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(54) **NONVIRAL GENERATION OF GENOME EDITED CHIMERIC ANTIGEN RECEPTOR T CELLS**

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(21) Appl. No.: **17/407,606**

(22) Filed: **Aug. 20, 2021**

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(60) Provisional application No. 62/982,847, filed on Feb. 28, 2020.

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A61K 35/17	(2006.01)

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

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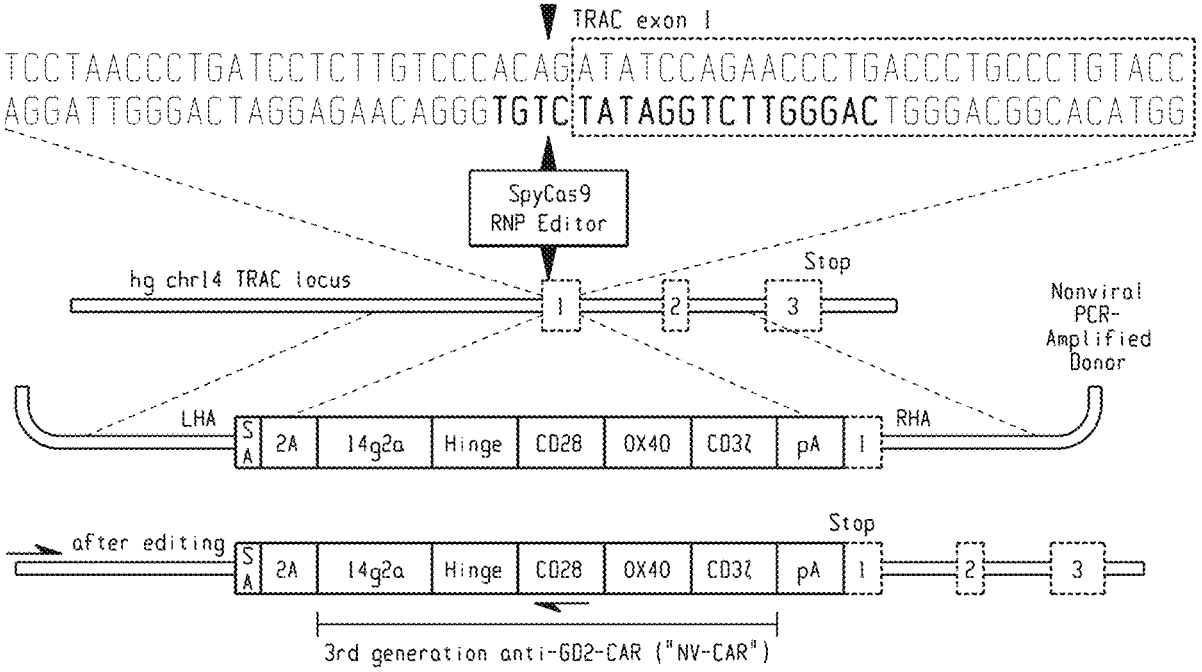
(57) **ABSTRACT**

Described herein are non-viral, ex vivo methods of site-specifically inserting a transgene containing a chimeric antigen receptor (CAR) gene into a T cell genome by introducing into a population of unmodified T cells a Cas9 ribonucleoprotein (RNP) and a non-viral double-stranded homology-directed repair (HDR) template, to provide genome-edited T cells. The Cas9 ribonucleoprotein includes a Cas9 protein and a guide RNA that directs double stranded DNA cleavage of a cleavage site in a T cell expressed gene. The non-viral double-stranded HDR template comprises the synthetic DNA sequence flanked by homology arms that are complementary to sequences on both sides of the cleavage site in the T cell expressed gene. The transgene is specifically integrated into the cleavage site of the T cell expressed gene created by the Cas9 RNP in the genome-edited T cells, and the cells are then cultured.

Specification includes a Sequence Listing.



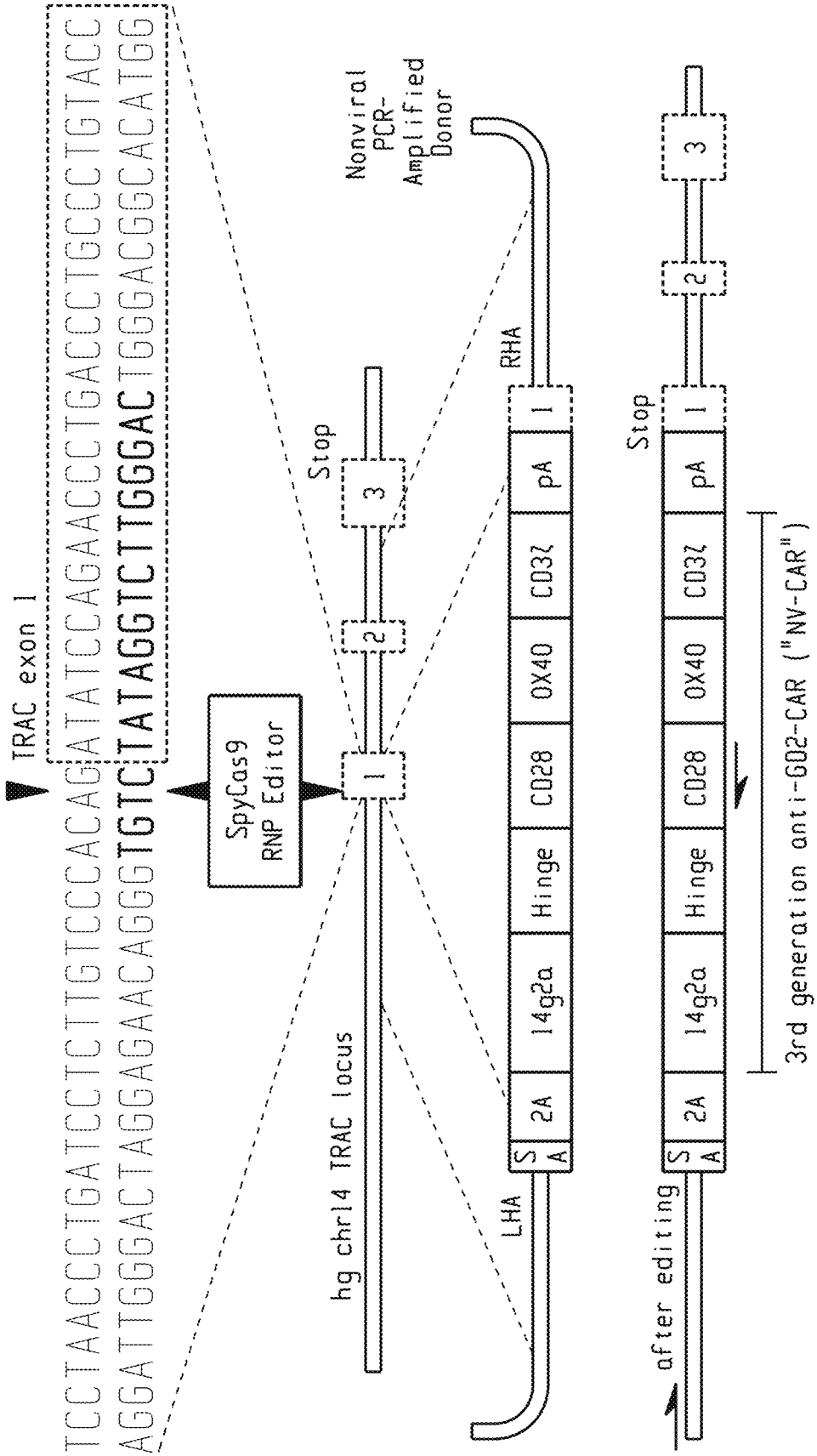

TRAC exon 1
TCCTAACCCTGATCCTCTTGTCCCACAG
AGGATTGGGACTAGGAGAACAGGGTGTCTATAGGTCTTGGGACTGGGACACATGG
SpyCas9
RNP Editor
Nonviral
PCR-
Amplified
Donor
Stop
1
2
3
hg chr14 TRAC locus
LHA
SA
2A
14g2a
Hinge
CD28
OX40
CD3ζ
pA
RHA
after editing
3rd generation anti-GD2-CAR ("NV-CAR")

Fig. 1A

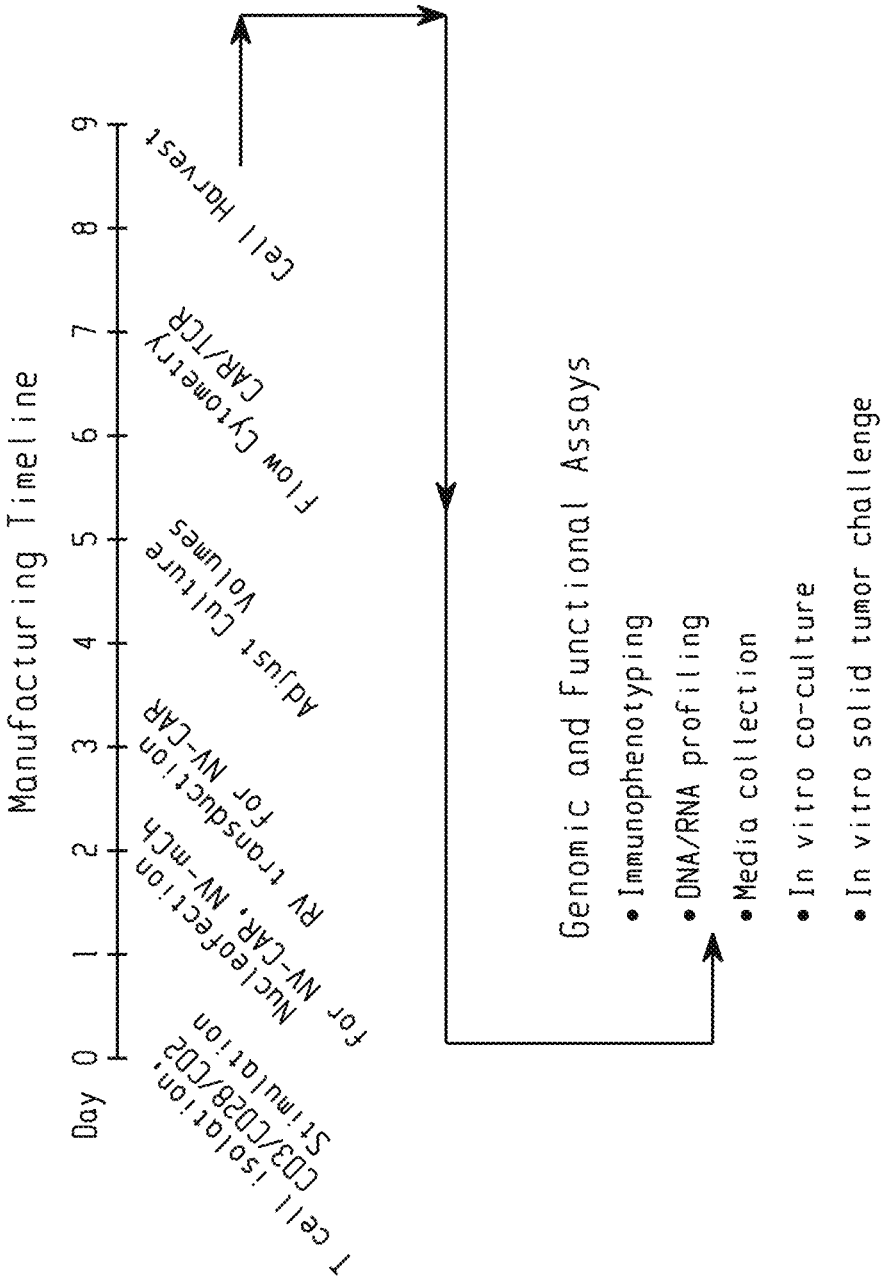

Manufacturing Timeline
Day 0
1
2
3
4
5
6
7
8
9
T cell isolation, CD3/CD28/CD2 Stimulation
Nucleofection for NV-CAR, NV-mCh
RV transduction for NV-CAR
Adjust Culture Volumes
Flow Cytometry CAR/TCR
Cell Harvest
Genomic and Functional Assays
• Immunophenotyping
• DNA/RNA profiling
• Media collection
• In vitro co-culture
• In vitro solid tumor challenge

Fig. 1B

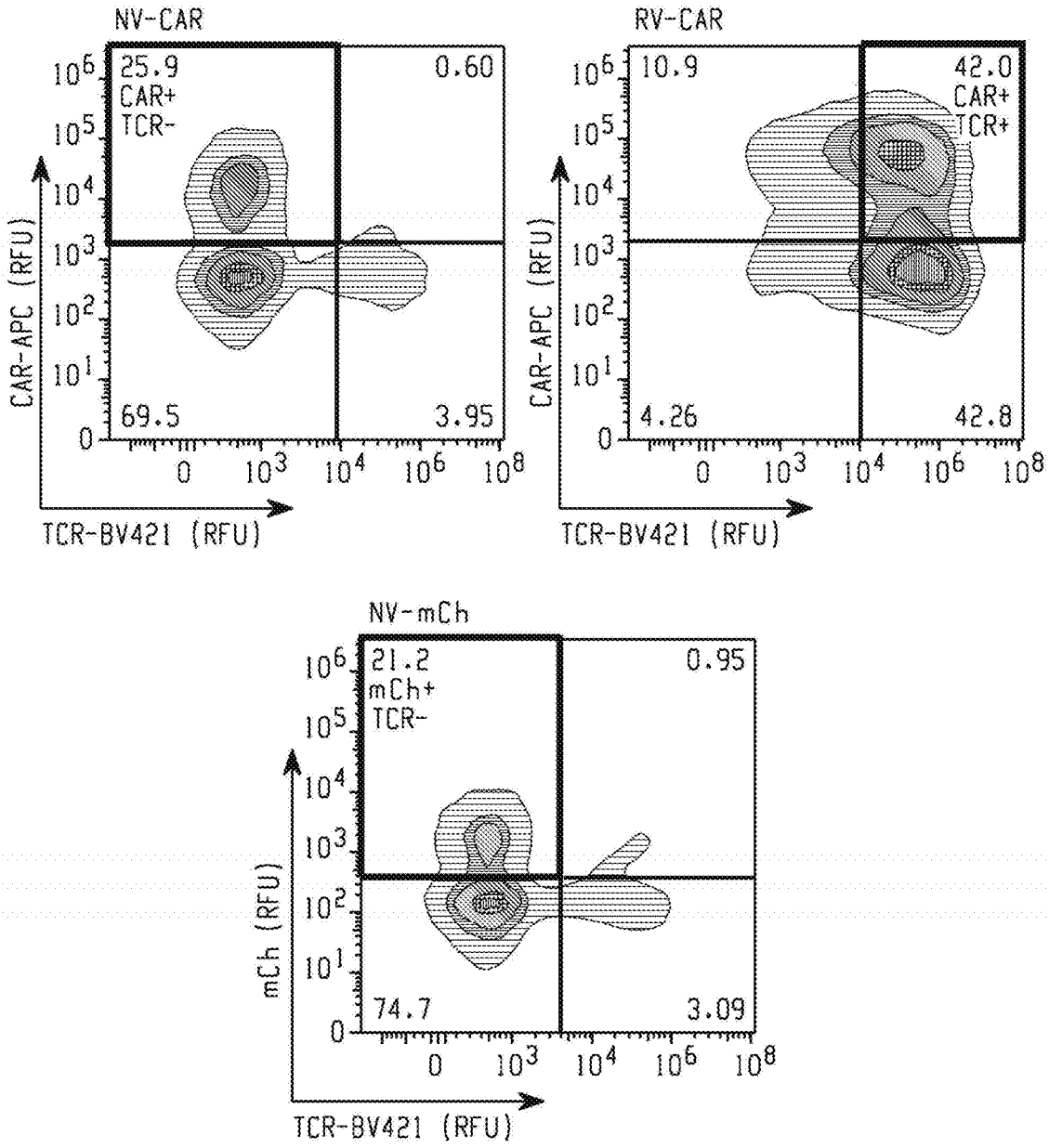

NV-CAR
25.9
CAR+
TCR-
0.60
69.5
3.95
CAR-APC (RFU)
TCR-BV421 (RFU)
RV-CAR
10.9
42.0
CAR+
TCR+
4.26
42.8
NV-mCh
21.2
mCh+
TCR-
0.95
74.7
3.09
mCh (RFU)

