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

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C12N 9/22	(2006.01)
C12N 15/11	(2006.01)

(52) **U.S. Cl.**

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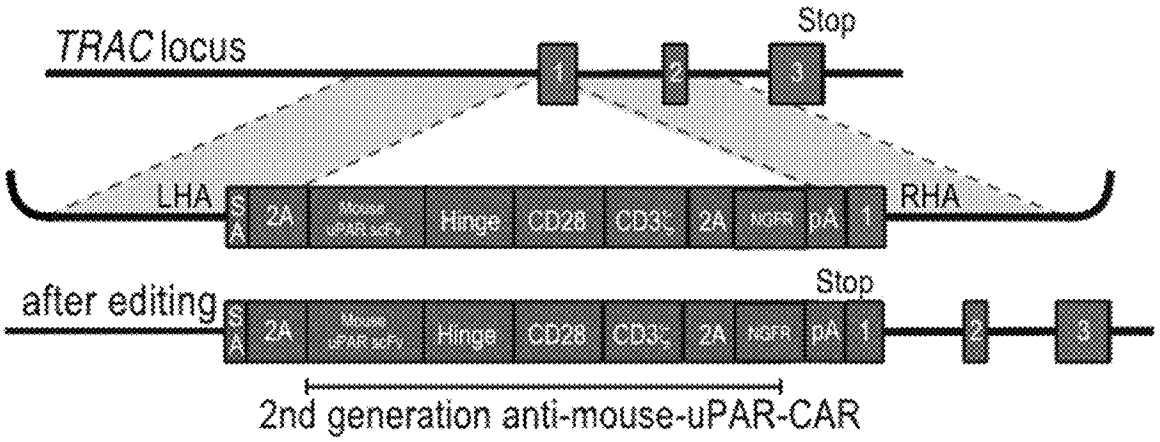
(57) **ABSTRACT**

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, craniocerebral trauma and/or accident, or elderly individuals in need of treatment for aging.

Specification includes a Sequence Listing.

A



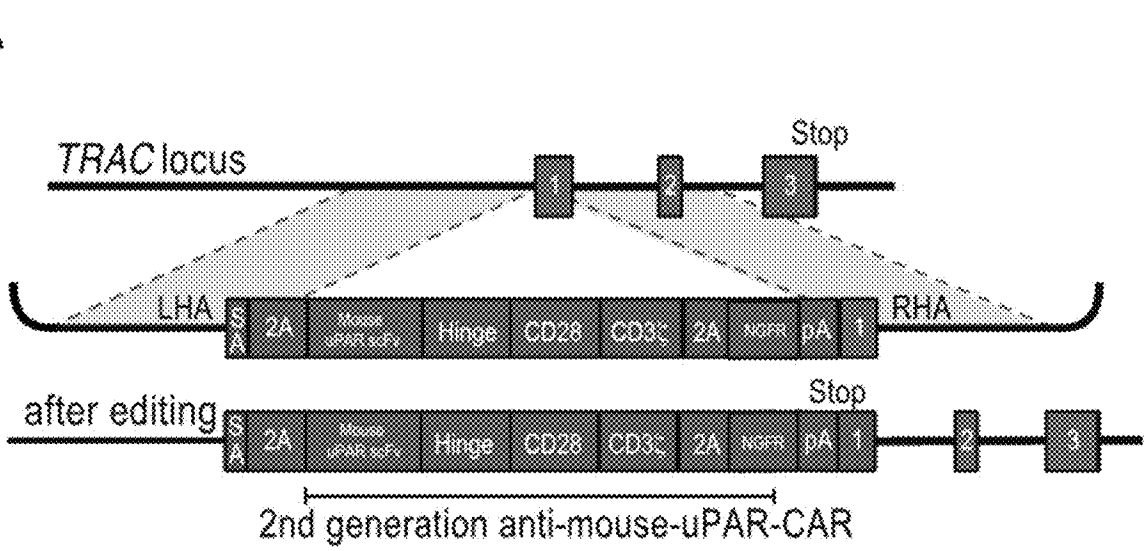

A
TRAC locus
Stop
1
2
3
LHA
SA
2A
Hinge
CD28
CD3ζ
2A
pA
RHA
after editing
Stop
2nd generation anti-mouse-uPAR-CAR

FIG. 1A

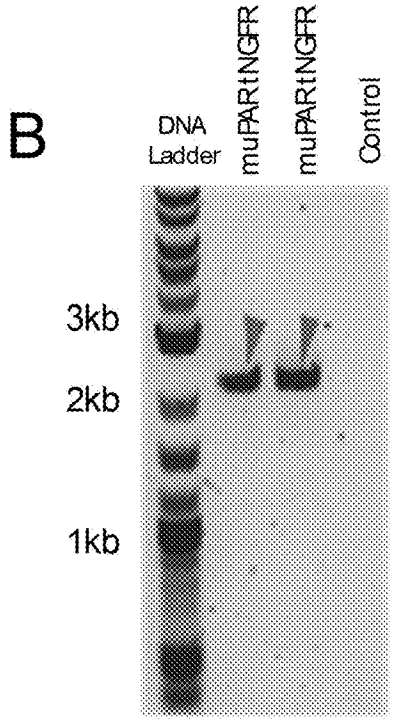

B
DNA Ladder
muPARtNGFR
muPARtNGFR
Control
3kb
2kb
1kb

FIG. 1B

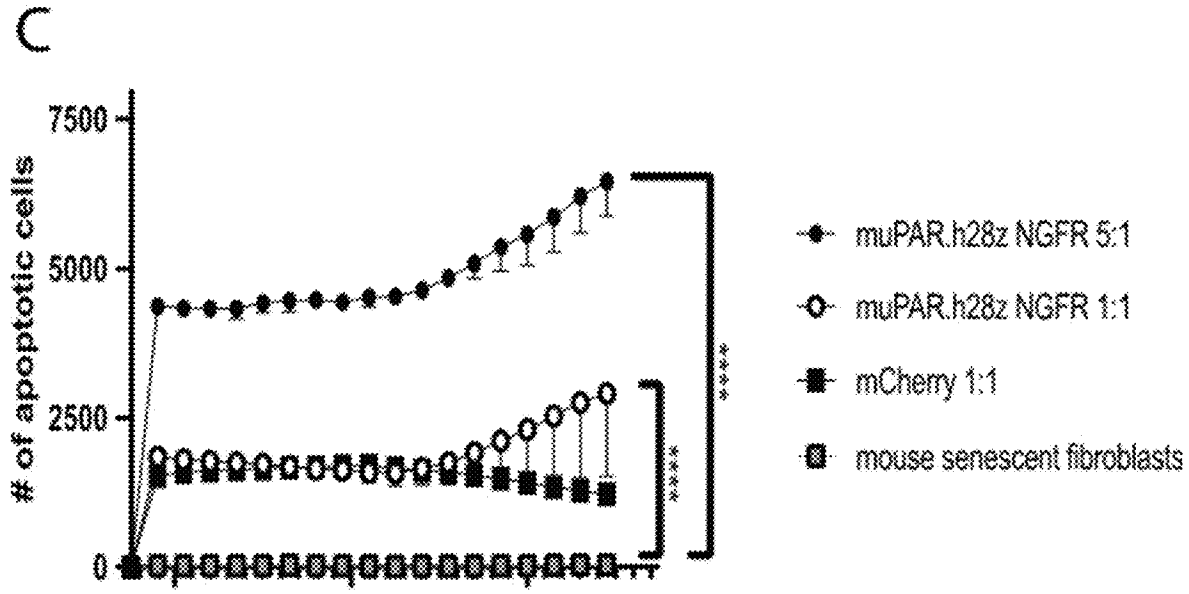

C
7500
5000
2500
0
# of apoptotic cells
****
****
muPAR.h28z NGFR 5:1
muPAR.h28z NGFR 1:1
mCherry 1:1
mouse senescent fibroblasts

FIG. 1C

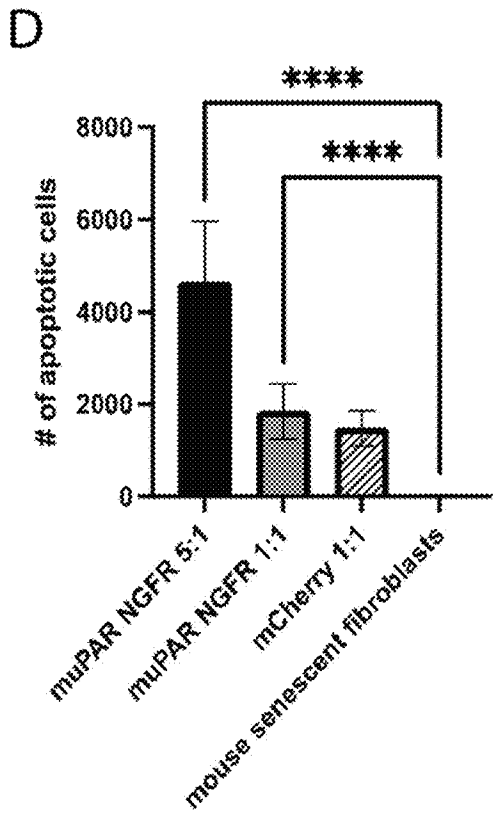

D
****
****
8000
6000
4000
2000
0
# of apoptotic cells
muPAR NGFR 5:1
muPAR NGFR 1:1
mCherry 1:1
mouse senescent fibroblasts

