucleotide or IRES)-(optional first secreted factor or first selection marker polynucleotide)-(optional third self-cleaving peptide polynucleotide or IRES)-(optional second secreted factor or second selection marker polynucleotide)-(polyA terminator)-(right HA);

or

(left HA)-(SA)-(first self-cleaving peptide polynucleotide or IRES) (uPAR binding fragment polynucleotide)-(hinge domain polynucleotide)-(transmembrane domain polynucleotide)-(intracellular domain polynucleotide)-(optional second self-cleaving peptide polynucleotide or IRES)-(optional inducible control sequence)-(optional first secreted factor or first selection marker polynucleotide)-(optional third self-cleaving peptide polynucleotide or IRES)-(optional second secreted factor or second selection marker polynucleotide)-(polyA terminator)-(right HA);

or

(left HA)-(SA)-(first self-cleaving peptide polynucleotide or IRES) (uPAR binding fragment polynucleotide)-(hinge domain polynucleotide)-(transmembrane domain polynucleotide)-(intracellular domain polynucleotide)-(optional second self-cleaving peptide polynucleotide or IRES)-(optional first secreted factor or first selection marker polynucleotide)-(optional third self-cleaving peptide polynucleotide or IRES)-(optional inducible control sequence)-(optional second

secreted factor or second selection marker polynucleotide)-(polyA terminator)-(right HA);

wherein the left HA and the right HA are homology arms complementary to sequences on both sides of a cleavage site in the T cell expressed gene;

wherein SA is a splice acceptor site;

wherein the first, second and third self-cleaving peptide polynucleotide or IRES are polynucleotides encoding a first, second and third self-cleaving peptide or an internal ribosome entry site (IRES), respectively;

wherein the optional inducible control sequence is a regulatory sequence which provides control of protein expression in response to a small molecule inducer;

wherein the uPAR binding fragment polynucleotide is a polynucleotide encoding a polypeptide that specifically binds uPAR;

wherein the hinge domain polynucleotide encodes a CD28 or CD8α hinge domain;

wherein the transmembrane domain polynucleotide encodes a transmembrane domain;

wherein the intracellular domain polynucleotide encodes one or more intracellular domains;

wherein the first and second secreted factor polynucleotides are coding sequences for a neurotrophic factor, growth factor, or cytokine;

wherein the first and second selection marker polynucleotides are coding sequences for a detectable protein; and

wherein the polyA terminator is a sequence-based element that defines the end of a transcriptional unit.

2. The template of claim 1, wherein the left homology arm comprises 383 to 588 bp of the TRAC locus directly upstream of the cutsite, and the right homology arm includes 391 to 499 bp of the TRAC locus directly downstream of the cutsite.

3. The template of claim 1, wherein the first, second and third self-cleaving peptides independently comprise a porcine teschovirus-1 (P2A) peptide, a Thosea asigna virus (T2A) peptide, an equine rhinitis A virus (E2A) peptide, or a foot-and-mouth disease virus (F2A) peptide.
4. The template of claim 1, wherein the uPAR binding fragment is an antibody fragment.
5. The template of claim 1, wherein the uPAR binding fragment is a single-chain variable fragment comprising a heavy variable fragment and a light chain variable fragment.
6. The template of claim 1, wherein the transmembrane domain is from CD28 and the intracellular domain is a portion of CD3-zeta.
7. The template of claim 1, further comprising a polynucleotide encoding a costimulatory domain between the transmembrane domain polynucleotide and the intracellular domain polynucleotide.
8. The template of claim 7, wherein the costimulatory domain is OX40, 41BB, ICOS, CD27, CD40, CD40L or a TLR.
9. The template of claim 1, wherein the first and second secreted factors are each independently a pro-regenerative secreted factor, a pro-memory secreted factor, growth factor, or a factor that attracts pro-regenerative immune cells.
10. The template of claim 1, wherein the first selection marker, second selection marker or both are a coding sequence for a fluorescent protein.
11. A plasmid containing a sequence coding for the HDR template of claim 1.
12. The plasmid of claim 11 comprising a virus-free plasmid.
13. An ex vivo, virus-free method of site-specifically inserting a transgene containing a chimeric antigen receptor (CAR) gene into a T cell expressed gene to generate CAR T cells, comprising:
 - preparing the homology-directed repair (HDR) DNA template of claim 1,
 - introducing into a population of unmodified T cells a Cas9 ribonucleoprotein (RNP) and the HDR template to provide the CAR T cells,

wherein the Cas9 RNP comprises a Cas9 protein and a guide RNA that directs double stranded DNA cleavage of a cleavage site in the T cell expressed gene, and

wherein the transgene is specifically integrated into the cleavage site of the T cell expressed gene locus created by the Cas9 RNP in the cells, and

culturing the CAR T cells in xeno-free medium to provide a cultured population of CAR T cells having the transgene specifically integrated in the T cell expressed gene,

wherein, in the cultured population of CAR T cells, an endogenous promoter of the T cell expressed gene drives expression of the transgene, or wherein the transgene includes a promoter that drives expression of the transgene, and

wherein the CAR gene encodes a fusion protein comprising the translated anti-uPAR binding motif, hinge, transmembrane domain, and one or more intracellular domain(s).

14. The method of claim 13, wherein the unmodified T cells are autologous T cells isolated from a patient, or T cells from an allogeneic healthy donor.

15. The method of claim 13, further comprising administering the cultured population of CAR T cells to a patient in need of treatment for a neurodegenerative disease, stroke, craniocerebral trauma and/or accident, or an elderly patient in need of treatment for aging.

16. The method of claim 15, wherein the neurodegenerative disease is Alzheimer's disease, dementia, Parkinson's disease, Lewy body disease, ataxia, Huntington's disease, amyotrophic lateral sclerosis, Down syndrome, or spinal muscular atrophy.

17. The method of claim 13, wherein administering is by intravenous or intracerebroventricular infusion or intracerebral injection.

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