Fig. 1C

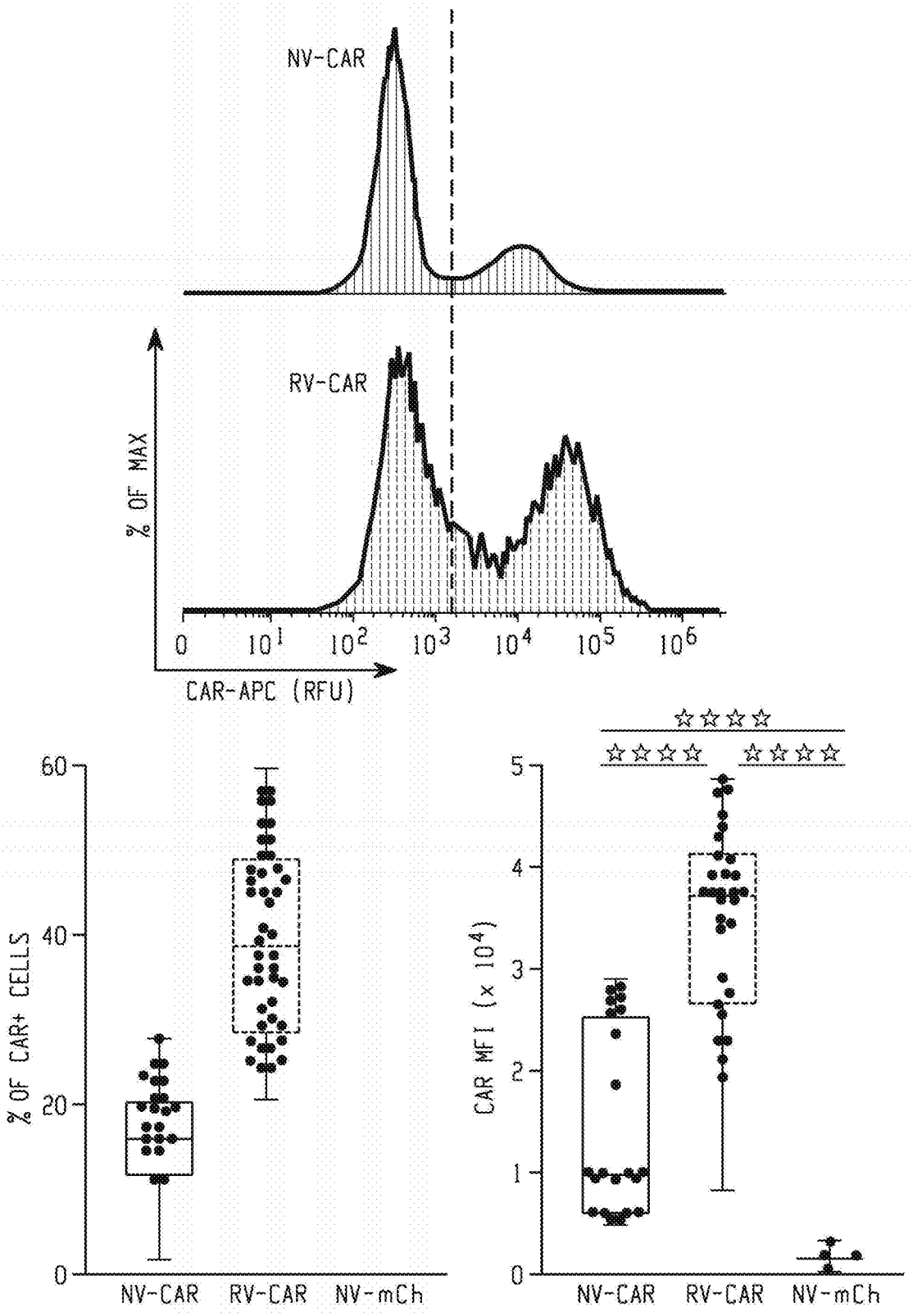

NV-CAR
RV-CAR
% OF MAX
0
10^1
10^2
10^3
10^4
10^5
10^6
CAR-APC (RFU)
☆☆☆☆
☆☆☆☆
☆☆☆☆
% OF CAR+ CELLS
60
40
20
0
CAR MFI (x 10^4)
5
4
3
2
1
0
NV-CAR
RV-CAR
NV-mCh
NV-CAR
RV-CAR
NV-mCh

Fig. 1D

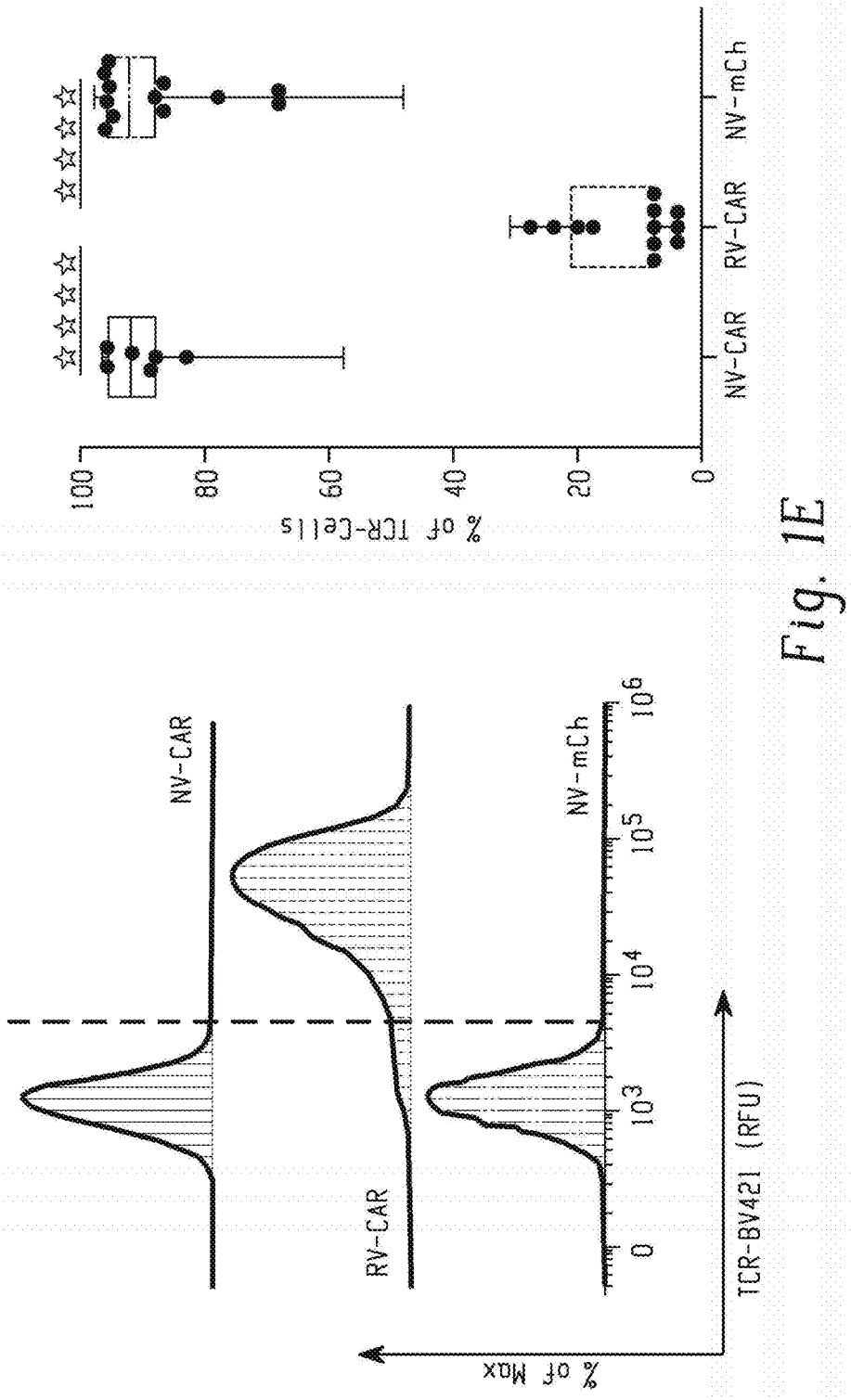

% of TCR-Cells
100
80
60
40
20
0
NV-CAR
RV-CAR
NV-mCh
% of Max
NV-CAR
RV-CAR
NV-mCh
0
10^3
10^4
10^5
10^6
TCR-BV421 (RFU)

Fig. 1E

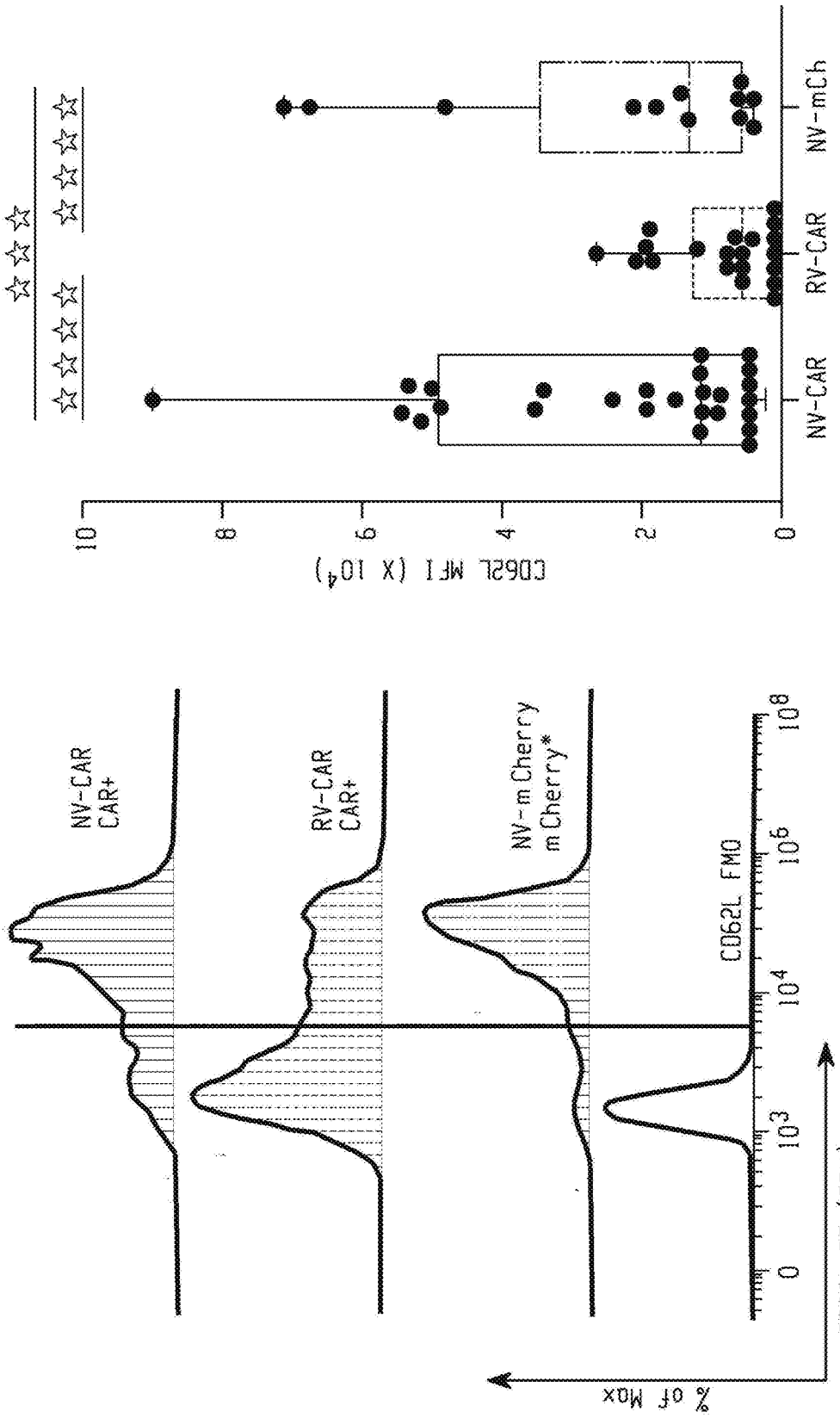

CD62L MFI (X 10^4)
0
2
4
6
8
10
NV-CAR
RV-CAR
NV-mCh
NV-CAR CAR+
RV-CAR CAR+
NV-mCherry mCherry*
CD62L FMO
0
10^3
10^4
10^6
10^8
CD62L-PE (RFU)
% of Max

Fig. 1F

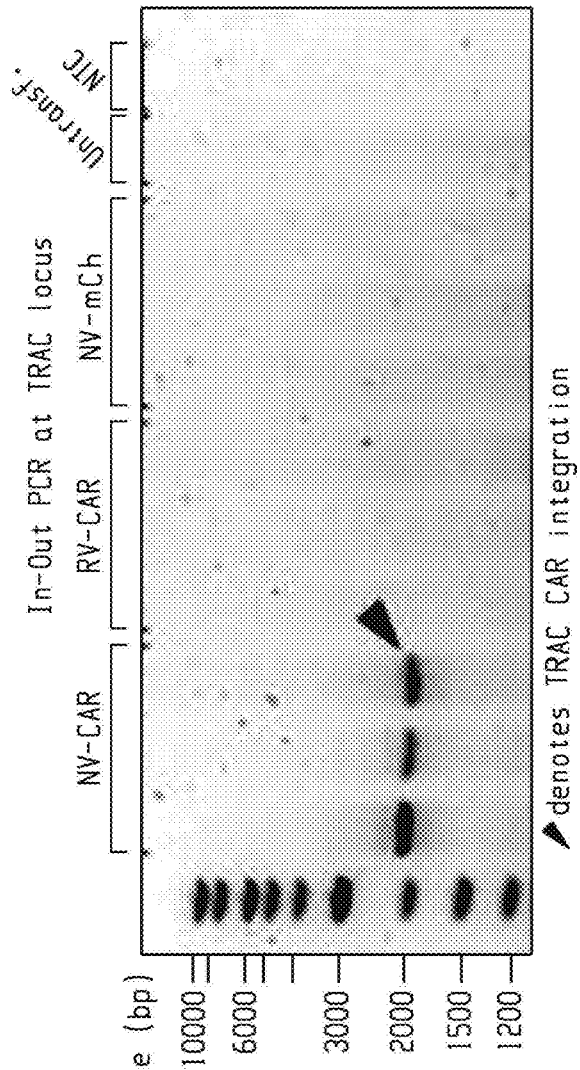

In-Out PCR at TRAC locus
NV-CAR
RV-CAR
NV-mCh
Untransf.
NTC
Size (bp)
10000
6000
3000
2000
1500
1200
denotes TRAC CAR integration

Fig. 1G

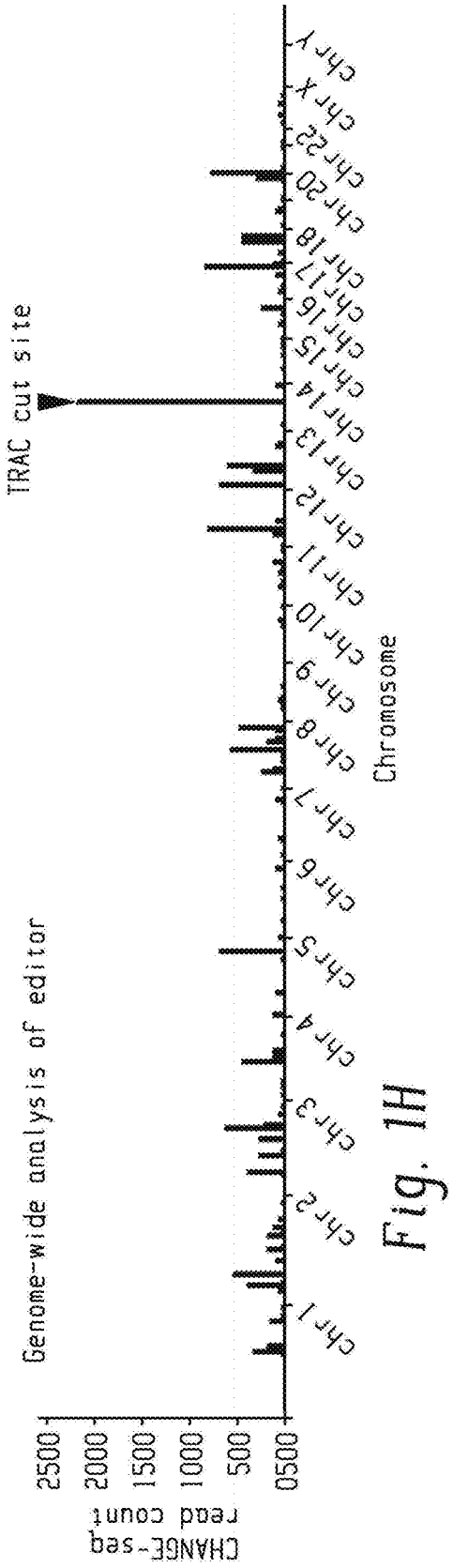

Genome-wide analysis of editor
TRAC cut site
CHANGE-seq read count
2500
2000
1500
1000
500
0500
chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr20
chr22
chrX
chrY
Chromosome