FIG. 1D

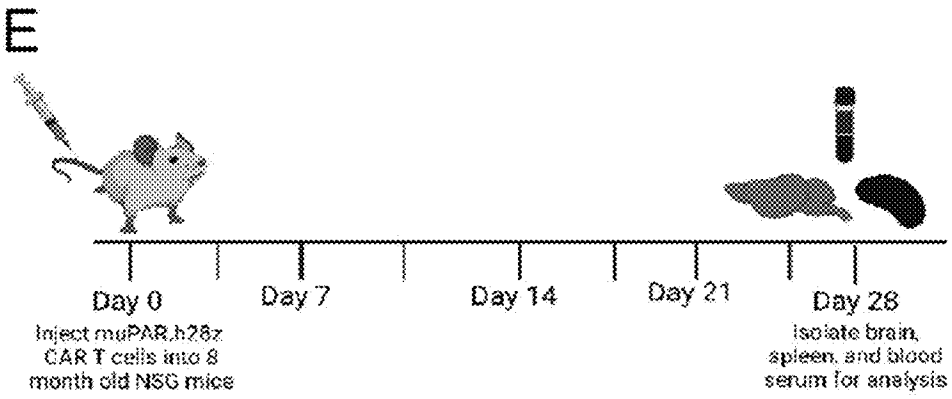


FIG. 1E

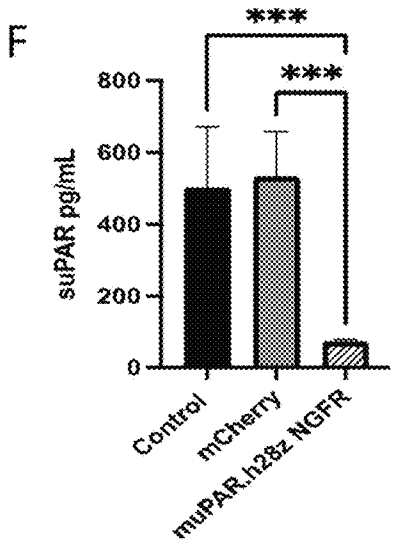


FIG. 1F

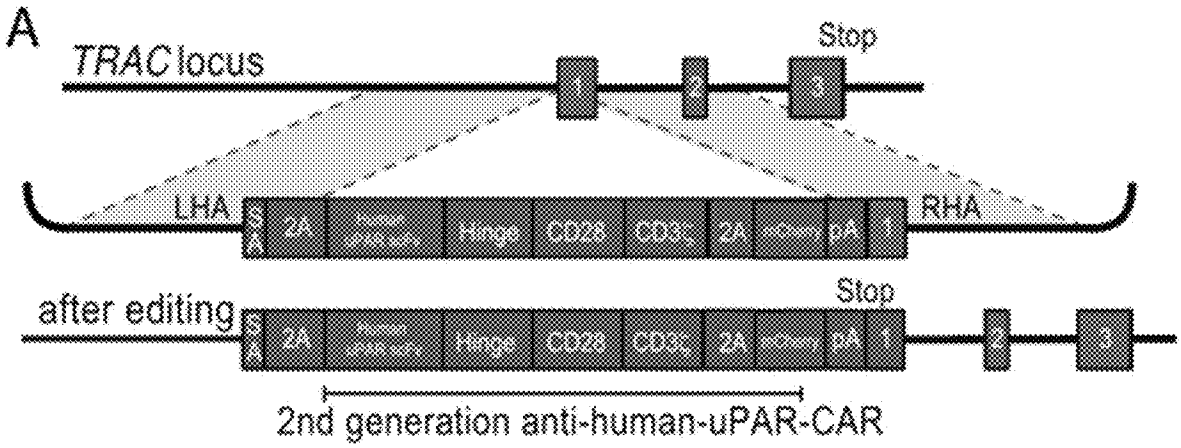


FIG. 2A

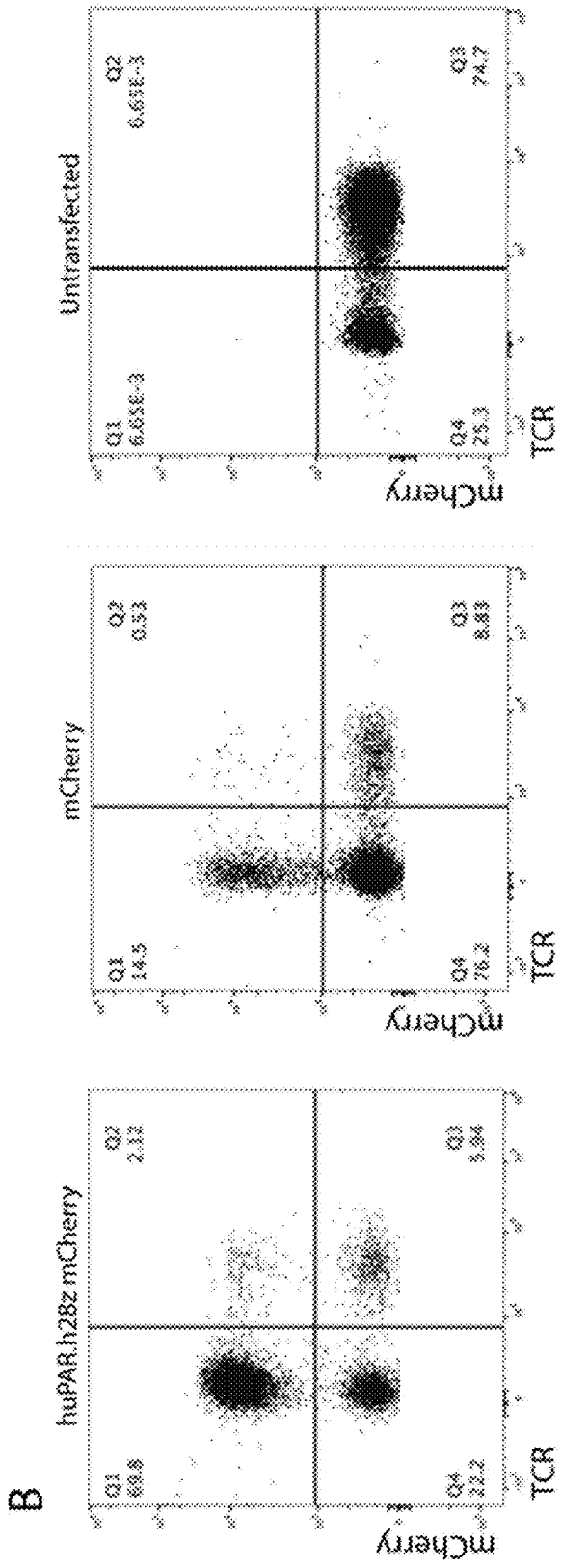

B
huPAR.h28z mCherry
Q1
69.8
Q2
2.12
Q3
5.98
Q4
22.2
mCherry
TCR
mCherry
Q1
14.5
Q2
0.53
Q3
8.83
Q4
76.2
mCherry
TCR
Untransfected
Q1
6.05E-3
Q2
6.61E-3
Q3
74.7
Q4
25.3
mCherry
TCR

FIG. 2B

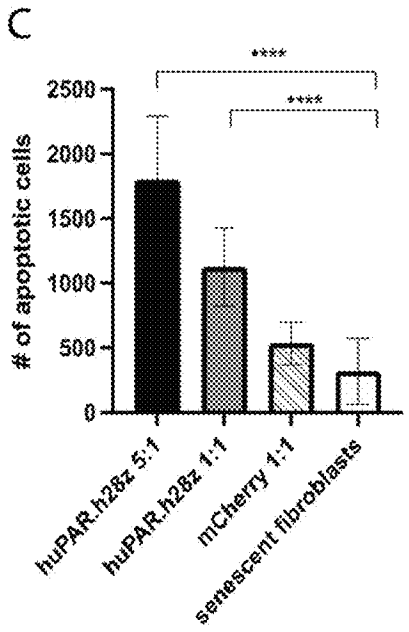


FIG. 2C

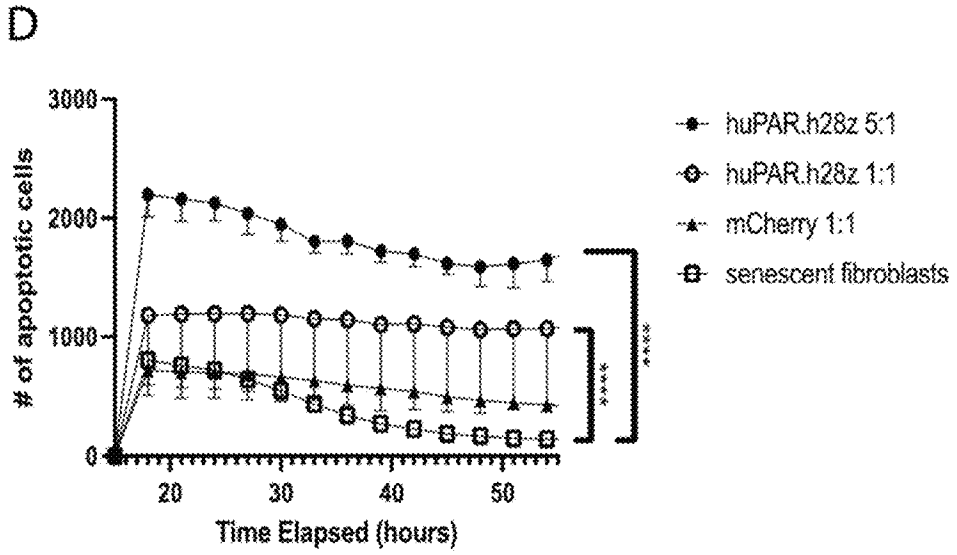


FIG. 2D

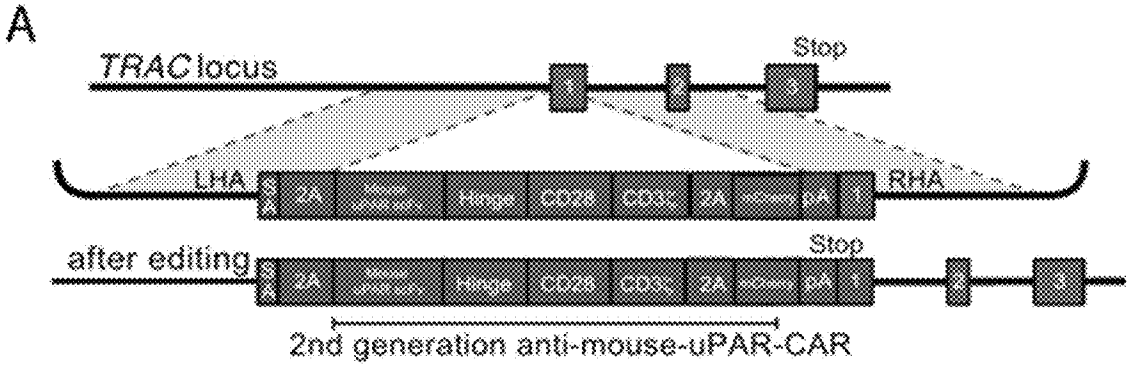


FIG. 3A

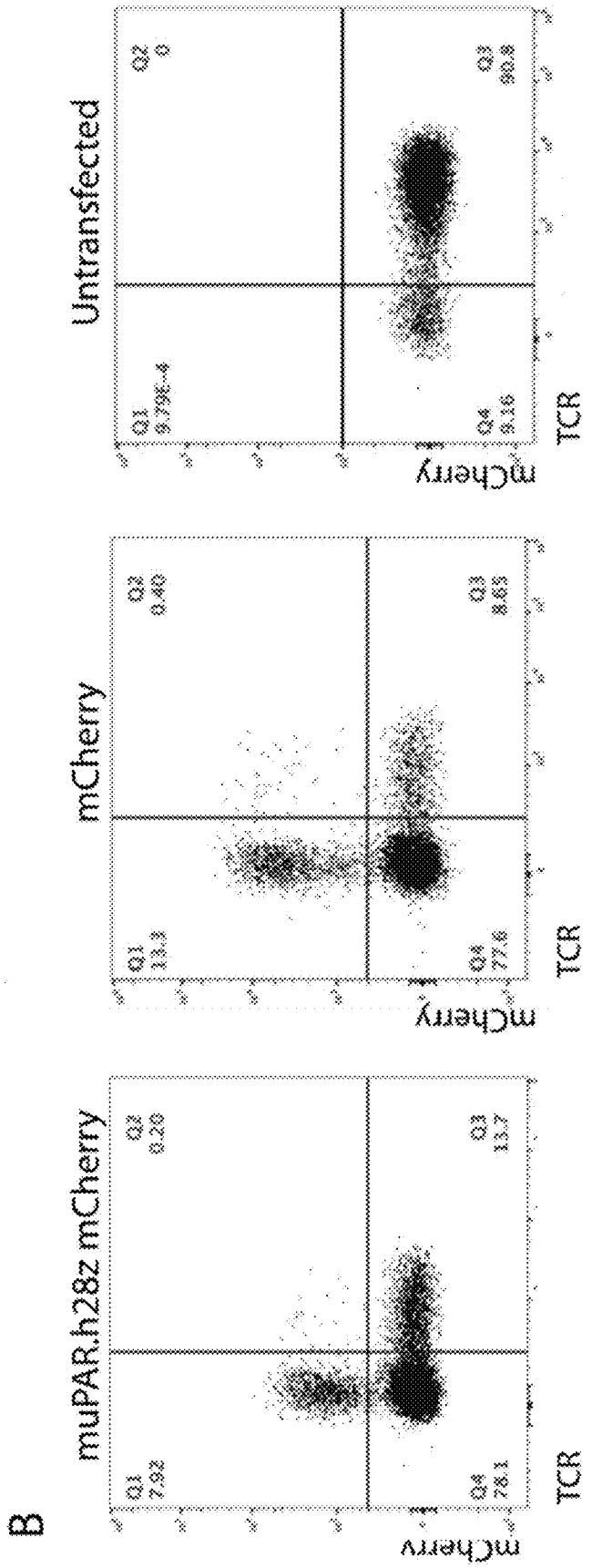

B
muPAR.h28z mCherry
Q2 0.20
Q3 13.7
Q1 7.92
Q4 78.1
mCherry
TCR
mCherry
Q2 0.40
Q3 8.83
Q1 13.3
Q4 77.6
mCherry
TCR
Untransfected
Q2 0
Q3 90.8
Q1 9.79E-4
Q4 9.10
mCherry
TCR