Fig. 1H

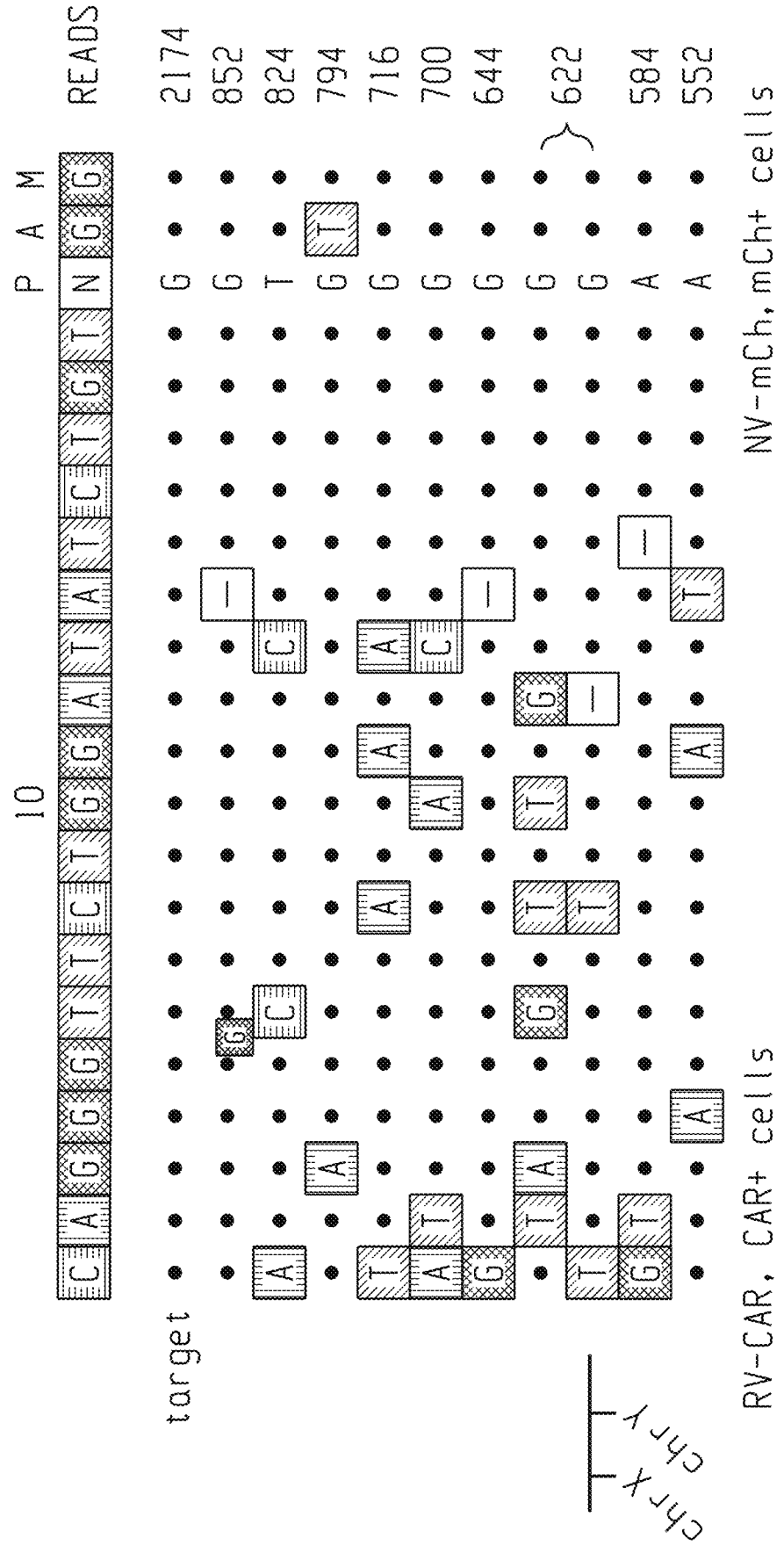

10
PAM
READS
target
2174
852
824
794
716
700
644
622
584
552
NV-mCh, mCh+ cells
RV-CAR, CAR+ cells
Chr X
Chr Y

Fig. 11

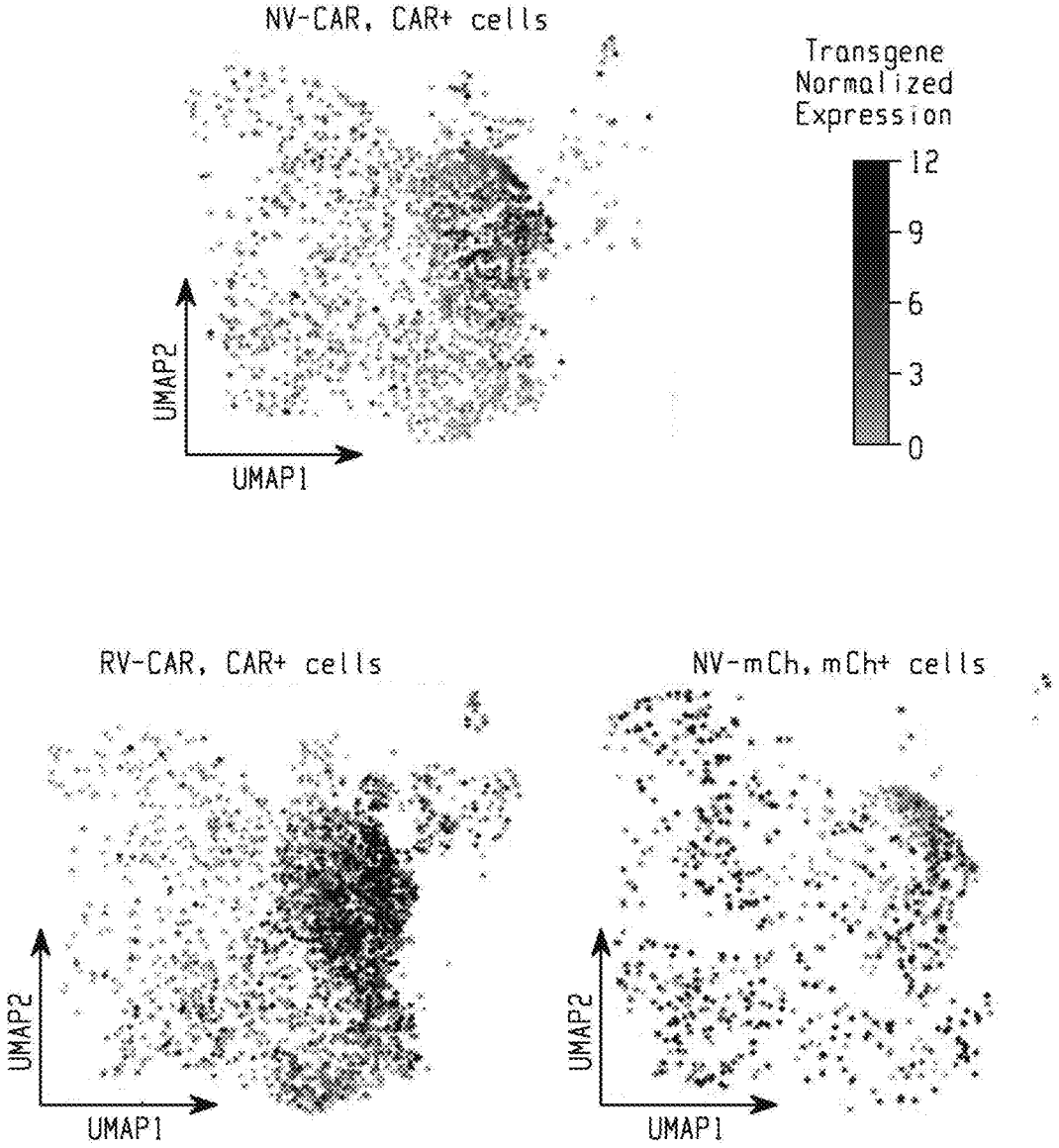
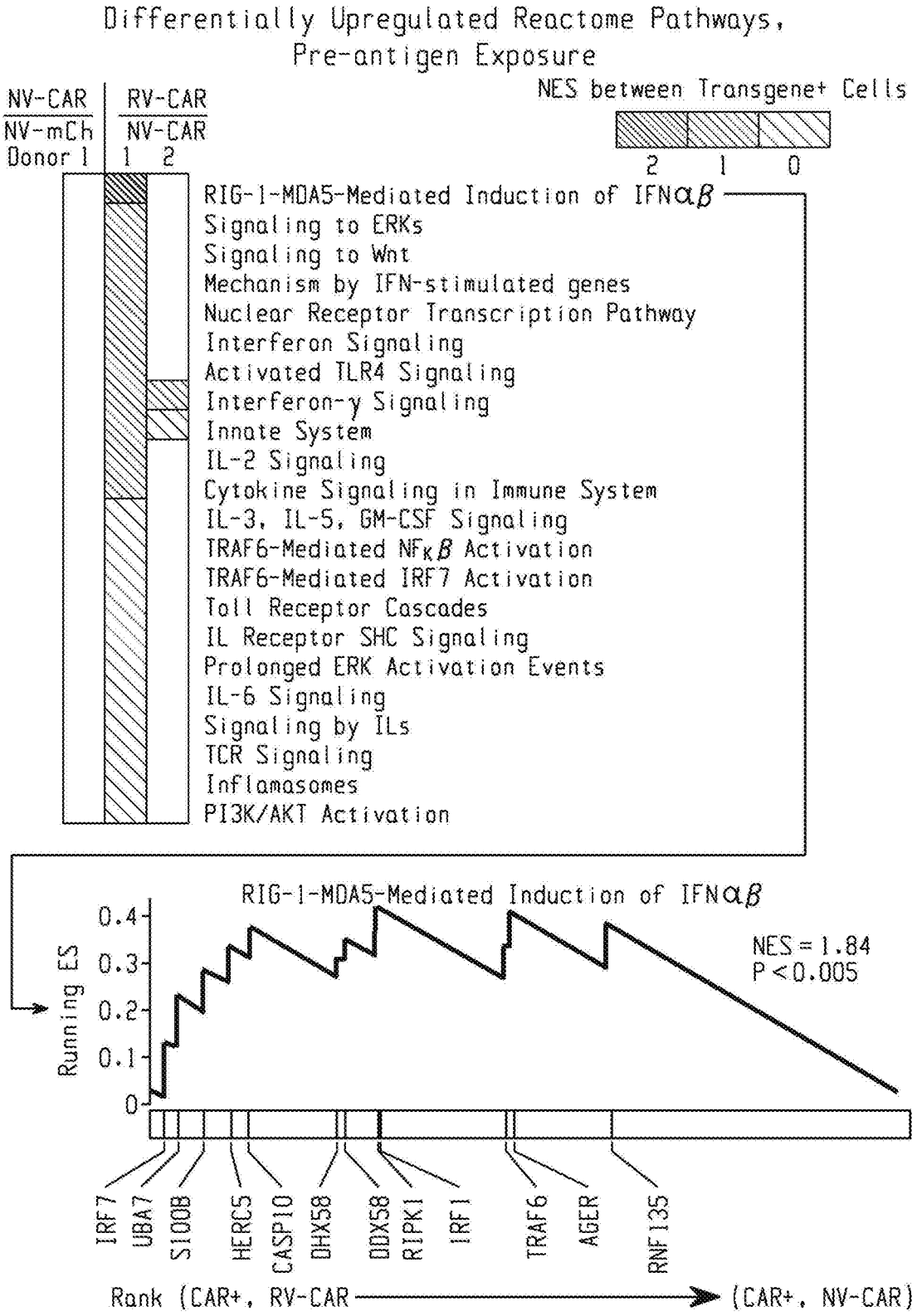

NV-CAR, CAR+ cells
Transgene
Normalized
Expression
12
9
6
3
0
UMAP2
UMAP1
RV-CAR, CAR+ cells
NV-mCh, mCh+ cells
UMAP2
UMAP1
UMAP2
UMAP1

Fig. 1J

Fig. 1K.1


Differentially Upregulated Reactome Pathways,
Pre-antigen Exposure
NES between Transgene+ Cells
2
1
0
NV-CAR
NV-mCh
Donor 1
RV-CAR
NV-CAR
1 2
RIG-1-MDA5-Mediated Induction of IFNαβ
Signaling to ERKs
Signaling to Wnt
Mechanism by IFN-stimulated genes
Nuclear Receptor Transcription Pathway
Interferon Signaling
Activated TLR4 Signaling
Interferon-γ Signaling
Innate System
IL-2 Signaling
Cytokine Signaling in Immune System
IL-3, IL-5, GM-CSF Signaling
TRAF6-Mediated NFκβ Activation
TRAF6-Mediated IRF7 Activation
Toll Receptor Cascades
IL Receptor SHC Signaling
Prolonged ERK Activation Events
IL-6 Signaling
Signaling by ILs
TCR Signaling
Inflamasomes
PI3K/AKT Activation
RIG-1-MDA5-Mediated Induction of IFNαβ
Running ES
0.4
0.3
0.2
0.1
0
NES = 1.84
P < 0.005
IRF7
UBA7
S100B
HERC5
CASP10
DHX58
DDX58
RIPK1
IRF1
TRAF6
AGER
RNF135
Rank (CAR+, RV-CAR
(CAR+, NV-CAR)

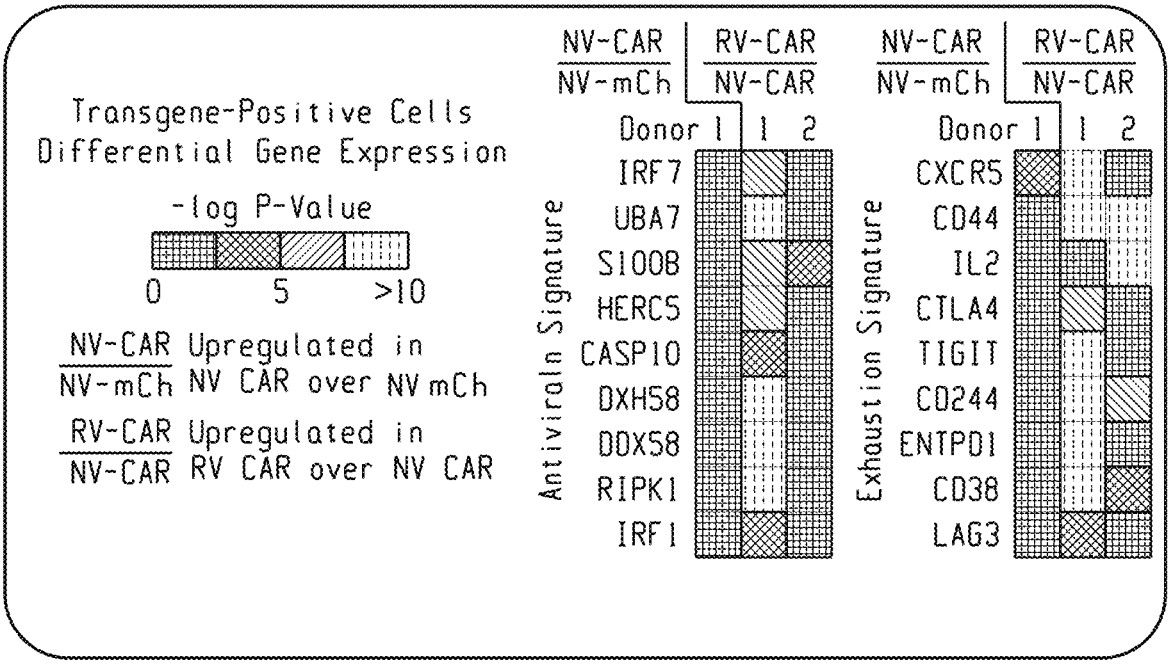

Transgene-Positive Cells
Differential Gene Expression
-log P-Value
0
5
>10
NV-CAR / NV-mCh Upregulated in NV CAR over NV mCh
RV-CAR / NV-CAR Upregulated in RV CAR over NV CAR
NV-CAR / NV-mCh
RV-CAR / NV-CAR
Donor 1
1
2
Antiviraln Signature
IRF7
UBA7
S100B
HERC5
CASP10
DXH58
DDX58
RIPK1
IRF1
Exhaustion Signature
CXCR5
CD44
IL2
CTLA4
TIGIT
CD244
ENTPD1
CD38
LAG3

Fig. 1K.2

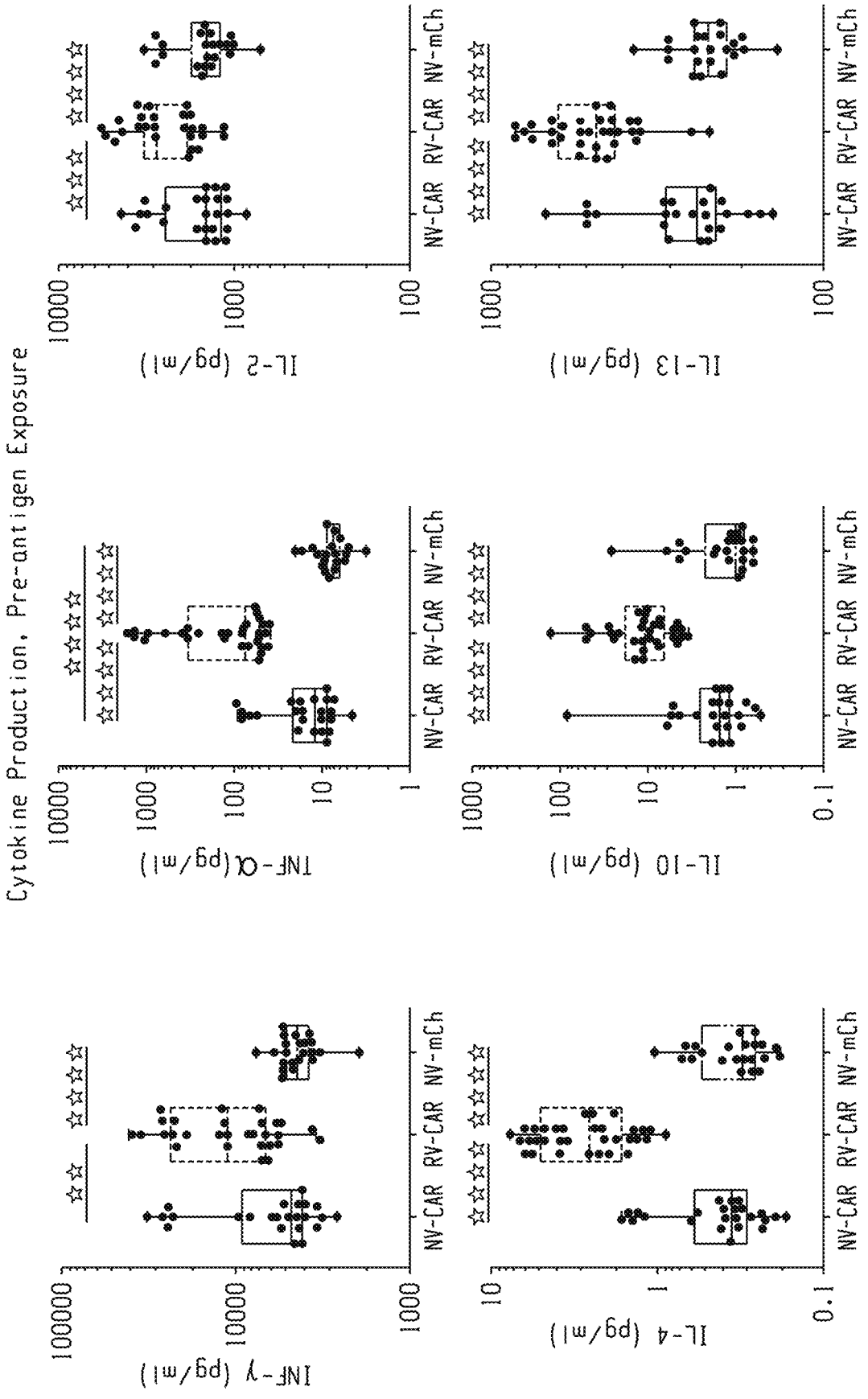

Cytokine Production, Pre-antigen Exposure
IL-2 (pg/ml)
IL-13 (pg/ml)
TNF-α (pg/ml)
IL-10 (pg/ml)
INF-γ (pg/ml)
IL-4 (pg/ml)
NV-CAR
RV-CAR
NV-mCh

Fig. 1L

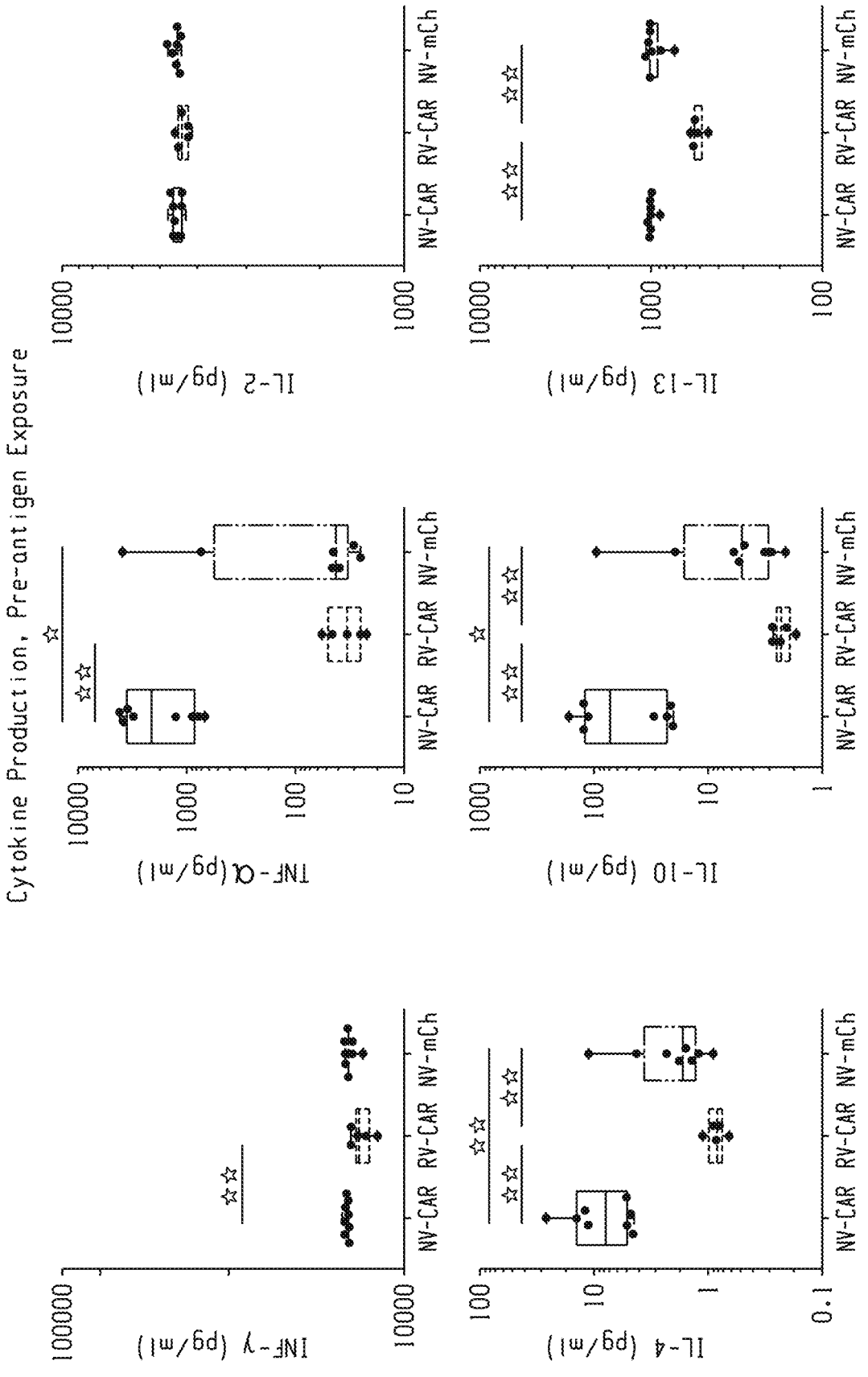

Cytokine Production, Pre-antigen Exposure
IL-2 (pg/ml)
IL-13 (pg/ml)
TNF-α (pg/ml)
IL-10 (pg/ml)
INF-γ (pg/ml)
IL-4 (pg/ml)
NV-CAR
RV-CAR
NV-mCh
☆
☆☆

Fig. 2A

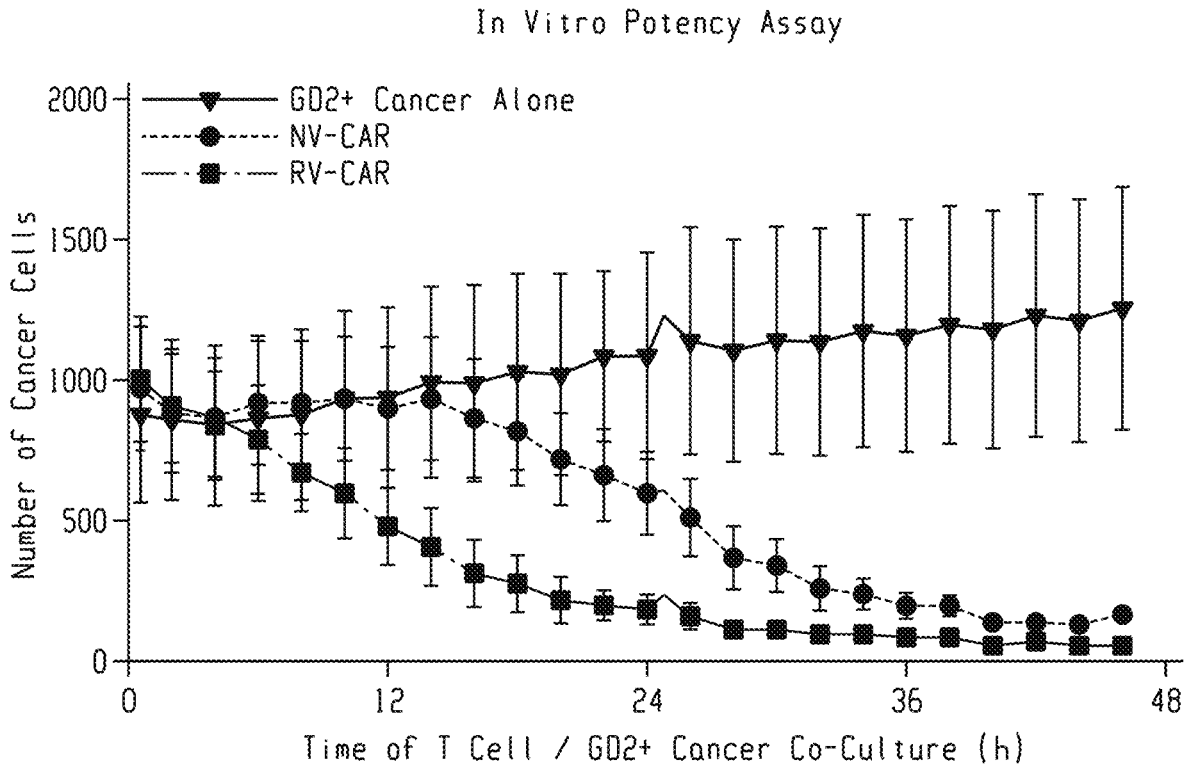

In Vitro Potency Assay
GD2+ Cancer Alone
NV-CAR
RV-CAR
Number of Cancer Cells
2000
1500
1000
500
0
0
12
24
36
48
Time of T Cell / GD2+ Cancer Co-Culture (h)

Fig. 2B

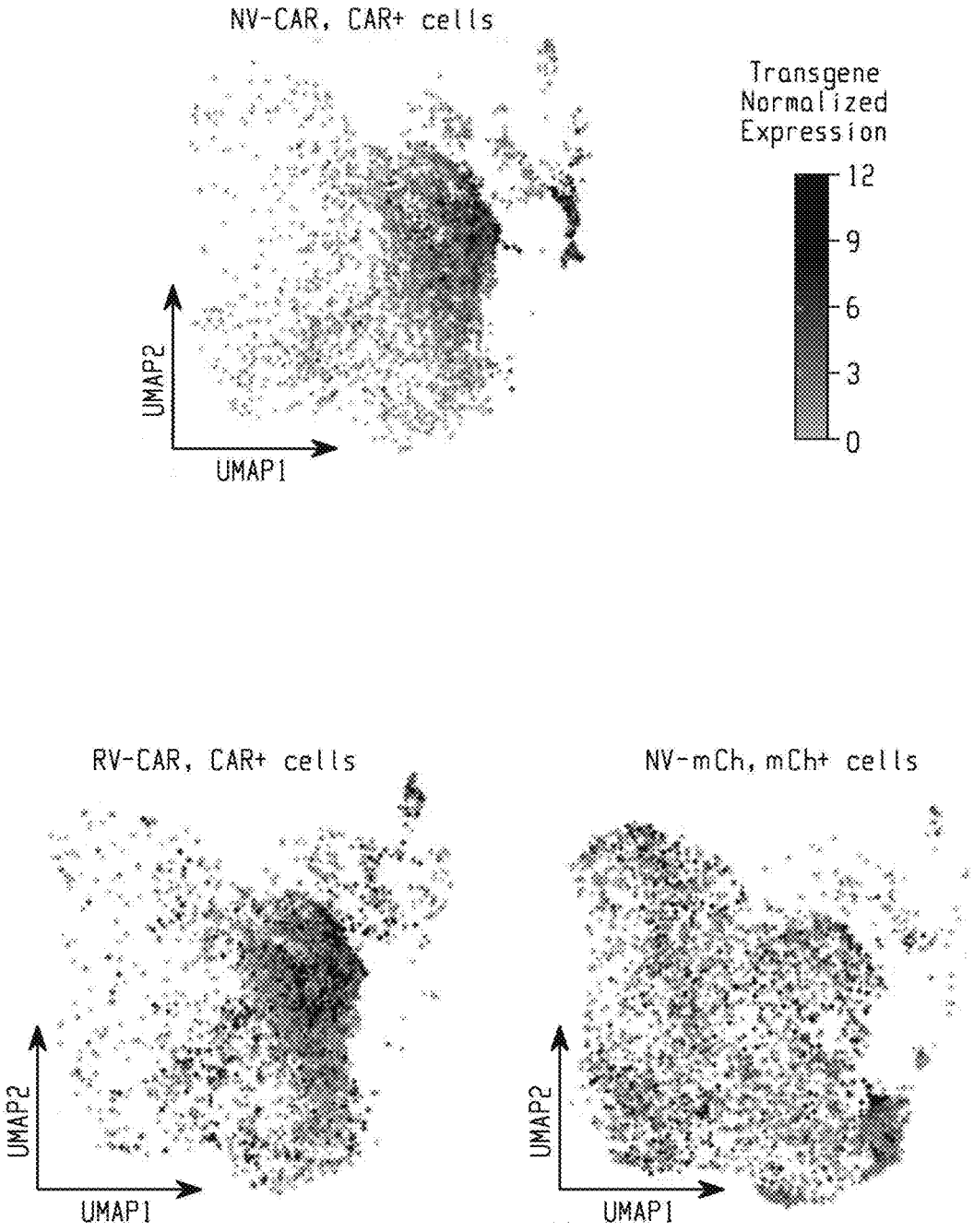
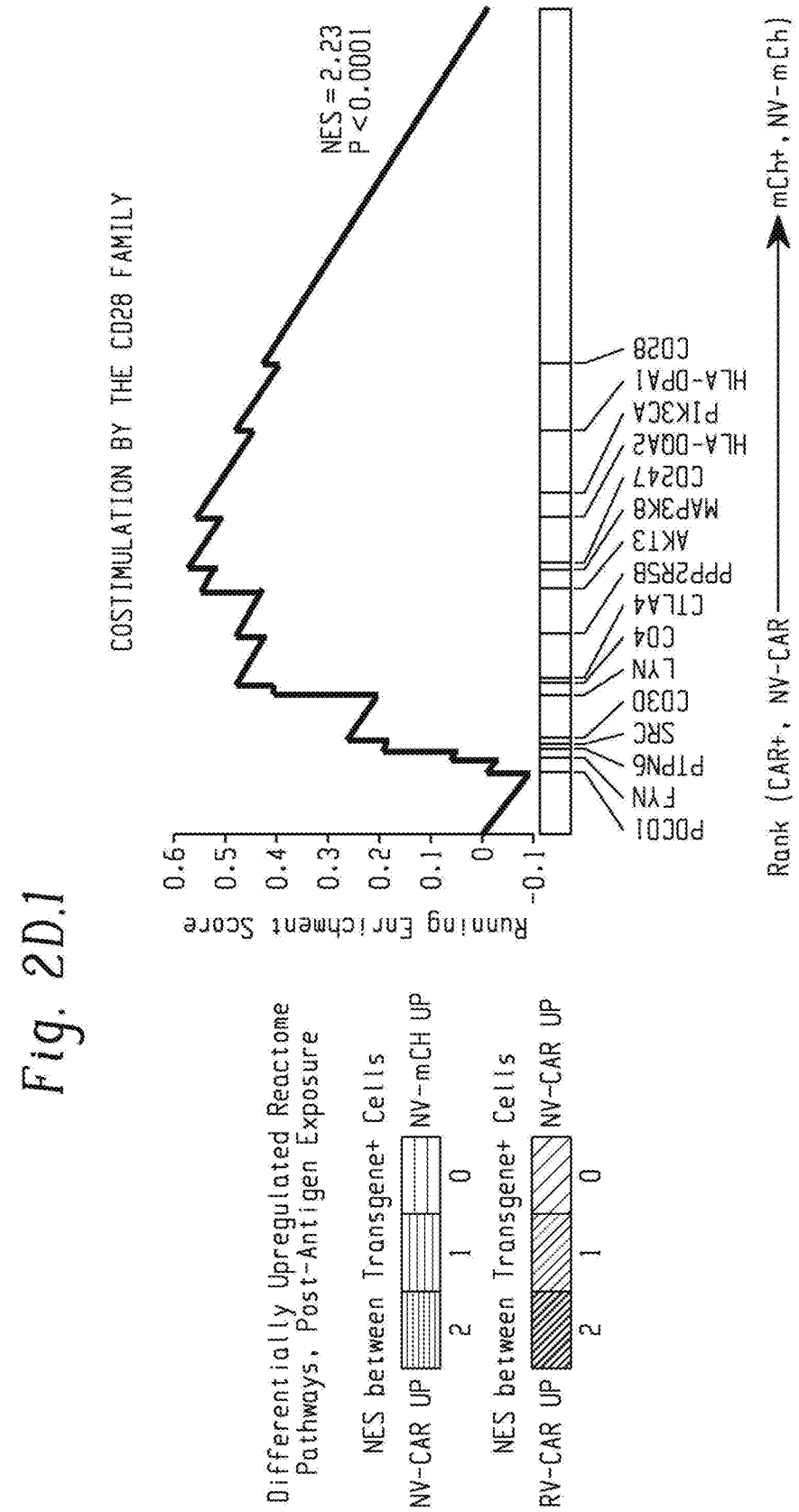