FIG. 3B

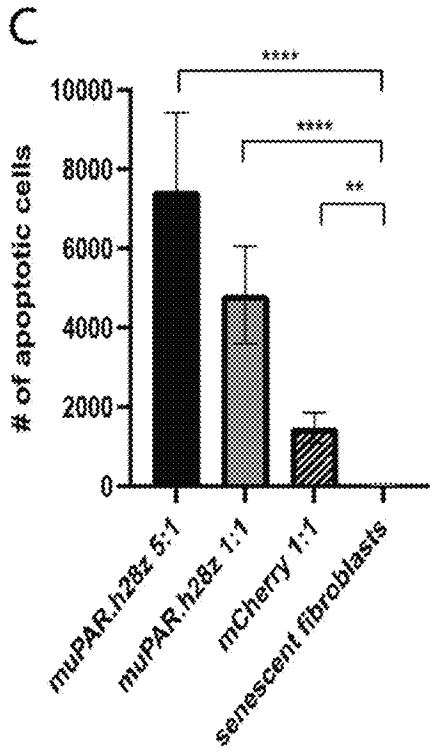

C
****
****
**
10000
8000
6000
4000
2000
0
# of apoptotic cells
muPAR.h28z 5:1
muPAR.h28z 1:1
mCherry 1:1
senescent fibroblasts

FIG. 3C

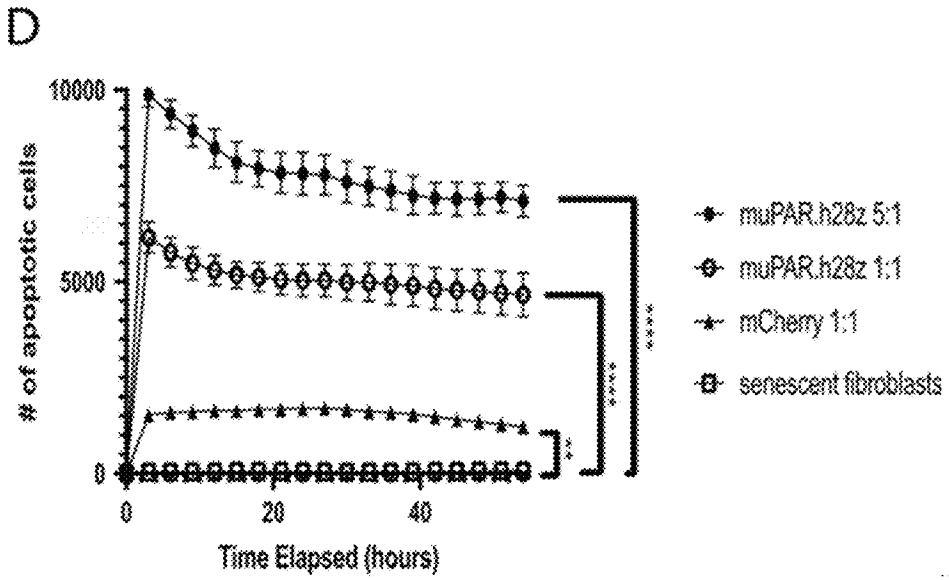

D
10000
5000
0
# of apoptotic cells
0
20
40
Time Elapsed (hours)
muPAR.h28z 5:1
muPAR.h28z 1:1
mCherry 1:1
senescent fibroblasts

FIG. 3D

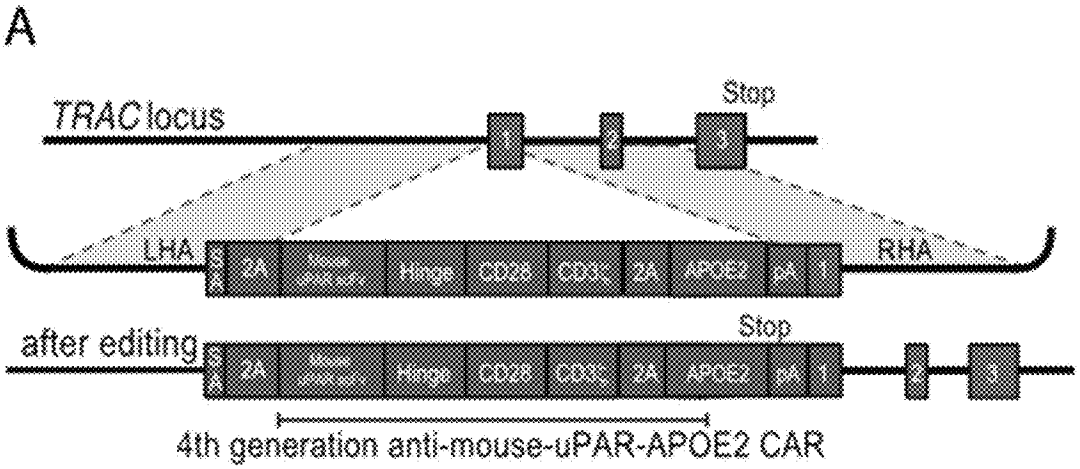

A
TRAC locus
Stop
LHA
RHA
after editing
Stop
4th generation anti-mouse-uPAR-APOE2 CAR

FIG. 4A

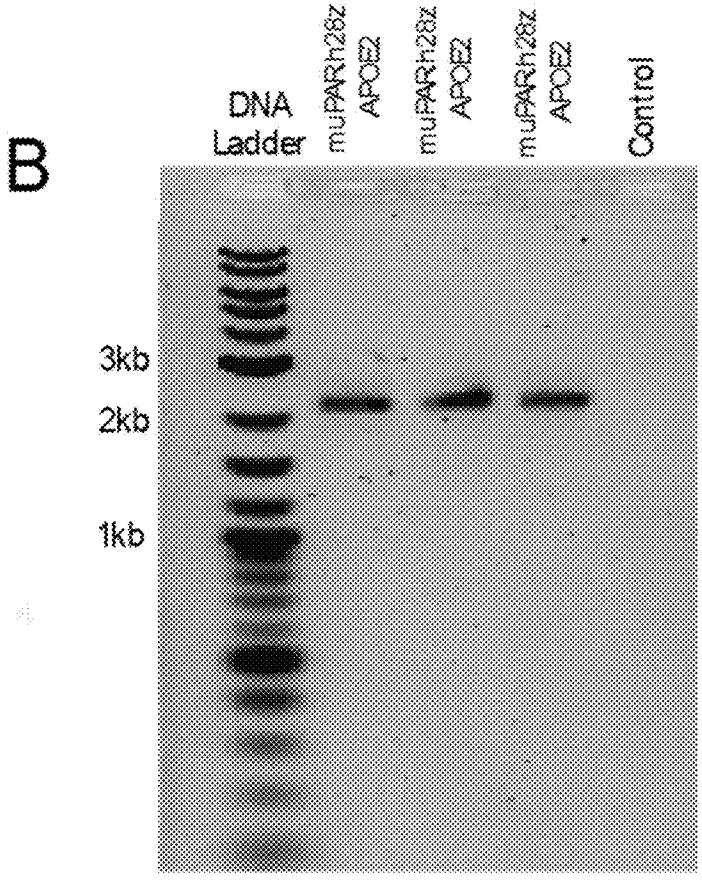

B
DNA Ladder
muPARh28z APOE2
muPARh28z APOE2
muPARh28z APOE2
Control
3kb
2kb
1kb

FIG. 4B

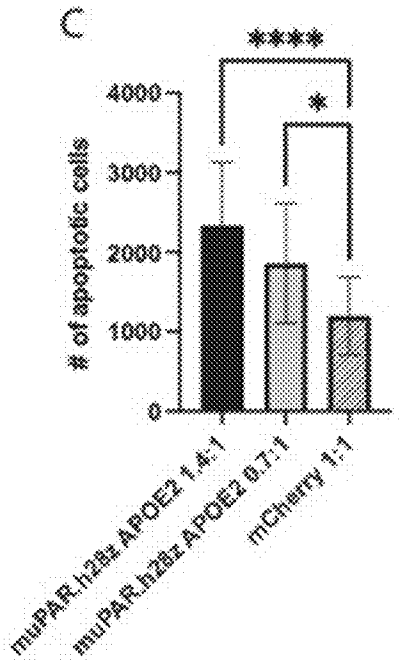

C
****
*
4000
3000
2000
1000
0
# of apoptotic cells
muPAR.h28z APOE2 1.4:1
muPAR.h28z APOE2 0.7:1
mCherry 1:1

FIG. 4C

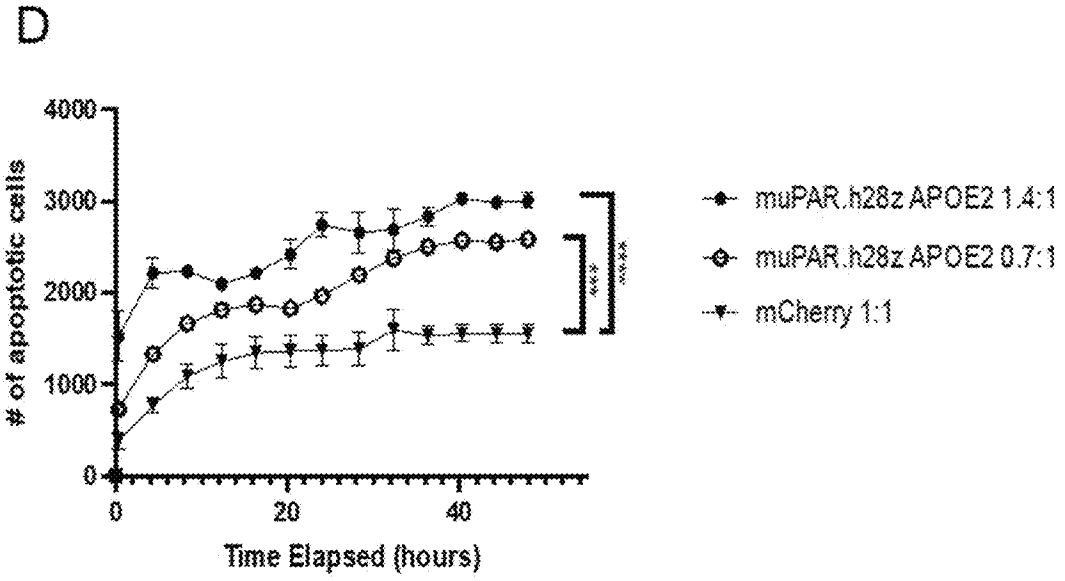

D
4000
3000
2000
1000
0
# of apoptotic cells
0
20
40
Time Elapsed (hours)
muPAR.h28z APOE2 1.4:1
muPAR.h28z APOE2 0.7:1
mCherry 1:1

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 CD8α 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. **1**A-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 Inucyte Live-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. **2**A-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 Inucyte Live-Cell Analysis over 48 hours. *$p<0.05$, one-way ANOVA. *$p<0.01$, one-way ANOVA.