NV-CAR, CAR+ cells
Transgene Normalized Expression
12
9
6
3
0
UMAP2
UMAP1
RV-CAR, CAR+ cells
UMAP2
UMAP1
NV-mCh, mCh+ cells
UMAP2
UMAP1

Fig. 2C

Fig. 2D.1



MATCH TO FIG. 2D.2

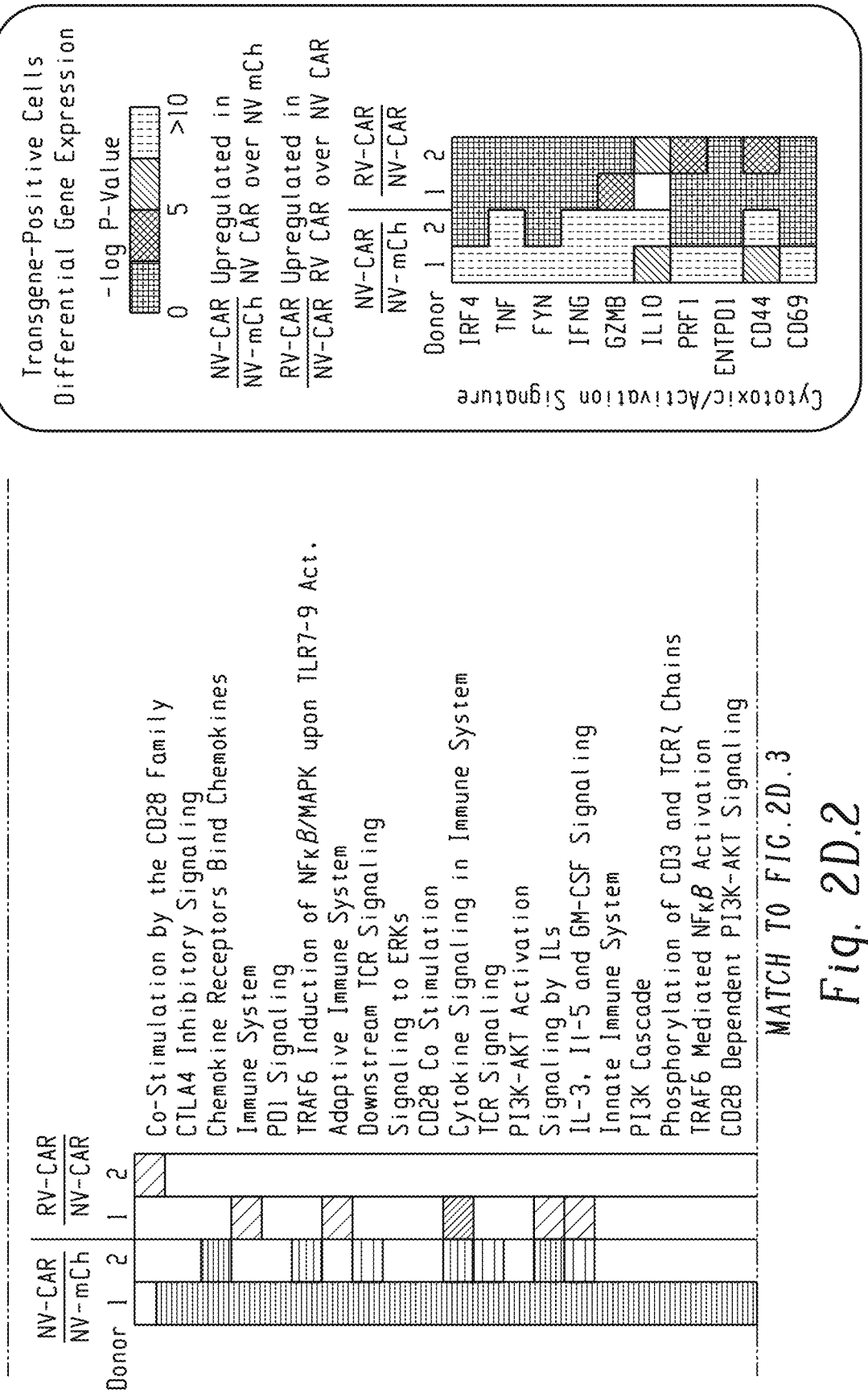
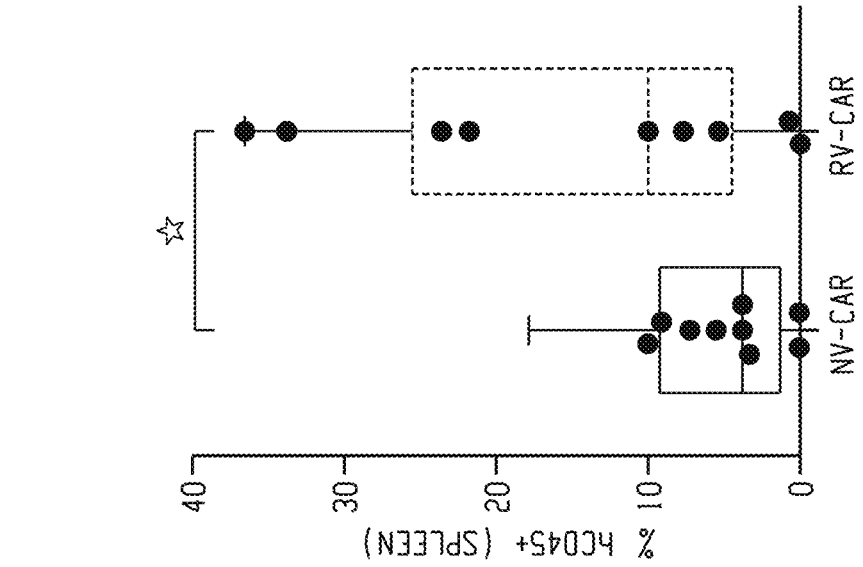

MATCH TO FIG.2D.1
NV-CAR / NV-mCh
RV-CAR / NV-CAR
Donor 1 2 1 2
Co-Stimulation by the CD28 Family
CTLA4 Inhibitory Signaling
Chemokine Receptors Bind Chemokines
Immune System
PD1 Signaling
TRAF6 Induction of NFκB/MAPK upon TLR7-9 Act.
Adaptive Immune System
Downstream TCR Signaling
Signaling to ERKs
CD28 Co Stimulation
Cytokine Signaling in Immune System
TCR Signaling
PI3K-AKT Activation
Signaling by ILs
IL-3, Il-5 and GM-CSF Signaling
Innate Immune System
PI3K Cascade
Phosphorylation of CD3 and TCRζ Chains
TRAF6 Mediated NFκB Activation
CD28 Dependent PI3K-AKT Signaling
MATCH TO FIG.2D.3
Transgene-Positive Cells
Differential Gene Expression
-log P-Value
0 5 >10
NV-CAR / NV-mCh Upregulated in NV CAR over NV mCh
RV-CAR / NV-CAR Upregulated in RV CAR over NV CAR
Cytotoxic/Activation Signature
NV-CAR / NV-mCh | RV-CAR / NV-CAR
Donor 1 2 1 2
IRF4
TNF
FYN
IFNG
GZMB
IL10
PRF1
ENTPD1
CD44
CD69

Fig. 2D.2


% hCD45+ (SPLEEN)
40
30
20
10
0
NV-CAR
RV-CAR

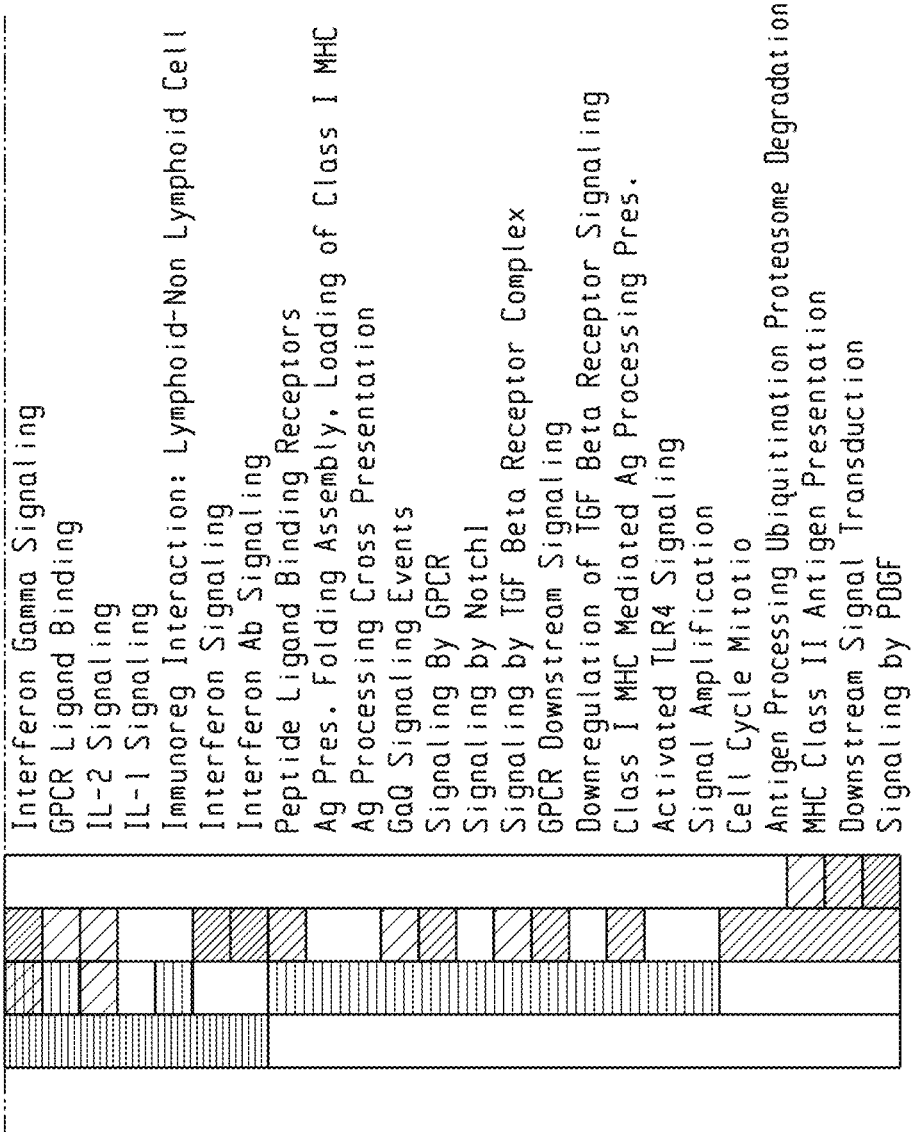

MATCH TO FIG.2D.2
Interferon Gamma Signaling
GPCR Ligand Binding
IL-2 Signaling
IL-1 Signaling
Immunoreg Interaction: Lymphoid-Non Lymphoid Cell
Interferon Signaling
Interferon Ab Signaling
Peptide Ligand Binding Receptors
Ag Pres. Folding Assembly, Loading of Class I MHC
Ag Processing Cross Presentation
GaQ Signaling Events
Signaling By GPCR
Signaling by Notch1
Signaling by TGF Beta Receptor Complex
GPCR Downstream Signaling
Downregulation of TGF Beta Receptor Signaling
Class I MHC Mediated Ag Processing Pres.
Activated TLR4 Signaling
Signal Amplification
Cell Cycle Mitotio
Antigen Processing Ubiquitination Proteasome Degradation
MHC Class II Antigen Presentation
Downstream Signal Transduction
Signaling by PDGF

Fig. 2D.3

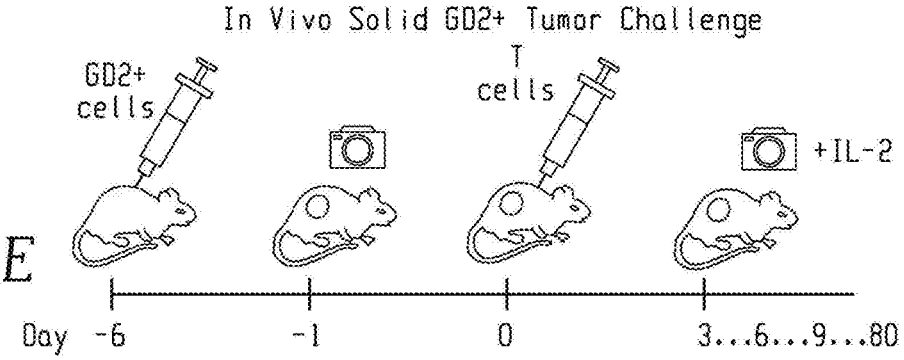


Fig. 2E

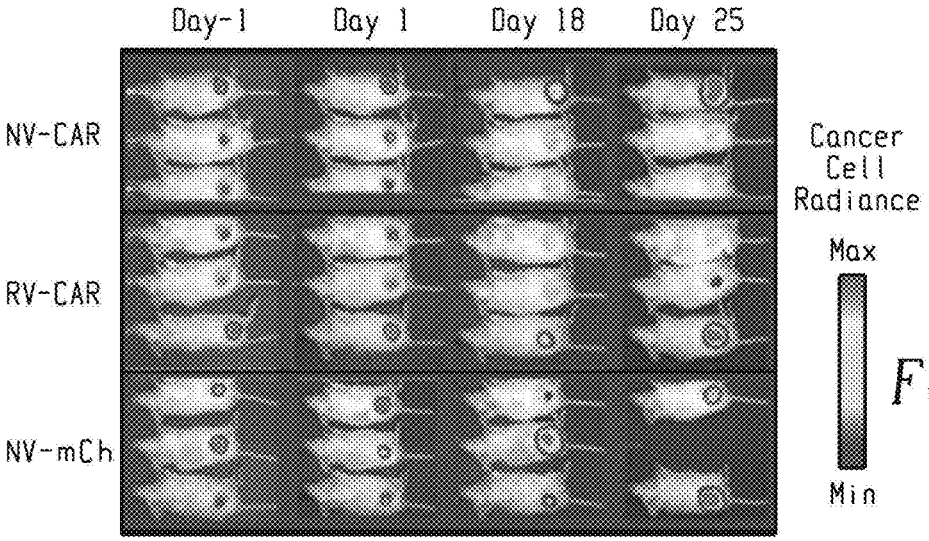


Fig. 2F

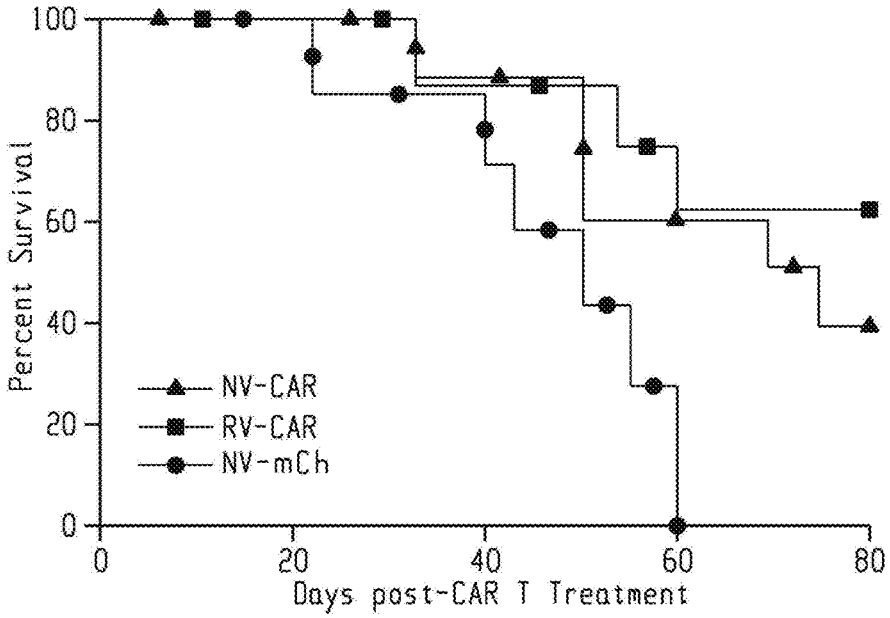


Fig. 2G

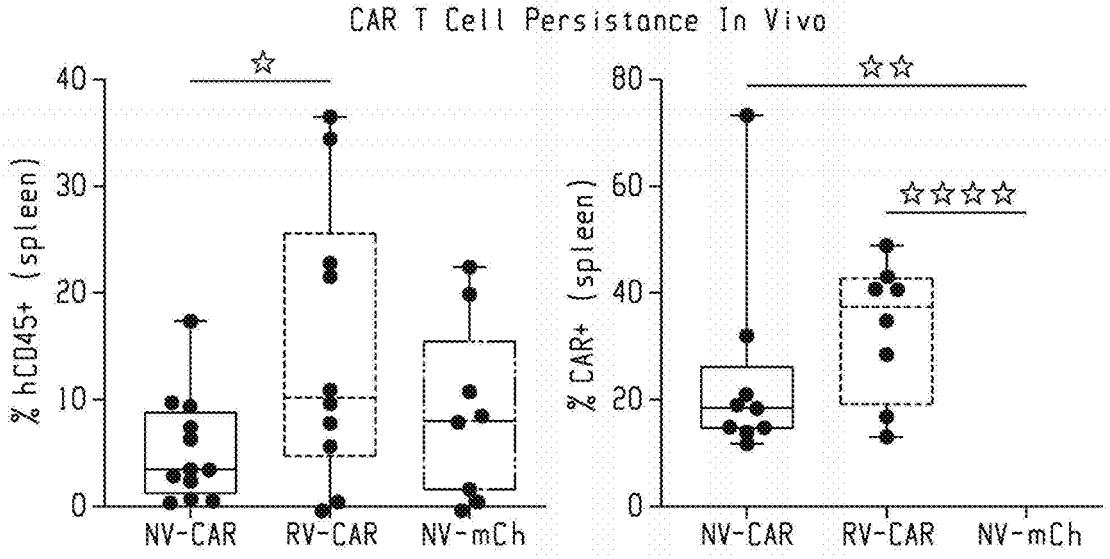

CAR T Cell Persistance In Vivo
% hCD45+ (spleen)
40
30
20
10
0
☆
NV-CAR
RV-CAR
NV-mCh
% CAR+ (spleen)
80
60
40
20
0
☆☆
☆☆☆☆
NV-CAR
RV-CAR
NV-mCh

Fig. 2H

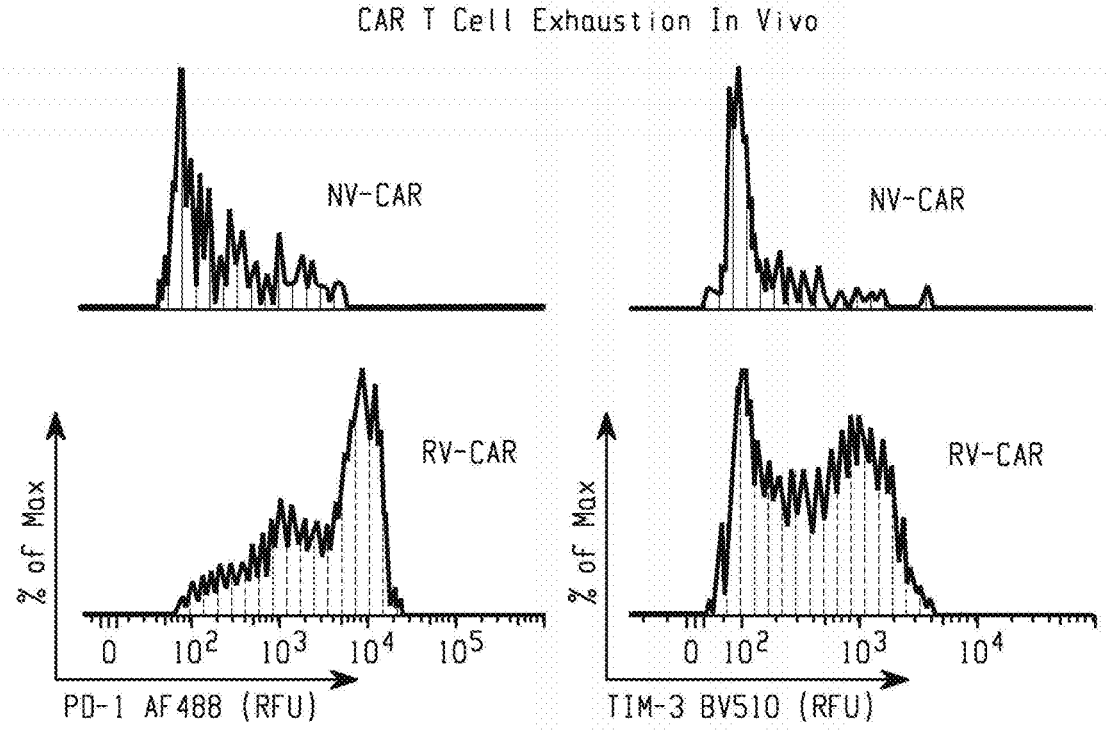

CAR T Cell Exhaustion In Vivo
NV-CAR
NV-CAR
RV-CAR
RV-CAR
% of Max
% of Max
0
10^2
10^3
10^4
10^5
PD-1 AF488 (RFU)
0
10^2
10^3
10^4
TIM-3 BV510 (RFU)

Fig. 2I

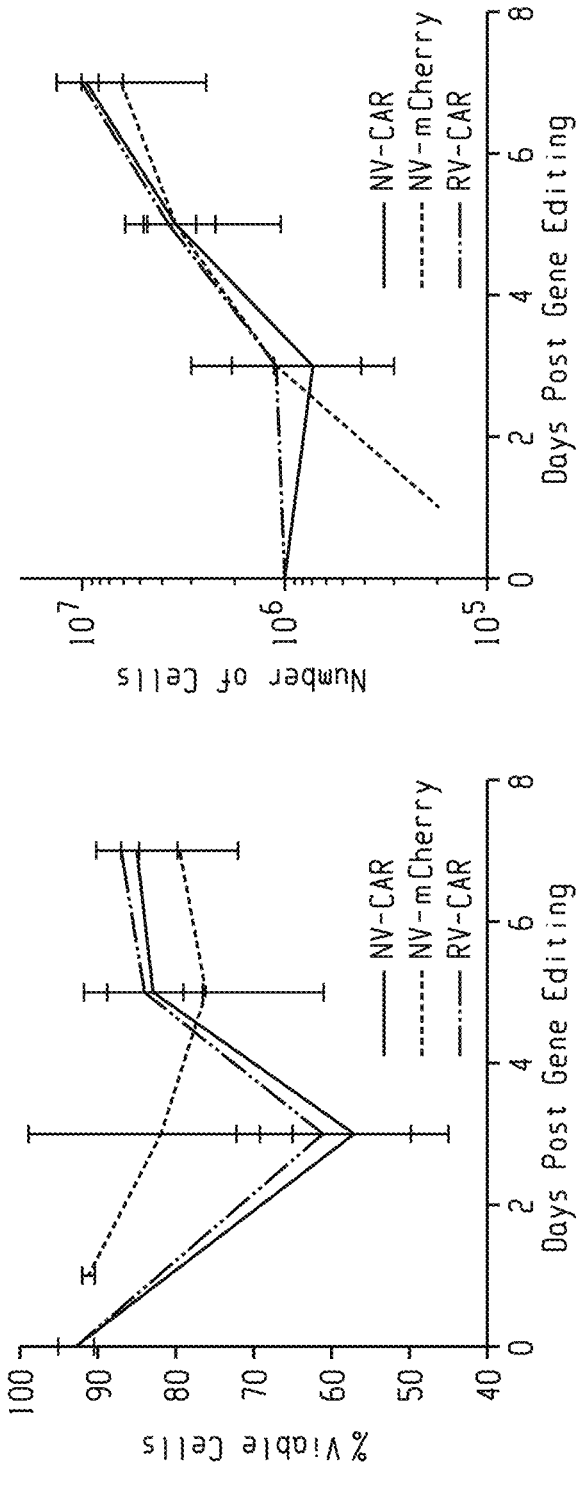

% Viable Cells
100
90
80
70
60
50
40
0
2
4
6
8
Days Post Gene Editing
NV-CAR
NV-mCherry
RV-CAR
Number of Cells
10^7
10^6
10^5

Fig. 3A

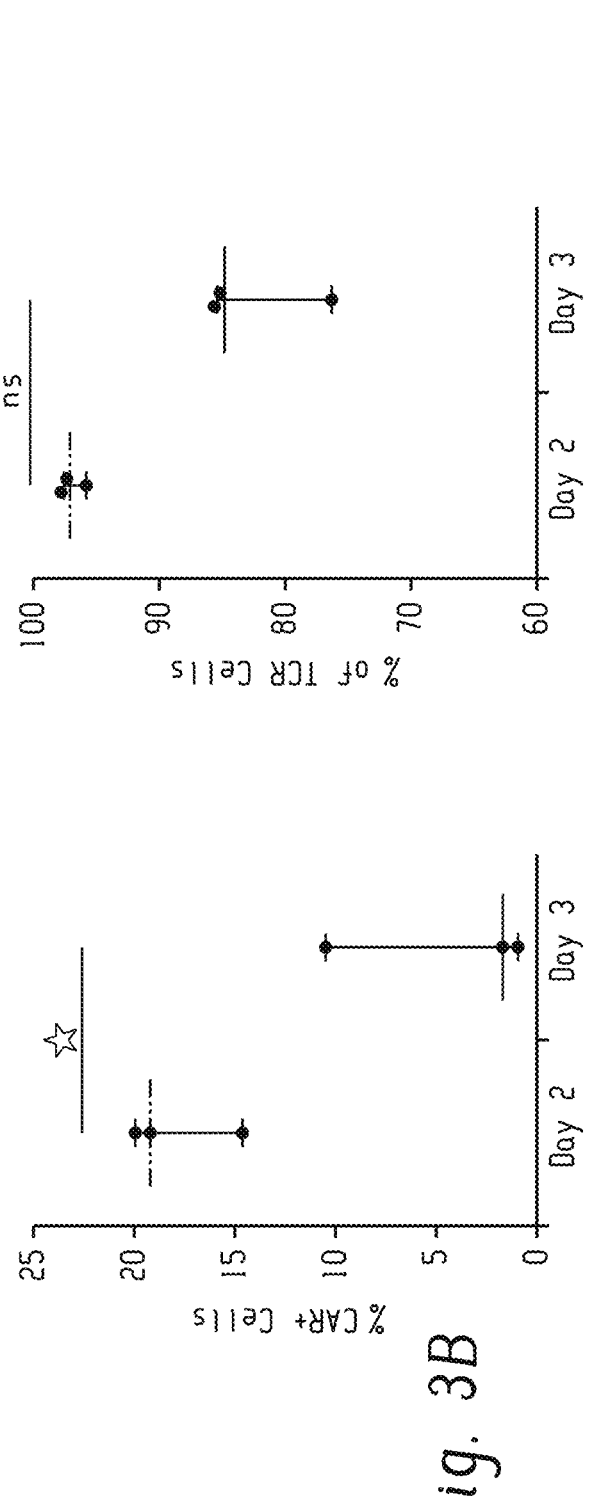

% CAR+ Cells
25
20
15
10
5
0
Day 2
Day 3
ns
% of TCR Cells
100
90
80
70
60
Day 2
Day 3