[0035] FIG. **3**A-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 Inucyte Live-Cell Analysis over 48 hours. *$p<0.05$, one-way ANOVA. *$p<0.01$, one-way ANOVA.

[0036] FIGS. **4**A-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: Apolioprotein 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 Inucyte 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 Thosea 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 VHCDRI 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); RASESVDSYGNSFMH (SEQ ID NO: 36), RASNLKS (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)

EVQLVESGGGLVQPGRSLKLSCAASGFTFSNYAMAWVRQA

PTKGLEWVASISTGGG NT YYRD S VKGRFTISRDNAK

NTL YLQMD SLRSEDT AT YYCARQGGGYSD SFD YW

G QGVMVTVSS,
or

(SEQ ID NO: 43)

Q VTLKE S GPGILQP SQTLSLTCSFSGESLSTS GMG

V GWIRQP S GKGLE 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 YRASNL KSGIP

ARFSGSGSGTDFTLTINPVEADDVATYCCQQSNEDPWTFG

GGTKLEIKR,

or

(SEQ ID NO: 46)

NIVMT Q SPKSMSMS VGERVTLT CKASENVVTYV SW

Y QQKPEQ SPKLLIYGASNRYT GVPDRFTGSGSATDFT

LTISSVQAEDLADYHCGQGYSYPYTFGGGTKLEIKR.

[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)

EVQLVESGGGLVQPGRSLKLSCAASGFTFSNYAMAWVRQA

PTKGLEWVASISTGGG NT YYRD S VKGRFTISRDNAK

NTL YLQMD SLRSEDT AT YYCARQGGGYSD SFD YW

G QGVMVTVSSGGGGSGGGGSGGGGSDVQMTQSPSNLAAS

PGESVSINCKASKSISKYL AWYQQKPGK ANKLLIY S

GS TLQS GTP SRF S GS GS GTDFTLTIRNLEPEDF

GL YY C QQH NE YPLTF GSGTKLEIKR;

(SEQ ID NO: 48)

Q VTLKE S GPGILQP SQTLSLTCSFSGFSLSTS GMG

V GWIRQP S GKGLE WLAHI WWDD DKRYNPALKSRL

TISKDPSSNQVFLKIASVDTADIATYYCVRIGGSSGYMDY

WGQGT SVTVSSGGGGSGGGGSGGGGSDIVLTQSPASLAV

SLGQRATISCR ASESVDSYGNSF MHWYQQKPGQPPKLL

IYRASNLKSGIPARFSGSGSGTDFTLTINPVEADDVATYC

CQ Q SNEDP WTFGGGTKLEIKR;
and

(SEQ ID NO: 49)

Q VTLKE S GPGILQP SQTLSLTCSFSGFSLSTS GMG

V GWIRQP S GKGLE 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 YV S W YQQKPEQSPKLLIYGASNRYTGVPDRF

TGSGSATDFTLTISSVQAEDLADYHCGQGY S YP YTF

GGGTKLEIKR.

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

(SEQ ID NO: 50)

GAAGTCCAACTCGTTGAAAGCGGCGGTGGTCTTGTCCAGC

CAGGCAGATCACTG AAACTGTCATGCGCCGCCAGTGGCT

TCACTTTCTCCAATTACGCAATGGCGTGGG TT AGAC A

GGCCCCC ACGAAAGGCTTGGAGT GGGTCGC ATC AAT

CAGT AC AGGAG GT GGAAAC ACTT ACT ATCGCGA

T AGT GTTAAGGGGAGATTC ACGATTAGCCGGG AC A

ACGCGAAAAAC ACGTTGTATCTGC AGATGGACTC ACT

T AGATCCGAGGAC A C AGCGACTT ACT ACTGTGCG

AGGC AGGGCGGAGGGT AT AGT GAT AGCTTT GATT

ACTGGGGCCAGGGCGTAATGGTAACTGTTAGTTCTGGTGG

AGGTGGATCAGGTG GAGGTGGATCTGGTGGAGGTGGATC

TGATGTGCAGATGACACAGAGTCCTTCAAATTTGGCCGCT

TCACCCGGAGAATCAGTAAGTATCAACTGTAAAGCGTCCA

AGTCC ATTTC AAAGT ATTTGGC ATGGTAT C AACA

GAAGCCGGGAAAGGCGAAC AAACTCCTGATTTATAGCGG

GAGTACCTTGCAGTCCGGCACGCCTAGTAGATTTTCAGGC

TCCGGTTCTGGGACCGACTTCACTTTGACGATTCGCAATT

TGGAACCAGAGGATTTTGGGCTGTACTATTGTCAGCAGCA

CAACGAATACCCGTTGACTTTTGGTAGTGGTACAAAGCTG

GAAATCAAGAGAGCGGCC;

(SEQ ID NO: 51)

CAGGTGACCCTGAAGGAGTCCGGCCCCGGCATCCTGCAGC

CCAGCCAGACCCTGAGCCTGACCTGCTCCTTCAGCGGCTT

CTCCCTGTCCACCTCCGGCATGGGCGTGGGCTGGATCAGA

CAGCCCAGCGGCAAGGGCCTGGAGTGGCTGGCCCACATCT

GGT GGGACGATGACAAGAGATACAACCCCGCTCTGAAGA

GCCGGCTGACAATCAGCAAGGACCCTAGCAGTAACCAGGT

GTTCCTGAAGATCGCTTCCGTGGACACAGCAGACATCGCA

ACATACTATTGCGTGCGGATCGGCGGAAGCAGTGGATACA

TGGACTACTGGGGAC AGGGAACC AGCGTGACCGT GAG

CAGT GGT GGAGGT GGAT CAGGTGGAGGTGGATCTGG

TGGAGGTGGATCTGACATCGTGCTGACCCAGAGCCCAGCT

AGCTTGGCAGTGAGCCTGGGACAGAGGGCTACCATCAGCT

GCAGAGCTTCAGAGAGCGTGGACAGCTACGGAAACAGCTT

CATGCACTGGTACCAGCAGAAGCCAGGACAGCC ACCT A

AGCT GCTGATCT ACCGGGCT AGC AACCT GAAGTCC

GGAATCCCTGCTCGGTTTAGCGGAAGCGGTAGCGGCACCG

-continued

ACTTCACCCTGACAATCAACCCAGTGGAGGCCGACGATGT

GGC AACCT ACTGCTGT C AGC AGAGC AACGAGGA

CCC AT GGACCTTCGGCGGT GGAACC AAACT GGAGA

T CAAGAGA;
and

(SEQ ID NO: 52)

CAGGTGACCCTGAAGGAGTCCGGCCCCGGCATCCTGCAGC

CCAGCCAGACCCTGAGCCTGACCTGCTCCTTCAGCGGCTT

CTCCCTGTCCACCTCCGGCATGGGCGTGGGCTGGATCAGA

CAGCCCAGCGGCAAGGGCCTGGAGTGGCTGGCCCACATCT

GGTGGGACGATGACAAGAGATACAACCCCGCTCTGAAGAG

CCGGCTGACAATCAGCAAGGACCCTAGCAGTAACCAGGTG

TTCCTGAAGATCGCTTCCGTGGACACAGCAGACATCGCAA

CATACTATTGCGTGCGGATCGGCGGAAGCAGTGGATACAT

GGACTACTGGGGACAGGGAACCAGCGTGACCGTGAGCAGT

GGTGGAGGTGGATCAGGTGGAGGTGGATCTGGTGGAGGTG

GATCTAACATCGTGATGACCCAGTCCCCTAAGAGCATGAG

CATGAGCGTGGGCGAGAGAGTGACCCTGACCTGCAAAGCC

TCCGAGAACGTGGTGACCTACGTGAGCTGGTACCAGCAGA

AGCCTGAGCAGAGCCCTAAGCTGCTGATCTACGGCGCTTC

CAACAGATACACCGGAGTGCCTGACAGATTCACCGGCAGC

GGAAGCGCAACCGACTTCACCTTGACCATCAGCAGCGTGC

AGGCTGAGGACCTGGCCGACTACCACTGCGGCCAGGGCTA

CAGCTACCCTTACACCTTCGGTGGAGGCACCAAGCTGGAG

ATCAAGCGG.

[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:

(SEQ ID NO: 53)

MRALL ARLLLC VLVV SD SKGSNELHQ VP SN CDC

LN GGT C V SNKYFSNIHW CN CPKKFGGQHCEIDKS

KTCYEGNGHFYRGKASTDTMGRPCLPWNSATVLQQTYHAH

RSDA LQLGLGKHNY CRNPDNRRRP W C YV Q V GL

KPL V QECMVHDCADGKKP;

-continued

or

(SEQ ID NO: 54)

MRALL ARLLLC VLVV SD SKGSNELHQ VP SN CDC

LN GGT C V SNKYFSNIHW CN CPKKFGGQHCEIDKS

KTCYEGNGHFYRGKASTDTMGRPCLPWNSATVLQQTYHAH

RSDA LQLGLGKHNY CRNPDNRRRP W.

[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:

(SEQ ID NO: 55)

ATGAGAGCCCTGCTGGCGCGCCTGCTTCTCTGCGTCCTGG

TCGTGAGCGACTCCA AAGGC AGC AAT GAACTTC AT

C AAGTTCC ATCGAACTGT GACTGTCTAAATGGAGGAA

CATGTGTGTCCAACAAGTACTTCTCCAACATTCACTGGTG

CAACTGCCCAAA GAAATTCGGAGGGC AGC ACTGT GA

AAT AG AT AAGTCAAAAACCTGCT ATGAGGGGAATGG

TCACTTTTACCGAGGAAAGGCCAGCACTGACACCATGGGC

CGGCCCTGCCTGCCCTGGAACTCTGCCACTGTCCTTCAGC

AAACGTACCATGCCCACAGATCT GAT GCTCTTC AGCT

GGGCCTGGGGAAAC AT AATT ACTGC AGGAACCC AG

AC AAC CGGAGGCGACCCTGGTGCTAT GT GC AGGT

GGGCCT AAAGCCGCTTGTCC AAGAG T GC AT GGT

GC ATGACTGCGC AGAT GGAAAAAAGCCC;
or

(SEQ ID NO: 56)

ATGAGAGCCCTGCTGGCGCGCCTGCTTCTCTGCGTCCTGG

TCGTGAGCGACTCCA AAGGC AGC AAT GAACTTC AT

C AAGTTCC ATCGAACTGT GACTGTCTAAATGGAGGAA

CATGTGTGTCCAACAAGTACTTCTCCAACATTCACTGGTG

CAACTGCCCAAA GAAATTCGGAGGGC AGC ACTGT GA

AAT AGAT AAGTCAAAAACCTGCT AT GAGGGGAATGG

TCACTTTTACCGAGGAAAGGCCAGCACTGACACCATGGGC

CGGCCCTGCCTGCCCTGGAACTCTGCCACTGTCCTTCAGC

AAACGTACCATGCCCACAGATCT GAT GCTCTTC AGCT

GGGCCTGGGGAAAC AT AATT ACTGC AGGAACCC A

GAC AAC CGGAGGCGACCCTGG

[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, CD8α, 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 CD8α 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. **1**A 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 Csnl) 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 NanoPhotemeter® N50. Concentrated template products were diluted in Ultra-Pure H20 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 µl 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 Dye™ 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 Attune™ 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 QuickExtract™ (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 Q5™ 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 Biosytems) 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 µl Fixing solution (Millipore Sigma) and incubated at room temperature for 10 minutes, washed twice with 1×PBS and stained with freshly prepared 1×SA-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 1×PBS. 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. **2**A). 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 Immunocult™ 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. **2**B).