Fig. 3B

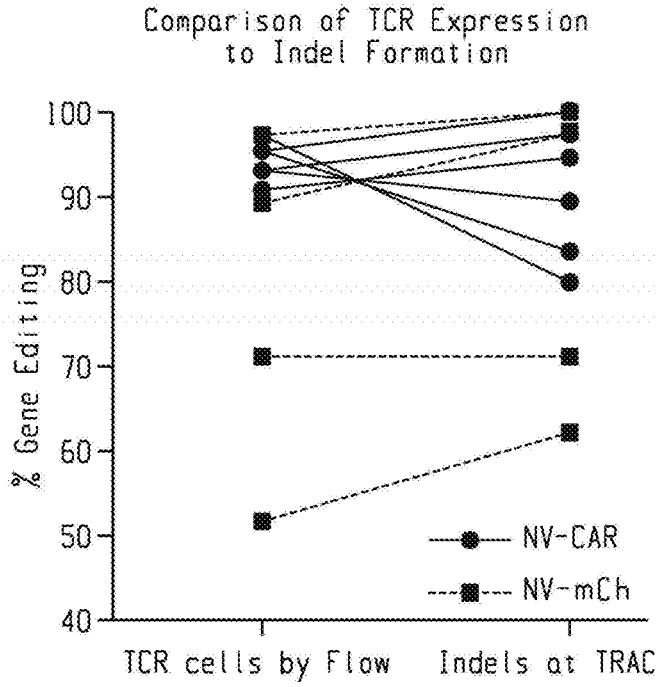


Fig. 3C

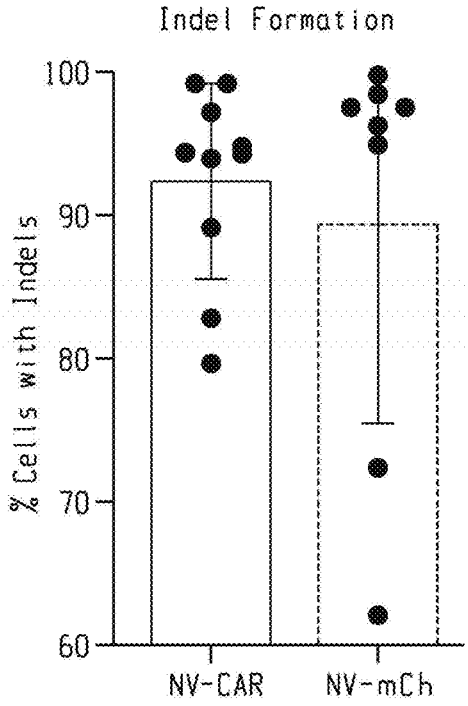


Fig. 3D

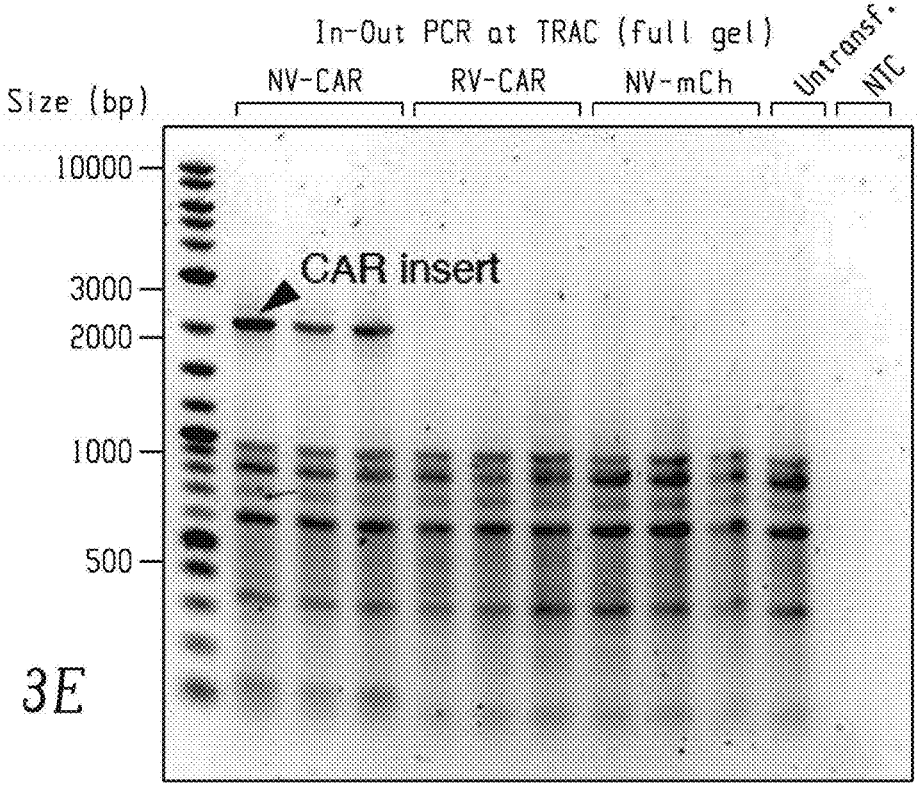


Fig. 3E

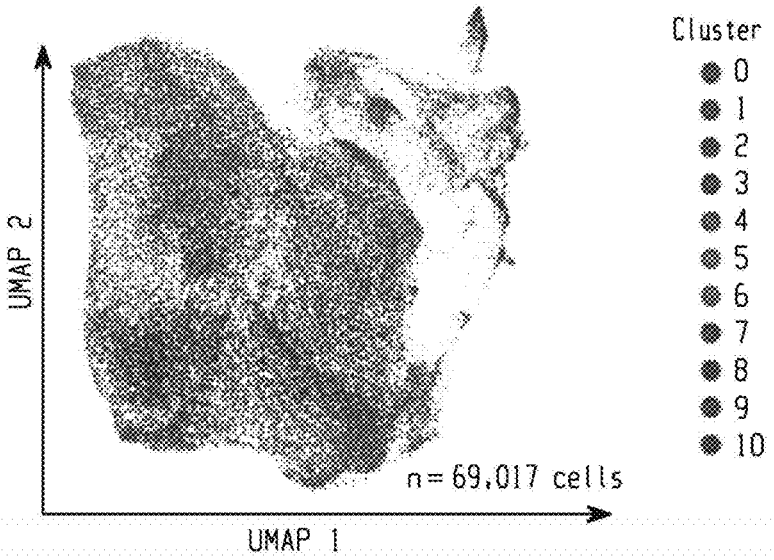

Cluster
0
1
2
3
4
5
6
7
8
9
10
UMAP 2
UMAP 1
n = 69,017 cells

Fig. 4A

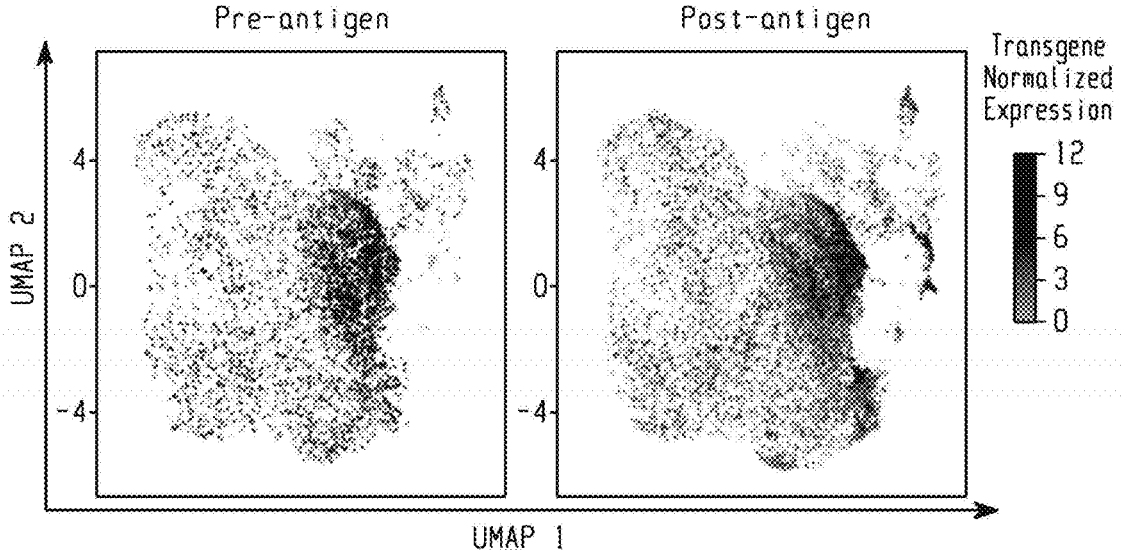

Pre-antigen
Post-antigen
Transgene
Normalized
Expression
12
9
6
3
0
4
0
-4
UMAP 2
UMAP 1

Fig. 4B

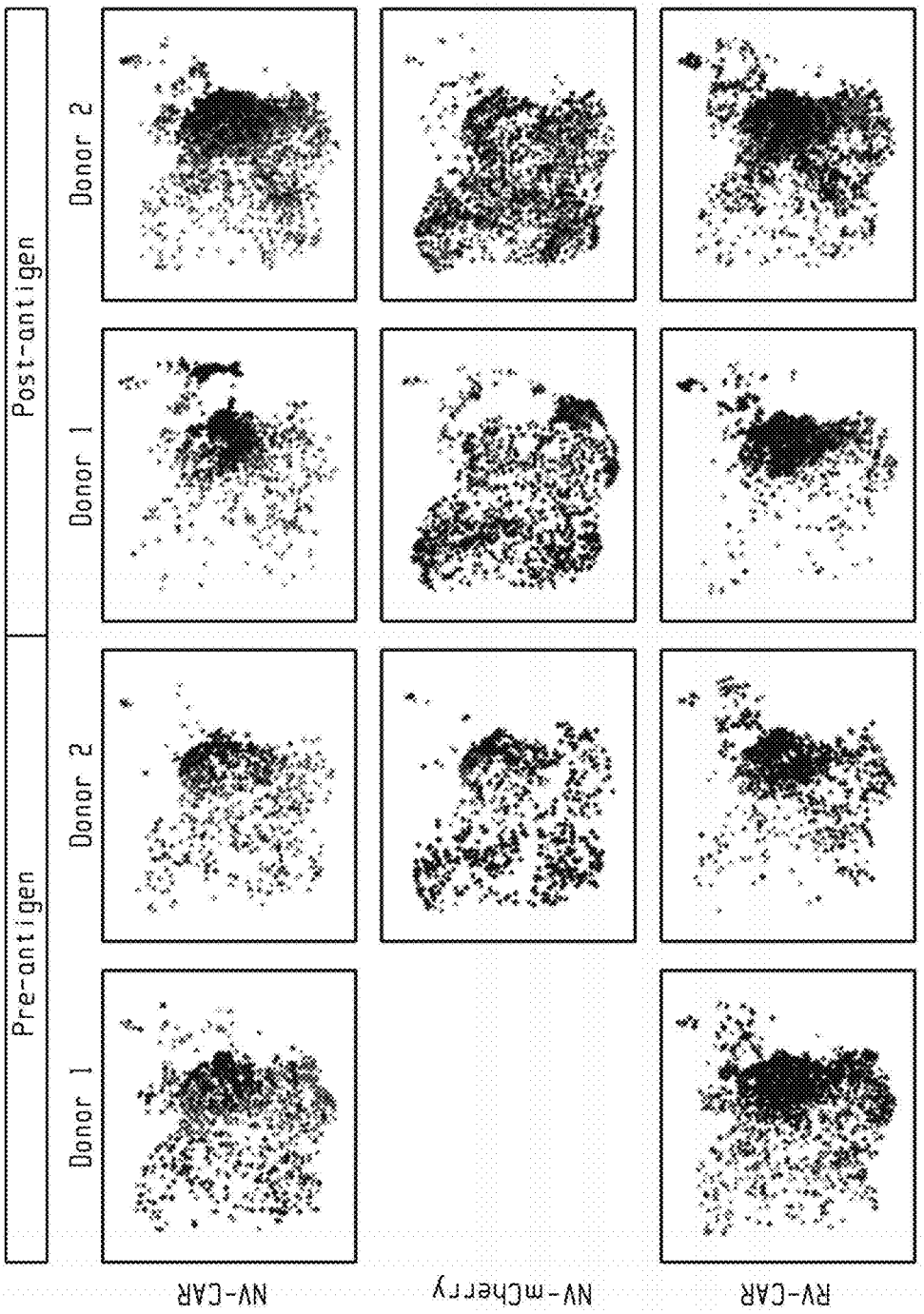

Post-antigen
Pre-antigen
Donor 2
Donor 1
Donor 2
Donor 1
NY-CAR
NY-mCherry
RY-CAR

Fig. 4C

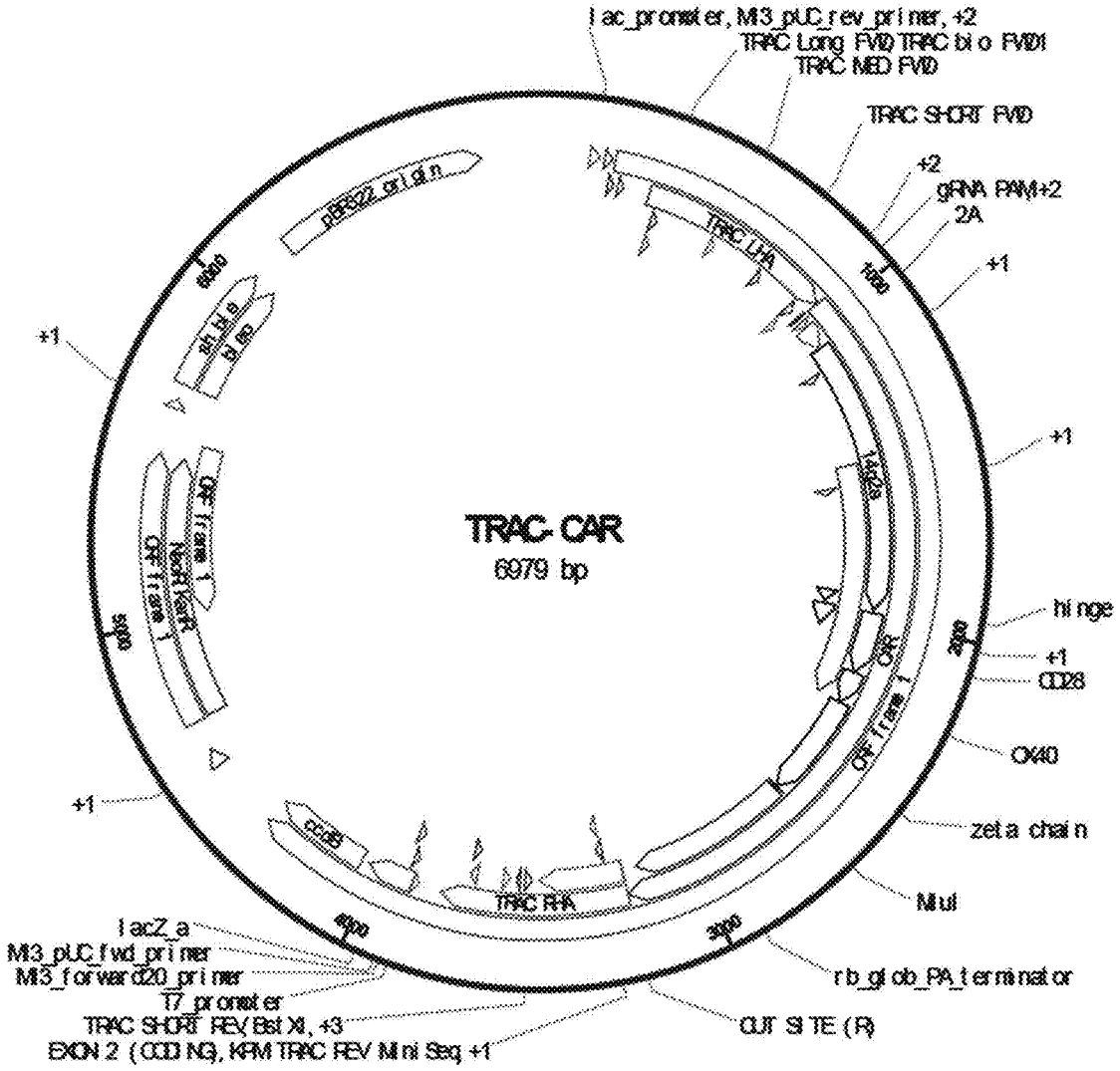

lac_promoter, M13_pUC_rev_primer, +2
TRAC Long FWD
TRAC bio FWD1
TRAC MED FWD
TRAC SHORT FWD
+2
gRNA PAM+2
2A
+1
TRAC LHA
TRAC-CAR
6979 bp
hinge
+1
CD28
OX40
zeta chain
MluI
rb_glob_PA_terminator
CUT SITE (R)
TRAC RHA
lacZ_a
M13_pUC_fwd_primer
M13_forward20_primer
T7_promoter
TRAC SHORT REV(Bst XI, +3
EXON 2 (CODING), KPM TRAC REV Mini Seq +1

Fig. 5

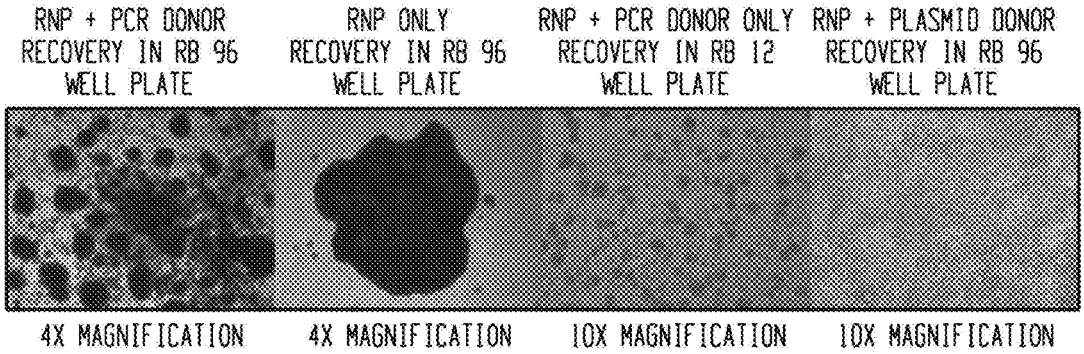

RNP + PCR DONOR RECOVERY IN RB 96 WELL PLATE
RNP ONLY RECOVERY IN RB 96 WELL PLATE
RNP + PCR DONOR ONLY RECOVERY IN RB 12 WELL PLATE
RNP + PLASMID DONOR RECOVERY IN RB 96 WELL PLATE
4X MAGNIFICATION
4X MAGNIFICATION
10X MAGNIFICATION
10X MAGNIFICATION

Fig. 6

NONVIRAL GENERATION OF GENOME EDITED CHIMERIC ANTIGEN RECEPTOR T CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a bypass continuation in part application of PCT/US2021/019806, filed on Feb. 26, 2021, which claims priority to U.S. Provisional Application 62/982,847 filed on Feb. 28, 2020, which are incorporated herein by reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH & DEVELOPMENT

[0002] This invention was made with government support under GM119644 and CA014520 awarded by the National Institutes of Health and under 1645123 and EEC1648035 awarded by the National Science Foundation. The government has certain rights in the invention.

FIELD OF THE DISCLOSURE

[0003] The present disclosure is related to method of preparing genome-edited T cells, particular chimeric antigen receptor (CAR) T cells.

BACKGROUND

[0004] Immunotherapy treatments using T cells expressing a chimeric antigen receptor (CAR T cells) targeted against tumor-associated antigens can provide complete remission for patients afflicted by cancer. Currently, there are over 850 clinical trials underway around the world with CAR T cell immunotherapies, and nearly all of them require the use of viral vectors to deliver the CAR gene into T cells. The use of viral vectors for CAR T cell manufacturing constitutes a bottleneck in the supply chain for biomanufacturing and can be problematic due to (1) batch-to-batch variability, (2) use of xenogeneic components during manufacturing of viral vectors, and (3) the high random integration of viral elements into the human genome. The poorly specified integration of the CAR transgene can lead to heterogeneous expression that can be readily silenced, in part by host cell recognition of viral genetic elements.

[0005] Methods to generate CAR T cells generally involve viral vectors, transposons or transient transfection. Autologous CAR T cells are traditionally generated using lentiviruses or retroviruses. They can also be generated using transposon-based systems. All of these systems randomly integrate the CAR transgene throughout the human genome. More recently, transfection with mRNA encoding the CAR has also been reported, however the limited half-life of mRNA ultimately does not provide a durable CAR therapy past a few days to weeks.