[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% confluency 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. **2**C-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. **1**A). 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 Immunocult™ 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. **1**B). 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% confluency 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. **1**C-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

Sequence total quantity: 65
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source 1..2362
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note = muPAR.h28z tNGFR
organism = synthetic construct
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SEQ ID NO: 2           moltype = AA  length = 788
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ASKSISKYLA WYQQKPGKAN KLLIYSGSTL QSGTPSRFSG SGSGTDFTLT IRNLEPEDFG  240
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APPRDFAAYR SRVKFSRSAD APAYQQGQNQ LYNELNLGRR EEYDVLDKRR GRDPEMGGKP  420
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PPRATNFSLL KQAGDVEENP GPMGAGATGR AMDGPRLLLL LLLGVSLGGA KEACPTGLYT  540
HSGECCKACN LGEGVAQPCG ANQTVCEPCL DSVTFSDVVS ATEPCKPCTE CVGLQSMSAP  600
CVEADDAVCR CAYGYYQDET TGRCEACRVC EAGSGLVFSC QDKQNTVCEE CPDGTYSDEA  660
NHVDPCLPCT VCEDTERQLR ECTRWADAEC EEIPGRWITR STPPEGSDST APSTQEPEAP  720
PEQDLIASTV AGVVTTVMGS SQPVVTRGTT DNLIPVYCSI LAAVVVGLVA YIAFKRWNSR  780
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SEQ ID NO: 3           moltype = DNA  length = 2373
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                       note = huPAR.h28z tNGFR
                       organism = synthetic construct
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SEQ ID NO: 4           moltype = AA  length = 791
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                       mol_type = protein
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                       organism = synthetic construct
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ASESVDSYGN SFMHWYQQKP GQPPKLLIYR ASNLKSGIPA RFSGSGSGTD FTLTINPVEA  240
DDVATYCCQQ SNEDPWTFGG GTKLEIKRIE VMYPPPYLDN EKSNGTIIHV KGKHLCPSPL  300
FPGPSKPFWV LVVVGGVLAC YSLLVTVAFI IFWVRSKRSR LLHSDYMNMT PRRPGPTRKH  360
YQPYAPPRDF AAYRSRVKFS RSADAPAYQQ GQNQLYNELN LGRREEYDVL DKRRGRDPEM  420
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MQALPPRATN FSLLKQAGDV EENPGPMGAG ATGRAMDGPR LLLLLLLGVS LGGAKEACPT  540
GLYTHSGECC KACNLGEGVA QPCGANQTVC EPCLDSVTFS DVVSATEPCK PCTECVGLQS  600
MSAPCVEADD AVCRCAYGYY QDETTGRCEA CRVCEAGSGL VFSCQDKQNT VCEECPDGTY  660
SDEANHVDPC LPCTVCEDTE RQLRECTRWA DAECEEIPGR WITRSTPPEG SDSTAPSTQE  720
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SEQ ID NO: 5           moltype = DNA  length = 2625
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                       mol_type = other DNA
                       note = huPAR.h28z mCherry
                       organism = synthetic construct
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SEQ ID NO: 6           moltype = AA  length = 874
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source                 1..874
                       mol_type = protein
                       note = huPAR.h28z mCherry
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ASESVDSYGN SFMHWYQQKP GQPPKLLIYR ASNLKSGIPA RFSGSGSGTD FTLTINPVEA  240
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FPGPSKPFWV LVVVGGVLAC YSLLVTVAFI IFWVRSKRSR LLHSDYMNMT PRRPGPTRKH  360
YQPYAPPRDF AAYRSRVKFS RSADAPAYQQ GQNQLYNELN LGRREEYDVL DKRRGRDPEM  420
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MEGSVNGHEF EIEGEGEGRP YEGTQTAKLK VTKGGPLPFA WDILSPQFMY GSKAYVKHPA  720
DIPDYLKLSF PEGFKWERVM NFEDGGVVTV TQDSSLQDGE FIYKVKLRGT NFPSDGPVMQ  780
KKTMGWEASS ERMYPEDGAL KGEIKQRLKL KDGGHYDAEV KTTYKAKKPV QLPGAYNVNI  840
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SEQ ID NO: 7           moltype = DNA  length = 2382
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source                 1..2382
                       mol_type = other DNA
                       note = muPAR.m28z tNGFR
                       organism = synthetic construct
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GGGYSDSFDY WGQGVMVTVS SGGGGSGGGG SGGGGSDVQM TQSPSNLAAS PGESVSINCK  180
ASKSISKYLA WYQQKPGKAN KLLIYSGSTL QSGTPSRFSG SGSGTDFTLT IRNLEPEDFG  240
LYYCQQHNEY PLTFGSGTKL EIKREQKLIS EEDLIEFMYP PPYLDNERSN GTIIHIKEKH  300
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RKPYQPYAPA RDFAAYRPRV KFSRSAEPPA YQQGQNQLYN ELNLGRREEY DVLDKRRGRD  420
PEMGGKPRRK NPQEGLYNEL QKDKMAEAYS EIGMKGERRR GKGHDGLYQG LSTATKDTYD  480
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LQSMSAPCVE ADDAVCRCAY GYYQDETTGR CEACRVCEAG SGLVFSCQDK QNTVCEECPD  660
GTYSDEANHV DPCLPCTVCE DTERQLRECT RWADAECEEI PGRWITRSTP PEGSDSTAPS  720
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ASKSISKYLA WYQQKPGKAN KLLIYSGSTL QSGTPSRFSG SGSGTDFTLT IRNLEPEDFG  240
LYYCQQHNEY PLTFGSGTKL EIKRIEVMYP PPYLDNEKSN GTIIHVKGKH LCPSPLFPGP  300
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VRLLLPGELA KHAVSEGTKA VTKYTSSKDP PVATMVSKGE EDNMAIIKEF MRFKVHMEGS  660
VNGHEFEIEG EGEGRPYEGT QTAKLKVTKG GPLPFAWDIL SPQFMYGSKA YVKHPADIPD  720
YLKLSFPEGF KWERVMNFED GGVVTVTQDS SLQDGEFIYK VKLRGTNFPS DGPVMQKKTM  780
GWEASSERMY PEDGALKGEI KQRLKLKDGG HYDAEVKTTY KAKKPVQLPG AYNVNIKLDI  840
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FPEGFKWERV MNFEDGGVVT VTQDSSLQDG EFIYKVKLRG TNFPSDGPVM QKKTMGWEAS  660
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SEQ ID NO: 13          moltype = DNA  length = 2484
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SEQ ID NO: 14          moltype = AA  length = 827
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source 1..833
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note = huPAR.h28z APOE2
organism = synthetic construct
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SEQ ID NO: 17 moltype = DNA length = 3279
FEATURE Location/Qualifiers
source 1..3279
mol_type = other DNA
note = miniXon huPAR.h28z mCherry
organism = synthetic construct
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SEQ ID NO: 18          moltype = AA  length = 1077
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SEQ ID NO: 19          moltype = DNA  length = 2889
FEATURE                Location/Qualifiers
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                       organism = synthetic construct
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                       note = miniXon muPAR.m28z mCherry
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SEQ ID NO: 21          moltype = DNA  length = 3138
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SEQ ID NO: 22 moltype = AA length = 1030
FEATURE Location/Qualifiers
source 1..1030
mol_type = protein
note = miniXon muPAR.m28z APOE2
organism = synthetic construct

SEQUENCE: 22

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ELLSSQVTQE LRALMDETMK ELKAYKSELE EQLTPVAEET RARLSKELQA AQARLGADME  840
DVCGRLVQYR GEVQAMLGQS TEELRVRLAS HLRKLRKRLL RDADDLQKCL AVYQAGAREG  900
AERGLSAIRE RLGPLVEQGR VRAATVGSLA GQPLQERAQA WGERLRARME EMGSRTRDRL  960
DEVKEQVAEV RAKLEEQAQQ IRLQAEAFQA RLKSWFEPLV EDMQRQWAGL VEKVQAAVGT  1020
SAAPVPSDNH                                                        1030
```

SEQ ID NO: 23 moltype = DNA length = 3156
FEATURE Location/Qualifiers
source 1..3156
mol_type = other DNA
note = miniXon huPAR.h28z APOE2
organism = synthetic construct

SEQUENCE: 23

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tttctgtaca acttaacctt gcagagagcc actggcatca gctttgccat tcttggaaac  60
ttttctggta agttctctcg ttaccatctt ttgaaatttt aagtgaatta atacatatct  120
tgcttagtct cttgtgcagg aaatgttttc catttatgac aaaacaggaa ttgtgtgaaa  180
tttcaaatat tggattagga aatacaaagt tactgaaagt gaggtactaa tgtttataaa  240
ataaaaactt tttcttgcca tttgcagatt taacattttt gagtcaatcc aagtgccacc  300
atgcaggagg ttcatgattg tgtagagtaa gacataattt tgttgaggtt taactctgaa  360
tacttaatgt ggtactgaat acttaatgtg gtactgagag gcagcctaac tgaccacaca  420
gcattcacgc ctggctaatt tttgtatttt tagtagagac ggggtttcac catggtggcc  480
aggctgggtt ctggttgttt atgatcttta ttttttggtg atctaggaac caaacaacaa  540
gaaattgttg tttcccgtgg gtaagaggat cccgagagat ctggcagcgg agagggcaga  600
ggaagtcttc taacatgcgg tgacgtggag gagaatcccg gccctaggct cgagatggcc  660
ctgccagtaa cggctctgct gctgccactt gctctgctcc tccatgcagc caggcctcag  720
gtgaccctga aggagtccgg ccccggcatc ctgcagccca gccagaccct gagcctgacc  780
tgctccttca gcggcttctc cctgtccacc tccggcatgg gcgtgggctg gatcagacag  840
cccagcggca agggcctgga gtggctggcc cacatctggt gggacgatga caagagatac  900
aaccccgctc tgaagagccg gctgacaatc agcaaggacc ctagcagtaa ccaggtgttc  960
ctgaagatcg cttccgtgga cacagcagac atcgcaacat actattgcgt gcggatcggc  1020
```

-continued

```
ggaagcagtg gatacatgga ctactgggga cagggaacca gcgtgaccgt gagcagtggt  1080
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agcccagcta gcttggcagt gagcctggga cagagggcta ccatcagctg cagagcttca  1200
gagagcgtgg acagctacgg aaacagcttc atgcactggt accagcagaa gccaggacag  1260
ccacctaagc tgctgatcta ccgggctagc aacctgaagt ccggaatccc tgctcggttt  1320
agcggaagcg gtagcggcac cgacttcacc ctgacaatca acccagtgga ggccgacgat  1380
gtggcaacct actgctgtca gcagagcaac gaggacccat ggaccttcgg cggtggaacc  1440
aaactggaga tcaagagaat tgaagttatg tatcctcctc cttacctaga caatgagaag  1500
agcaatggaa ccattatcca tgtgaaaggg aaacaccttt gtccaagtcc cctatttccc  1560
ggaccttcta agcccttttg ggtgctggtg gtggttggtg gagtcctggc ttgctatagc  1620
ttgctagtaa cagtggcctt tattattttc tgggtgagga gtaagaggag caggctcctg  1680
cacagtgact acatgaacat gactccccgc cgccccgggc ccacccgcaa gcattaccag  1740
ccctatgccc caccacgcga cttcgcagcc tatcgctcca gagtgaagtt cagcaggagc  1800
gcagacgccc ccgcgtacca gcagggccag aaccagctct ataacgagct caatctagga  1860
cgaagagagg agtacgatgt tttggacaag agacgtggcc gggaccctga gatgggggga  1920
aagccgagaa ggaagaaccc tcaggaaggc ctgtacaatg aactgcagaa agataagatg  1980
gcggaggcct acagtgagat tgggatgaaa ggcgagcgcc ggaggggcaa ggggcacgat  2040
ggcctttacc agggtctcag tacagccacc aaggacacct acgacgccct tcacatgcag  2100
gccctgcccc ctcgcgcgac caactttagc ctgctgaaac aggcgggcga tgtggaagaa  2160
aacccgggcc cggaacagaa actgattagc gaagaagatc tgatgaaagt tttgtgggcc  2220
gctttgttgg taacgttctt ggcaggctgt caggcgaagg ttgaacaagc agtcgaaacg  2280
gagccagagc cagagctccg acagcagacc gaatggcaat ctggtcaaag gtgggaactt  2340
gcgttgggcc gattttggga ttaccttaga tgggtgcaga cactttcaga acaggttcag  2400
gaggaattgc ttagctcaca ggtaactcag gagttgcgcg cacttatgga cgagacgatg  2460
aaagaactca aggcgtacaa gagcgagctg gaagagcagc tcacacctgt agctgaagaa  2520
acacgcgcac ggttgtctaa agaactccag gctgctcagg cccgcttggg agcagatatg  2580
gaggacgtct gtggaagact cgtccagtat cggggcgagg tgcaggccat gttgggacaa  2640
agtacggaag agcttcgggt aagattggca agccacctca ggaaactgag aaagagactc  2700
ctgagagacg cggatgacct gcagaaatgt cttgcagtgt accaagctgg agctcgcgaa  2760
ggcgctgaac ggggactgag tgcgattaga gaacgattgg gccctcttgt tgaacagggg  2820
agggttagag cggcgactgt cggttctctg gcagggcagc ctctgcaaga gcgcgctcaa  2880
gcttggggtg aacgccttag agcccgaatg gaagagatgg gctctcggac ccgagatcga  2940
cttgatgagg tgaaggagca agtggcggaa gttcgagcta agctggagga acaggcccaa  3000
caaatccgac tccaagccga ggcttttcaa gcaaggctga aaagctggtt tgaacccttg  3060
gtcgaagaca tgcagcgcca gtgggcggga ttggttgaaa aagtccaagc cgcggttggc  3120
acgtccgccg cccccgtgcc aagtgacaat cactaa                             3156

SEQ ID NO: 24          moltype = AA  length = 1036
FEATURE                Location/Qualifiers
source                 1..1036
                       mol_type = protein
                       note = miniXon huPAR.h28z APOE2
                       organism = synthetic construct
SEQUENCE: 24
FLYNLTLQRA TGISFAILGN FSGKFSRYHL LKFVNYISCL VSCAGNVFHL QNRNCVKFQI  60
LDEIQSYKGT NVYKIKTFSC HLQIHFVNPS ATMQEVHDCV EDIILLRFNS EYLMWYILNV  120
VLRGSLTDHT AFTPGFLYFR RGFTMVARLG SGCLSLFFGD LGTKQQEIVV SRGEDPERSG  180
SGEGRGSLLT CGDVEENPGP RLEMALPVTA LLLPLALLLH AARPQVTLKE SGPGILQPSQ  240
TLSLTCSFSG FSLSTSGMGV GWIRQPSGKG LEWLAHIWWD DDKRYNPALK SRLTISKDPS  300
SNQVFLKIAS VDTADIATYY CVRIGGSSGY MDYWGQGTSV TVSSGGGGSG GGGSGGGGSD  360
IVLTQSPASL AVSLGQRATI SCRASESVDS YGNSFMHWYQ QKPGQPPKLL IYRASNLKSG  420
IPARFSGSGS GTDFTLTINP VEADDVATYC CQQSNEDPWT FGGGTKLEIK RIEVMYPPPY  480
LDNEKSNGTI IHVKGKHLCP SPLFPGPSKP FWVLVVVGGV LACYSLLVTV AFIIFWVRSK  540
RSRLLHSDYM NMTPRRPGPT RKHYQPYAPP RDFAAYRSRV KFSRSADAPA YQQGQNQLYN  600
ELNLGRREEY DVLDKRRGRD PEMGGKPRRK NPQEGLYNEL QKDKMAEAYS EIGMKGERRR  660
GKGHDGLYQG LSTATKDTYD ALHMQALPPR ATNFSLLKQA GDVEENPGPE QKLISEEDLM  720
KVLWAALLVT FLAGCQAKVE QAVETEPEPE LRQQTEWQSG QRWELALGRF WDYLRWVQTL  780
SEQVQEELLS SQVTQELRAL MDETMKELKA YKSELEEQLT PVAEETRARL SKELQAAQAR  840
LGADMEDVCG RLVQYRGEVQ AMLGQSTEEL RVRLASHLRK LRKRLLRDAD DLQKCLAVYQ  900
AGAREGAERG LSAIRERLGP LVEQGRVRAA TVGSLAGQPL QERAQAWGER LRARMEEMGS  960
RTRDRLDEVK EQVAEVRAKL EEQAQQIRLQ AEAFQARLKS WFEPLVEDMQ RQWAGLVEKV  1020
QAAVGTSAAP VPSDNH                                                  1036

SEQ ID NO: 25          moltype = DNA  length = 2484
FEATURE                Location/Qualifiers
source                 1..2484
                       mol_type = other DNA
                       note = muPAR.h28z APOE2
                       organism = synthetic construct
SEQUENCE: 25
atggccctgc cagtaacggc tctgctgctg ccacttgctc tgctcctcca tgcagccagg  60
cctgaagtgc agctggtgga aagcggcggc ggcctggtgc agccgggccg cagcctgaaa  120
ctgagctgcg cggcgagcgg ctttaccttt agcaactatg cgatggcgtg ggtgcgccag  180
gcgccgacca aaggcctgga atgggtggcg agcattagca ccggcggcgg caacacctat  240
tatcgcgata gcgtgaaagg ccgctttacc attagccgcg ataacgcgaa aaacaccctg  300
tatctgcaga tggatagcct gcgcagcgaa gataccgcga cctattattg cgcgcgccag  360
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ggcggcggct atagcgatag ctttgattat tggggccagg gcgtgatggt gaccgtgagc  420
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acccagagcc cgagcaacct ggcggcgagc ccgggcgaaa gcgtgagcat taactgcaaa  540
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agcggcagcg gcaccgattt taccctgacc attcgcaacc tggaaccgga agattttggc  720
ctgtattatt gccagcagca taacgaatat ccgctgacct ttggcagcgg caccaaactg  780
gaaattaaac gcattgaagt tatgtatcct cctccttacc tagacaatga gaagagcaat  840
ggaaccatta tccatgtgaa agggaaacac ctttgtccaa gtcccctatt tcccggacct  900
tctaagccct tttgggtgct ggtggtggtt ggtggagtcc tggcttgcta tagcttgcta  960
gtaacagtgg cctttattat tttctgggtg aggagtaaga ggagcaggct cctgcacagt 1020
gactacatga acatgactcc ccgccgcccc gggcccaccc gcaagcatta ccagccctat 1080
gccccaccac gcgacttcgc agcctatcgc tccagagtga agttcagcag gagcgcagac 1140
gccccgcgt accagcaggg ccagaaccag ctctataacg agctcaatct aggacgaaga 1200
gaggagtacg atgttttgga caagagacgt ggccgggacc ctgagatggg gggaaagccg 1260
agaaggaaga accctcagga aggcctgtac aatgaactgc agaaagataa gatggcggag 1320
gcctacagtg agattgggat gaaaggcgag cgccggaggg gcaaggggca cgatggcctt 1380
taccagggtc tcagtacagc caccaaggac acctacgacg cccttcacat gcaggccctg 1440
ccccctcgcg cgaccaactt tagcctgctg aaacaggcgg gcgatgtgga agaaaacccg 1500
ggcccggact acaaagacga tgacgacaag atgaaagttt tgtgggccgc tttgttggta 1560
acgttcttgg caggctgtca ggcgaaggtt gaacaagcag tcgaaacgga gccagagcca 1620
gagctccgac agcagaccga atggcaatct ggtcaaaggt gggaacttgc gttgggccga 1680
ttttgggatt accttagatg ggtgcagaca ctttcagaac aggttcagga ggaattgctt 1740
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gcgtacaaga gcgagctgga agagcagctc acacctgtag ctgaagaaac acgcgcacgg 1860
ttgtctaaag aactccaggc tgctcaggcc cgcttgggag cagatatgga ggacgtctgt 1920
ggaagactcg tccagtatcg gggcgaggtg caggccatgt tgggacaaag tacggaagag 1980
cttcgggtaa gattggcaag ccacctcagg aaactgagaa agagactcct gagagacgcg 2040
gatgacctgc agaaatgtct tgcagtgtac caagctggag ctcgcgaagg cgctgaacgg 2100
ggactgagtg cgattagaga acgattgggc cctcttgttg aacaggggag ggttagagcg 2160
gcgactgtcg gttctctggc agggcagcct ctgcaagagc gcgctcaagc ttggggtgaa 2220
cgccttagag cccgaatgga agagatgggc tctcggaccc gagatcgact tgatgaggtg 2280
aaggagcaag tggcggaagt tcgagctaag ctggaggaac aggcccaaca aatccgactc 2340
caagccgagg cttttcaagc aaggctgaaa agctggtttg aacccttggt cgaagacatg 2400
cagcgccagt gggcgggatt ggttgaaaaa gtccaagccg cggttggcac gtccgccgcc 2460
cccgtgccaa gtgacaatca ctaa                                        2484
```

```
SEQ ID NO: 26          moltype = AA  length = 827
FEATURE                Location/Qualifiers
source                 1..827
                       mol_type = protein
                       note = muPAR.h28z APOE2
                       organism = synthetic construct
SEQUENCE: 26
MALPVTALLL PLALLLHAAR PEVQLVESGG GLVQPGRSLK LSCAASGFTF SNYAMAWVRQ  60
APTKGLEWVA SISTGGGNTY YRDSVKGRFT ISRDNAKNTL YLQMDSLRSE DTATYYCARQ  120
GGGYSDSFDY WGQGVMVTVS SGGGGSGGGG SGGGGSDVQM TQSPSNLAAS PGESVSINCK  180
ASKSISKYLA WYQQKPGKAN KLLIYSGSTL QSGTPSRFSG SGSGTDFTLT IRNLEPEDFG  240
LYYCQQHNEY PLTFGSGTKL EIKRIEVMYP PPYLDNEKSN GTIIHVKGKH LCPSPLFPGP  300
SKPFWVLVVV GGVLACYSLL VTVAFIIFWV RSKRSRLLHS DYMNMTPRRP GPTRKHYQPY  360
APPRDFAAYR SRVKFSRSAD APAYQQGQNQ LYNELNLGRR EEYDVLDKRR GRDPEMGGKP  420
RRKNPQEGLY NELQKDKMAE AYSEIGMKGE RRRGKGHDGL YQGLSTATKD TYDALHMQAL  480
PPRATNFSLL KQAGDVEENP GPDYKDDDDK MKVLWAALLV TFLAGCQAKV EQAVETEPEP  540
ELRQQTEWQS GQRWELALGR FWDYLRWVQT LSEQVQEELL SSQVTQELRA LMDETMKELK  600
AYKSELEEQL TPVAEETRAR LSKELQAAQA RLGADMEDVC GRLVQYRGEV QAMLGQSTEE  660
LRVRLASHLR KLRKRLLRDA DDLQKCLAVY QAGAREGAER GLSAIRERLG PLVEQGRVRA  720
ATVGSLAGQP LQERAQAWGE RLRARMEEMG SRTRDRLDEV KEQVAEVRAK LEEQAQQIRL  780
QAEAFQARLK SWFEPLVEDM QRQWAGLVEK VQAAVGTSAA PVPSDNH                827
```

```
SEQ ID NO: 27          moltype = AA  length = 7
FEATURE                Location/Qualifiers
source                 1..7
                       mol_type = protein
                       note = VHCDRI sequence
                       organism = synthetic construct
SEQUENCE: 27
GFTFSNY                                                            7
```

```
SEQ ID NO: 28          moltype = AA  length = 6
FEATURE                Location/Qualifiers
source                 1..6
                       mol_type = protein
                       note = VHCDR2 sequence
                       organism = synthetic construct
SEQUENCE: 28
STGGGN                                                             6
```

-continued

```
SEQ ID NO: 29          moltype = AA  length = 11
FEATURE                Location/Qualifiers
source                 1..11
                       mol_type = protein
                       note = VHCDR3 sequence
                       organism = synthetic construct
SEQUENCE: 29
QGGGYSDSFD Y                                                    11

SEQ ID NO: 30          moltype = AA  length = 9
FEATURE                Location/Qualifiers
source                 1..9
                       mol_type = protein
                       note = VHCDRI sequence
                       organism = synthetic construct
SEQUENCE: 30
GFSLSTSGM                                                       9

SEQ ID NO: 31          moltype = AA  length = 5
FEATURE                Location/Qualifiers
source                 1..5
                       mol_type = protein
                       note = VHCDR2 sequence
                       organism = synthetic construct
SEQUENCE: 31
WWDDD                                                           5

SEQ ID NO: 32          moltype = AA  length = 10
FEATURE                Location/Qualifiers
source                 1..10
                       mol_type = protein
                       note = VHCDR3 sequence
                       organism = synthetic construct
SEQUENCE: 32
IGGSSGYMDY                                                      10

SEQ ID NO: 33          moltype = AA  length = 11
FEATURE                Location/Qualifiers
source                 1..11
                       mol_type = protein
                       note = VHCDRI sequence
                       organism = synthetic construct
SEQUENCE: 33
KASKSISKYL A                                                    11

SEQ ID NO: 34          moltype = AA  length = 7
FEATURE                Location/Qualifiers
source                 1..7
                       mol_type = protein
                       note = VHCDR2 sequence
                       organism = synthetic construct
SEQUENCE: 34
SGSTLQS                                                         7

SEQ ID NO: 35          moltype = AA  length = 9
FEATURE                Location/Qualifiers
source                 1..9
                       mol_type = protein
                       note = VHCDR3 sequence
                       organism = synthetic construct
SEQUENCE: 35
QQHNEYPLT                                                       9

SEQ ID NO: 36          moltype = AA  length = 15
FEATURE                Location/Qualifiers
source                 1..15
                       mol_type = protein
                       note = VHCDRI sequence
                       organism = synthetic construct
SEQUENCE: 36
RASESVDSYG NSFMH                                                15

SEQ ID NO: 37          moltype = AA  length = 7
FEATURE                Location/Qualifiers
source                 1..7
```

-continued

```
                     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
QQSNEDPWT                                                        9

SEQ ID NO: 39        moltype = AA  length = 11
FEATURE              Location/Qualifiers
source               1..11
                     mol_type = protein
                     note = VHCDRI 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 LVQPGRSLKL SCAASGFTFS NYAMAWVRQA PTKGLEWVAS ISTGGGNTYY  60
RDSVKGRFTI SRDNAKNTLY 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 TCSFSGFSLS 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
RFSGSGSGTD FTLTIRNLEP 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 SYGNSFMHWY QQKPGQPPKL LIYRASNLKS  60
GIPARFSGSG SGTDFTLTIN PVEADDVATY CCQQSNEDPW TFGGGTKLEI 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
EVQLVESGGG LVQPGRSLKL SCAASGFTFS NYAMAWVRQA PTKGLEWVAS ISTGGGNTYY  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
QVTLKESGPG ILQPSQTLSL TCSFSGFSLS TSGMGVGWIR QPSGKGLEWL AHIWWDDDKR  60
YNPALKSRLT ISKDPSSNQV FLKIASVDTA DIATYYCVRI GGSSGYMDYW GQGTSVTVSS  120
GGGGSGGGGS GGGGSDIVLT QSPASLAVSL GQRATISCRA SESVDSYGNS FMHWYQQKPG  180
QPPKLLIYRA SNLKSGIPAR 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
QVTLKESGPG ILQPSQTLSL TCSFSGFSLS TSGMGVGWIR QPSGKGLEWL AHIWWDDDKR  60
YNPALKSRLT ISKDPSSNQV FLKIASVDTA DIATYYCVRI GGSSGYMDYW GQGTSVTVSS  120
GGGGSGGGGS GGGGSNIVMT QSPKSMSMSV GERVTLTCKA SENVVTYVSW YQQKPEQSPK  180
LLIYGASNRY TGVPDRFTGS GSATDFTLTI SSVQAEDLAD YHCGQGYSYP YTFGGGTKLE  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
gaagtccaac tcgttgaaag cggcggtggt cttgtccagc caggcagatc actgaaactg  60
tcatgcgccg ccagtggctt cactttctcc aattacgcaa tggcgtgggt tagacaggcc  120
cccacgaaag gcttggagtg ggtcgcatca atcagtacag gaggtggaaa cacttactat  180
cgcgatagtg ttaaggggag attcacgatt agccgggaca acgcgaaaaa cacgttgtat  240
ctgcagatgg actcacttag atccgaggac acagcgactt actactgtgc gaggcagggc  300
ggagggtata gtgatagctt tgattactgg ggccagggcg taatggtaac tgttagttct  360
ggtggaggtg gatcaggtgg aggtggatct ggtggaggtg gatctgatgt gcagatgaca  420
cagagtcctt caaatttggc cgcttcaccc ggagaatcag taagtatcaa ctgtaaagcg  480
tccaagtcca tttcaaagta tttggcatgg tatcaacaga agccgggaaa ggcgaacaaa  540
ctcctgattt atagcgggag taccttgcag tccggcacgc ctagtagatt ttcaggctcc  600
ggttctggga ccgacttcac tttgacgatt cgcaatttgg aaccagagga ttttgggctg  660
tactattgtc agcagcacaa cgaatacccg ttgacttttg gtagtggtac 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 cggccccggc atcctgcagc ccagccagac cctgagcctg  60
acctgctcct tcagcggctt ctccctgtcc acctccggca tgggcgtggg ctggatcaga  120
cagcccagcg gcaagggcct ggagtggctg gcccacatct ggtgggacga tgacaagaga  180
tacaaccccg ctctgaagag ccggctgaca atcagcaagg accctagcag taaccaggtg  240
ttcctgaaga tcgcttccgt ggacacagca gacatcgcaa catactattg cgtgcggatc  300
ggcggaagca gtggatacat ggactactgg ggacagggaa ccagcgtgac cgtgagcagt  360
ggtggaggtg gatcaggtgg aggtggatct ggtggaggtg gatctgacat cgtgctgacc  420
cagagcccag ctagcttggc agtgagcctg ggacagaggg ctaccatcag ctgcagagct  480
tcagagagcg tggacagcta cggaaacagc ttcatgcact ggtaccagca gaagccagga  540
cagccaccta agctgctgat ctaccgggct agcaacctga agtccggaat ccctgctcgg  600
tttagcggaa gcggtagcgg caccgacttc accctgacaa tcaacccagt ggaggccgac  660
gatgtggcaa cctactgctg tcagcagagc aacgaggacc catggacctt cggcggtgga  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 cggccccggc atcctgcagc ccagccagac cctgagcctg  60
acctgctcct tcagcggctt ctccctgtcc acctccggca tgggcgtggg ctggatcaga  120
cagcccagcg gcaagggcct ggagtggctg gcccacatct ggtgggacga tgacaagaga  180
tacaaccccg ctctgaagag ccggctgaca atcagcaagg accctagcag taaccaggtg  240
ttcctgaaga tcgcttccgt ggacacagca gacatcgcaa catactattg cgtgcggatc  300
ggcggaagca gtggatacat ggactactgg ggacagggaa ccagcgtgac cgtgagcagt  360
ggtggaggtg gatcaggtgg aggtggatct ggtggaggtg gatctaacat cgtgatgacc  420
cagtccccta agagcatgag catgagcgtg ggcgagagag tgaccctgac ctgcaaagcc  480
tccgagaacg tggtgaccta cgtgagctgg taccagcaga agcctgagca gagccctaag  540
ctgctgatct acggcgcttc caacagatac accggagtgc ctgacagatt caccggcagc  600
ggaagcgcaa ccgacttcac cttgaccatc agcagcgtgc aggctgagga cctggccgac  660
taccactgcg gccagggcta cagctaccct tacaccttcg gtggaggcac caagctggag  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 CDCLNGGTCV SNKYFSNIHW CNCPKKFGGQ  60
HCEIDKSKTC YEGNGHFYRG KASTDTMGRP CLPWNSATVL QQTYHAHRSD ALQLGLGKHN  120
YCRNPDNRRR PWCYVQVGLK 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 CDCLNGGTCV SNKYFSNIHW CNCPKKFGGQ  60
HCEIDKSKTC YEGNGHFYRG KASTDTMGRP CLPWNSATVL QQTYHAHRSD ALQLGLGKHN  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 cctgcttctc tgcgtcctgg tcgtgagcga ctccaaaggc  60
agcaatgaac ttcatcaagt tccatcgaac tgtgactgtc taaatggagg aacatgtgtg  120
tccaacaagt acttctccaa cattcactgg tgcaactgcc caaagaaatt cggagggcag  180
cactgtgaaa tagataagtc aaaaacctgc tatgagggga atggtcactt ttaccgagga  240
aaggccagca ctgacaccat gggccggccc tgcctgccct ggaactctgc cactgtcctt  300
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cagcaaacgt accatgccca cagatctgat gctcttcagc tgggcctggg gaaacataat  360
tactgcagga acccagacaa ccggaggcga ccctggtgct atgtgcaggt gggcctaaag  420
ccgcttgtcc aagagtgcat ggtgcatgac tgcgcagatg gaaaaaagcc 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
atgagagccc tgctggcgcg cctgcttctc tgcgtcctgg tcgtgagcga ctccaaaggc  60
agcaatgaac ttcatcaagt tccatcgaac tgtgactgtc taaatggagg aacatgtgtg  120
tccaacaagt acttctccaa cattcactgg tgcaactgcc caaagaaatt cggagggcag  180
cactgtgaaa tagataagtc aaaaacctgc tatgagggga atggtcactt ttaccgagga  240
aaggccagca ctgacaccat gggccggccc tgcctgccct ggaactctgc cactgtcctt  300
cagcaaacgt accatgccca cagatctgat gctcttcagc tgggcctggg gaaacataat  360
tactgcagga acccagacaa ccggaggcga 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 KFKVLGNTDR HSIKKNLIGA LLFDSGETAE  60
ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG  120
NIVDEVAYHE KYPTIYHLRK KLVDSTDKAD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD  180
VDKLFIQLVQ TYNQLFEENP INASGVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN  240
LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAI  300
LLSDILRVNT EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA  360
GYIDGGASQE EFYKFIKPIL EKMDGTEELL VKLNREDLLR KQRTFDNGSI PHQIHLGELH  420
AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE  480
VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL  540
SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI  600
IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG  660
RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL  720
HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER  780
MKRIEEGIKE LGSQILKEHP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDH  840
IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL  900
TKAERGGLSE LDKAGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVKVITLKS  960
KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK  1020
MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF  1080
ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA  1140
YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKNPID FLEAKGYKEV KKDLIIKLPK  1200
YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE  1260
QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA  1320
PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ 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
MLFNKCIIIS INLDFSNKEK CMTKPYSIGL DIGTNSVGWA VITDNYKVPS KKMKVLGNTS  60
KKYIKKNLLG VLLFDSGITA EGRRLKRTAR RRYTRRRNRI LYLQEIFSTE MATLDDAFFQ  120
RLDDSFLVPD DKRDSKYPIF GNLVEEKVYH DEFPTIYHLR KYLADSTKKA DLRLVYLALA  180
HMIKYRGHFL IEGEFNSKNN DIQKNFQDFL DTYNAIFESD LSLENSKQLE EIVKDKISKL  240
EKKDRILKLF PGEKNSGIFS EFLKLIVGNQ ADFRKCFNLD EKASLHFSKE SYDEDLETLL  300
GYIGDDYSDV FLKAKKLYDA ILLSGFLTVT DNETEAPLSS AMIKRYNEHK EDLALLKEYI  360
RNISLKTYNE VFKDDTKNGY AGYIDGKTNQ EDFYVYLKNL LAEFEGADYF LEKIDREDFL  420
RKQRTFDNGS IPYQIHLQEM RAILDKQAKF YPFLAKNKER IEKILTFRIP YYVGPLARGN  480
SDFAWSIRKR NEKITPWNFE DVIDKESSAE AFINRMTSFD LYLPEEKVLP KHSLLYETFN  540
VYNELTKVRF IAESMRDYQF LDSKQKKDIV RLYFKDKRKV TDKDIIEYLH AIYGYDGIEL  600
KGIEKQFNSS LSTYHDLLNI INDKEFLDDS SNEAIIEEII HTLTIFEDRE MIKQRLSKFE  660
NIFDKSVLKK LSRRHYTGWG KLSAKLINGI RDEKSGNTIL DYLIDDGISN RNFMQLIHDD  720
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ALSFKKKIQK AQIIGDEDKG NIKEVVKSLP GSPAIKKGIL QSIKIVDELV KVMGGRKPES  780
IVVEMARENQ YTNQGKSNSQ QRLKRLEKSL KELGSKILKE NIPAKLSKID NNALQNDRLY  840
LYYLQNGKDM YTGDDLDIDR LSNYDIDHII PQAFLKDNSI DNKVLVSSAS NRGKSDDFPS  900
LEVVKKRKTF WYQLLKSKLI SQRKFDNLTK AERGGLLPED KAGFIQRQLV ETRQITKHVA  960
RLLDEKFNNK KDENNRAVRT VKIITLKSTL VSQFRKDFEL YKVREINDFH HAHDAYLNAV  1020
IASALLKKYP KLEPEFVYGD YPKYNSFRER KSATEKVYFY SNIMNIFKKS ISLADGRVIE  1080
RPLIEVNEET GESVWNKESD LATVRRVLSY PQVNVVKKVE EQNHGLDRGK PKGLFNANLS  1140
SKPKPNSNEN LVGAKEYLDP KKYGGYAGIS NSFAVLVKGT IEKGAKKKIT NVLEFQGISI  1200
LDRINYRKDK LNFLLEKGYK DIELIIELPK YSLFELSDGS RRMLASILST NNKRGEIHKG  1260
NQIFLSQKFV KLLYHAKRIS NTINENHRKY VENHKKEFEE LFYYILEFNE NYVGAKKNGK  1320
LLNSAFQSWQ NHSIDELCSS FIGPTGSERK GLFELTSRGS AADFEFLGVK IPRYRDYTPS  1380
SLLKDATLIH QSVTGLYETR IDLAKLGEG                                    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 SVGWAMVEID EEENPIRLID LGVRVFERAE VPKTGDSLAM  60
VRRLARSVRR LTRRRAHRLL RARRLLKREG VLQAADFDEN GLIKSLPNTP WQLRAAALDR  120
KLTPLEWSAV LLHLIKHRGY LSQRKNEGET ADKELGALLK GVADNAHALQ TGDFRTPAEL  180
ALNKFEKESG HIRNQRGDYS HTFSRKDLQA ELILLFEKQK EFGNPHISGG LKEGIETLLM  240
TQRPALSGDA VQKMLGHCTF EPAEPKAAKN TYTAERFIWL TKLNNLRILE QGSERPLTDT  300
ERATLMDEPY RKSKLTYAQA RKLLGLEDTA FFKGLRYGKD NAEASTLMEM KAYHAISRAL  360
EKEGLKDKKS PLNLSPELQD EIGTAFSLFK TDEDITGRLK DRIQPEILEA LLKHISFDKF  420
VQISLKALRR IVPLMEQGKR YDEACAEIYG DHYGKKNTEE KIYLPPIPAD EIRNPVVLRA  480
LSQARKVING VVRRYGSPAR IHIETAREVG KSFKDRKEIE KRQEENRKDR EKAAAKFREY  540
FPNFVGEPKS KDILKLRLYE QQHGKCLYSG KEINLGRLNE KGYVEIDHAL PFSRTWDDSF  600
NNKVLVLGSE NQNKGNQTPY EYFNGKDNSR EWQEFKARVE TSRFPRSKKQ RILLQKFDED  660
GFKERNLNDT RYVNRFLCQF VADRMRLTGK GKKRVFASNG QITNLLRGFW GLRKVRAEND  720
RHHALDAVVV ACSTVAMQQK ITRFVRYKEM NAFDGKTIDK ETGEVLHQKT HFPQPWEFFA  780
QEVMIRVFGK PDGKPEFEEA DTPEKLRTLL AEKLSSRPEA VHEYVTPLFV SRAPNRKMSG  840
QGHMETVKSA KRLDEGVSVL RVPLTQLKLK DLEKMVNRER EPKLYEALKA RLEAHKDDPA  900
KAFAEPFYKY DKAGNRTQQV KAVRVEQVQK TGVWVRNHNG IADNATMVRV DVFEKGDKYY  960
LVPIYSWQVA KGILPDRAVV QGKDEEDWQL IDDSFNFKFS LHPNDLVEVI TKKARMFGYF  1020
ASCHRGTGNI NIRIHDLDHK IGKNGILEGI GVKTALSFQK YQIDELGKEI RPCRLKKRPP  1080
VR                                                                 1082

SEQ ID NO: 61          moltype = AA  length = 1395
FEATURE                Location/Qualifiers
source                 1..1395
                       mol_type = protein
                       organism = Treponema denticola
SEQUENCE: 61
MKKEIKDYFL GLDVGTGSVG WAVTDTDYKL LKANRKDLWG MRCFETAETA EVRRLHRGAR  60
RRIERRKKRI KLLQELFSQE IAKTDEGFFQ RMKESPFYAE DKTILQENTL FNDKDFADKT  120
YHKAYPTINH LIKAWIENKV KPDPRLLYLA CHNIIKKRGH FLFEGDFDSE NQFDTSIQAL  180
FEYLREDMEV DIDADSQKVK EILKDSSLKN SEKQSRLNKI LGLKPSDKQK KAITNLISGN  240
KINFADLYDN PDLKDAEKNS ISFSKDDFDA LSDDLASILG DSFELLLKAK AVYNCSVLSK  300
VIGDEQYLSF AKVKIYEKHK TDLTKLKNVI KKHFPKDYKK VFGYNKNEKN NNNYSGYVGV  360
CKTKSKKLII NNSVNQEDFY KFLKTILSAK SEIKEVNDIL TEIETGTFLP KQISKSNAEI  420
PYQLRKMELE KILSNAEKHF SFLKQKDEKG LSHSEKIIML LTFKIPYYIG PINDNHKKFF  480
PDRCWVVKKE KSPSGKTTPW NFFDHIDKEK TAEAFITSRT NFCTYLVGES VLPKSSLLYS  540
EYTVLNEINN LQIIIDGKNI CDIKLKQKIY EDLFKKYKKI TQKQISTFIK HEGICNKTDE  600
VIILGIDKEC TSSLKSYIEL KNIFGKQVDE ISTKNMLEEI IRWATIYDEG EGKTILKTKI  660
KAEYGKYCSD EQIKKILNLK FSGWGRLSRK FLETVTSEMP GFSEPVNIIT AMRETQNNLM  720
ELLSSEFTFT ENIKKINSGF EDAEKQFSYD GLVKPLFLSP SVKKMLWQTL KLVKEISHIT  780
QAPPKKIFIE MAKGAELEPA RTKTRLKILQ DLYNNCKNDA DAFSSEIKDL SGKIENEDNL  840
RLRSDKLYLY YTQLGKCMYC GKPIEIGHVF DTSNYDIDHI YPQSKIKDDS ISNRVLVCSS  900
CNKNKEDKYP LKSEIQSKQR GFWNFLQRNN FISLEKLNRL TRATPISDDE TAKFIARQLV  960
ETRQATKVAA KVLEKMFPET KIVYSKAETV SMFRNKFDIV KCREINDFHH AHDAYLNIVV  1020
GNVYNTKFTN NPWNFIKEKR DNPKIADTYN YYKVFDYDVK RNNITAWEKG KTIITVKDML  1080
KRNTPIYTRQ AACKKGELFN QTIMKKGLGQ HPLKKEGPFS NISKYGGYNK VSAAYYTLIE  1140
YEEKGNKIRS LETIPLYLVK DIQKDQDVLK SYLTDLLGKK EFKILVPKIK INSLLKINGF  1200
PCHITGKTND SFLLRPAVQF CCSNNEVLYF KKIIRFSEIR SQREKIGKTI SPYEDLSFRS  1260
YIKENLWKKT KNDEIGEKEF YDLLQKKNLE IYDMLLTKHK DTIYKKRPNS ATIDILVKGK  1320
EKFKSLIIEN QFEVILEILK LFSATRNVSD LQHIGGSKYS GVAKIGNKIS SLDNCILIYQ  1380
SITGIFEKRI DLLKV                                                   1395

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

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SEQUENCE: 62
cagggttctg gatatctgt                                              19

SEQ ID NO: 63          moltype = DNA  length = 20
FEATURE                Location/Qualifiers
source                 1..20
                       mol_type = other DNA
                       note = guide sequence
                       organism = synthetic construct
SEQUENCE: 63
gggagtcaaa gtcggtgaac                                             20

SEQ ID NO: 64          moltype = DNA  length = 22
FEATURE                Location/Qualifiers
source                 1..22
                       mol_type = other DNA
                       note = primer
                       organism = synthetic construct
SEQUENCE: 64
atcttgtgcg catgtgaggg gc                                          22

SEQ ID NO: 65          moltype = DNA  length = 22
FEATURE                Location/Qualifiers
source                 1..22
                       mol_type = other DNA
                       note = primer
                       organism = synthetic construct
SEQUENCE: 65
gcaagccagg 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.

* * * * *