[0006] Genome editing has been used to generate CAR T cells with a site-specific integration of the CAR, however these methods rely on transduction of the T cells with AAVs. AAV6 has been used to deliver the homology directed repair template that encodes the CAR. This was recently demonstrated for a CD19 CAR appropriate for treatment of hematologic malignancies, but not solid tumors. To date, methods to generate CAR T cells have shown limited to no activity in solid tumors. What is needed are new methods for generating genetically modified T cells, such as CAR T cells, that would lead to measurable efficacy against either hematologic malignancies or solid tumors.

BRIEF SUMMARY

[0007] In one aspect, an ex vivo, non-viral 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

[0008] preparing a non-viral double-stranded homology-directed repair (HDR) template comprising the transgene flanked by homology arms that are complementary to sequences on both sides of a cleavage site in the T cell expressed gene,

[0009] introducing into a population of unmodified T cells a Cas9 ribonucleoprotein (RNP) and the double-stranded HDR template, to provide the CAR T cells

[0010] 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,

[0011] wherein the non-viral double-stranded HDR template contains the transgene sequence flanked by homology arms that are complementary to sequences on both sides of the cleavage site in the T cell expressed gene, and

[0012] wherein the transgene is specifically integrated into the cleavage site of the T cell expressed gene locus created by the Cas9 RNP in the CAR T cells, and

[0013] 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,

[0014] 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

[0015] wherein the CAR gene encodes a fusion protein comprising of one or more antigen-specific extracellular domains coupled to an intracellular domain by a transmembrane domain.

[0016] In another aspect, a non-viral produced CAR T cell has a genome having a CAR sequence specifically integrated into a T cell expressed gene, wherein the T cell is enriched for the CD62L and/or CD45RA markers indicative of naïve and stem cell memory phenotypes compared to viral-produced control CAR T cells.

[0017] In another aspect, a non-viral produced CAR T cell has a genome having a CAR sequence specifically integrated into a T cell expressed gene, wherein the T cell has reduced expression of TIM3 and/or LAG3 markers of T cell exhaustion compared to viral-produced control CAR T cells.

[0018] In another aspect, a method of treating a subject comprises administering any of the foregoing CART cells to a subject in need of adoptive T cell therapy.

[0019] Also included is a plasmid of SEQ ID NO: 1.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] FIGS. **1***a*-**1***k* show nonviral CRISPR-CAR T cells are efficiently manufactured in 9 days and exhibit decreased detrimental signaling and exhaustion before encountering their target antigen. FIG. **1***a* is a schematic showing the CAR genetic construct and nonviral strategy to insert the CAR into the first exon of the human TRAC locus. The seed sequence of the gRNA is identified and the protospacer

adjacent motif (PAM) for SpyCas9 is underlined (SEQ ID NOS. 2 and 3). LHA: left homology arm, SA: splice acceptor, 2A: self-cleaving peptide, pA: rabbit β-globin polyA terminator. FIG. **1***b* is a summary of manufacturing schedule and analyses for all cell products. RV-CAR, donor-matched CAR T cell product generated by retroviral transduction with the same third generation anti-GD2 CAR shown in a; NV-mCh, donor-matched control T cell product manufactured nonvirally as in FIG. **1***a* but with an mCherry fluorescent protein instead of the CAR. FIG. **1***c* shows representative density flow cytometry plots for transgene and TCR surface protein levels on the manufactured cell products. Y-axis shows CAR or mCherry transgene levels and X-axis shows TCR levels on day 7 post isolation (day 5 post-electroporation for NV-CAR and NV-mCh, and day 4 post viral transfection for control RV-CAR). Boxes show populations selected for downstream analysis in FIG. **1***d-f*. FIG. **1***d* shows histograms show CAR expression for the three test groups. Boxplots show the percentage of CAR positive cells in each sample, and mean fluorescence intensity (MFI) values for the CAR expression levels, respectively. NV-CAR N=31; RV-CAR N=39; NV-mCh N=27. FIG. **1***e* shows histograms show TCR expression on the three test groups. Boxplots show the percentage of CAR positive cells in each sample. NV-CAR N=31; RV-CAR N=39; NV-mCh N=27. FIG. **1***f* shows histograms show CD62L expression for the three test groups. Boxplots show mean fluorescence intensity (MFI) for CD62L expression. NV-CAR N=31; RV-CAR N=39; NV-mCh N=27. Replicates from 97 samples across 4 separate donors. FIG. **1***g* shows in-out PCR indicates proper on-target genomic integration of the CAR transgene in NV-CAR cells. Primer locations are shown in a by arrows upstream of the LHA and within the CD28 sequence of the CAR. Untransf., untransfected donor-matched T cells; NTC=non-template control. FIG. **1***h* shows a Manhattan plot of CHANGE-seq-detected on- and off-target sites organized by chromosomal position with bar heights representing CHANGE-seq read count. The on-target site is indicated with the arrow. FIG. 1*i* shows visualization of sites detected by CHANGE-seq. The intended target sequence (SEQ ID NO: 4) is shown in the top line. Cleaved sites (on- and off-target) are shown below and are ordered top to bottom by CHANGE-seq read count, with mismatches to the intended target sequence indicated. Insertions are shown in smaller lettering between genomic positions, deletions are shown by (-). FIG. **1***j* shows UMAP projections as in j showing only cells for which transgene-positive cells were detected. Transgene-positive cells cluster similarly for both NV-CAR and RV-CAR T cells, but not NV-mCh T cells. FIG. **1***k*.1, k.2 and k.3, show enrichment of Reactome pathway gene signatures (rows) in the transgene-positive cells from donors 1 and 2. NES, Normalized Enrichment Score. At right, representative gene set enrichment analysis (GSEA) plot of a signature within CAR-positive T cells from a RV-CAR sample, where genes differentially expressed in CAR-positive RV-CAR cells versus CAR-positive NV-CAR cells from donor 1 are listed and ranked. FDR <0.001 for each comparison, by gene-set permutation test. Below the GSEA plot is a heatmap representing transcripts with significant differential expression. Rows represent adjusted p-value using Bonferroni correction for all features in the dataset. FIG. **11** shows cytokine production from conditioned media taken from T cell products at the end of manufacturing (pre-antigen exposure). Values are pooled from all 4 donors. NV-CAR, N=24; RV-CAR, N=33; NV-mCherry N=22. * indicates p<=0.05; ** indicates p<=0.01; *** indicates p<=0.001; **** indicates p<=0.0001.

[0021] FIGS. **2***a-i* show nonviral CRISPR-CAR T cells exhibit a robust cytotoxic response to target antigen-positive tumor cells in vitro and induce tumor regression in vivo with a reduced exhaustion phenotype. FIG. **2***a* shows cytokine production in conditioned media after a 24 hour co-culture of manufactured T cell products with the target GD2-antigen on CHLA20 neuroblastoma cells. Values are pooled from 2 donors. NV-CAR N=8; RV-CAR (green) N=5; NV-mCh N=8. FIG. **2***b* shows IncuCyte in vitro assay of T cell potency, averaged across donors. AnnexinV was added as a marker of cell death; y-axis shows GFP-positive cancer cells in each well of a 96-well plate. The ratio of T cells to cancer cells is 5:1. The consistent decrease in CHLA20 cells after 15 hours indicates high potency of both NV-CAR and RV-CAR T cells. NV-CAR (blue) N=12; RV-CAR N=12; CHLA20 neuroblastoma alone N=9. FIG. **2***c* shows a UMAP projections as in c showing only cells for which transgene was detected. Transgene-positive cells cluster similarly for both NV-CAR and RV-CAR T cells, but not for NV-mCh T cells. FIGS. **2***d*.**1**, d.**2**, and d.**3** show enrichment of Reactome pathway gene signatures (rows) in the transgene-positive cells from donors 1 and 2 after co-culture with GD2-positive CHLA20 cancer cells. NES, Normalized Enrichment Score. At right, representative GSEA showing differential cytotoxicity signature of NV-CAR/NV-mCh paired samples for two donors, and NV-CAR/RV-CAR samples. NV-CAR T cells show significant upregulation of cytotoxicity markers relative to NV-mCh control cells after GD2 antigen exposure, while NV-CAR and RV-CAR T cells show no significant difference in activation signature upon GD2 antigen stimulation. FDR<0.001 for each comparison, by gene-set permutation test. Bottom GSEA plot is a heatmap representing transcripts with significant differential expression. Rows represent adjusted p-value using Bonferroni correction for all features in the dataset. FIG. **2***e* shows a schematic of the in vivo mouse dosing strategy using NSG mice harboring GD2-positive CHLA20 neuroblastoma tumors. FIG. **2***f* shows representative IVIS images of NSG mice with CHLA20 tumors that were treated with either 10 million NV-CAR, RV-CAR, or NV-mCh T cells. FIG. **2***g* shows Kaplan-Meyer survival curve for mice. NV-CAR N=10; RV-CAR N=8; NV-mCh N=7. FIG. **2***i* shows box plots on the amount of human T cells present in mouse spleens, as measured by the presence of human CD45 using flow cytometry, and the percentage of those cells in the spleen that were CAR-positive. FIG. **2***h* shows histograms showing the expression levels of PD-1 and TIM-3 on the human CD45+ cells in the mouse spleens. *indicates p<=0.05; ** indicates p<=0.01; *** indicates p<=0.001; **** indicates p<=0.0001.

[0022] FIG. **3***a-e* shows pre-antigen exposure characterization of NV-CART cells. FIG. **3***a* shows left, viability of cells throughout the manufacturing timeline, pooled for all 4 donors. Right, cell counts throughout the manufacture calendar, pooled for all 4 donors. NV-CAR N=36; RV-CAR N=27; NV-mCh N=25. FIG. **3***b* shows left, Percent of CAR+ cells as measured by flow cytometry when electroporated on day 2 or day 3 post-isolation. Right, Percent of cells with TCR knockout as measured by flow cytometry when electroporated on day 2 or day 3 post-isolation. All groups, N=3.

FIG. **3***c* shows the level of TCR disruption in NV-CAR and NV-mCh T cells measured by both TCR surface expression by flow cytometry (right) and presence of indels at the TRAC locus (left). NV-CAR N=10, NV-mCh N=8. FIG. **3***d* shows percent of cells with indels at the TRAC locus in both NV-CAR and NV-mCh conditions. NV-CA N=10; NV-mCh N=8, both for one donor. FIG. **3***e* shows in-out PCR confirming NV-CAR insertion, full gel from FIG. **1***g* shown. PCR was optimized to minimize off-target amplification which occurs only for fragments <1 kb across the genome. N=3 for all samples from one donor. Untransf., donor matched untransfected control T cells; NTC=non-template control.

[0023] FIG. **4***a-c* show single cell transcriptomic characterization across eleven samples shows distinct transcriptional signatures associated with CAR expression but not mCherry expression, both before and after antigen exposure. FIG. **4***a* shows a UMAP projection of single cell RNA-seq data showing cells across all eleven samples and two donors, both pre-and post-antigen exposure. N=69,017 single cells. FIG. **4** *b* shows a UMAP projection as in FIG. **4***a*, separated to show clustering of transgene positive cells prior to antigen exposure (left) and after 24 hours of in vitro exposure to GD2+ CHLA20 neuroblastoma. FIG. **4***c* shows a UMAP projection as in FIG. **4***a*, showing transgene positive cells for each individual sample. CAR-positive cells from NV-CAR and RV-CAR groups consistently cluster regardless of the presence of GD2 antigen, while NV-mCh cells do not, suggesting a distinct transcriptional profile associated with CAR signaling.

[0024] FIG. **5** shows a novel plasmid used to generate CAR HDR template via PCR. The PCR primers were designed to amplify the following: TRAC LHA-SA-2A-14g2a-hinge-CD28-OX40-zeta chain-rb_glob_PA_terminator-TRAC RHA. LHA: left homology arm, SA: splice acceptor: 2A: self-cleaving peptide, rb_glob_PA_terminator: rabbit beta globin polyA terminator. One example is shown here, but any synthetic gene sequence could be inserted between the homology arms.

[0025] FIG. **6** shows representative images of NV-CRISPR CAR T cells post-editing.

[0026] FIG. **7** shows a flow cytometry plot with representative gene editing. TCR expression is shown on the X axis, and CAR expression is on the Y axis, with 94% TCR knockout and 46% CAR knockin.

[0027] FIG. **8** shows average gene editing efficiency across 20 replicates per cell type. 20 replicate NV-CAR and NV-mCherry editing experiments yielded an average knockin efficiency of 35% in both conditions, as measured by flow cytometry. Unedited controls show no non-specific staining.

[0028] 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

[0029] Described herein are methods to generate genome edited T cells such as CAR T cells using site-specific genome editing where the editing machinery consists only of proteins and nucleic acids without any viral vectors. In an aspect, demonstrated herein is CRISPR-Cas9 mediated genomic insertion of CAR transgenes into the T cell receptor alpha constant, TRAC, locus in primary human T cells collected from healthy donors. These cells, termed nonviral-(NV)-TRAC-CAR T cells, exhibit proper TRAC-specific integration of the CAR transgene, robust gene expression of the CAR mRNA, and translated CAR proteins on the T cell surface. The NV TRAC-CAR T cells potently upregulate cytotoxic transcriptional programs and kill target-antigen-positive human cancer cells in vitro within co-culture assays. The NV TRAC-CAR T cells successfully cause tumor regression in vivo within human xenograft cancer models in mice at comparable efficiency to state-of-the-art, viral CAR T cells. NV-TRAC-CAR T cells can be manufactured in a xeno-free manner and have high potential to simplify and advance CAR T cell manufacturing by elimination of viral vectors.

[0030] In an aspect, an ex vivo method of site-specifically inserting a synthetic DNA sequence, e.g., a transgene containing a chimeric antigen receptor (CAR) gene, into a T cell genome comprises introducing into a population of unmodified T cells a Cas9 ribonucleoprotein (RNP) and a non-viral double-stranded homology-directed repair (HDR) template, to provide genome-edited T cells. The Cas9 RNP comprises a Cas9 protein and a guide RNA that directs double stranded DNA cleavage of a cleavage site in a T-cell expressed gene. The non-viral double-stranded HDR template comprises a synthetic DNA sequence flanked by homology arms that are complementary to sequences on both sides of the cleavage site in the T cell expressed gene. The synthetic DNA sequence is specifically integrated into the cleavage site of the T cell expressed gene by the Cas9 ribonucleoprotein in the genome-edited T cells. After integration, the method includes culturing the genome-edited T cells in xeno-free medium to provide a cultured population of genome-edited T cells having the synthetic DNA sequence specifically integrated in the T-cell expressed gene locus. In the cultured population of genome-edited T cells, an endogenous promoter of the T cell expressed gene drives expression of the synthetic DNA sequence, or the synthetic DNA sequence includes a promoter that drives expression of the synthetic DNA sequence.

[0031] In the methods described herein, a synthetic DNA sequence is site-specifically inserted into the genome of a T cell, specifically into a T cell expressed gene. As used herein, a synthetic DNA sequence is a DNA sequence that is not native to the genome of the T cell to be modified. An exemplary aspect of a synthetic DNA sequence is a "chimeric antigen receptor (CAR)". CAR refers to a recombinant fusion protein that has an antigen-specific extracellular domain coupled to an intracellular domain that directs the cell to perform a specialized function upon binding of an antigen to the extracellular domain. In an aspect, a CAR comprises an antigen-specific extracellular domain (e.g., a single chain variable fragment [scFV] that can bind a surface-expressed antigen of a malignancy) coupled to an intracellular domain (e.g., CD28, ICOS, CD27, 4-1BB, OX40, CD40L, or CD3-ζ) by a transmembrane domain (e.g., derived from a CD4, CD8α, CD28, IgG or CD3-ζ transmembrane domain).

[0032] 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.

[0033] The antigen-specific extracellular domain of a CAR recognizes and specifically binds an antigen, typically a surface-expressed antigen of a malignancy. An antigen-

specific extracellular domain specifically binds an antigen when, for example, it binds the antigen with an affinity constant or affinity of interaction (KD) between about 0.1 pM to about 10 μM, specifically about 0.1 pM to about 1 μM, more specifically about 0.1 pM to about 100 nM. Methods for determining the affinity of interaction are known in the art. An antigen-specific extracellular domain suitable for use in a CAR may be any antigen-binding polypeptide, one or more scFv, or another antibody based recognition domain (cAb VHH (camelid antibody variable domains) or humanized versions thereof, IgNAR VH (shark antibody variable domains) and humanized versions thereof, sdAb VH (single domain antibody variable domains) and "camelized" antibody variable domains are suitable for use. In some instances, T cell receptor (TCR) based recognition domains such as single chain TCR may be used as well as ligands for cytokine receptors.

[0034] The present disclosure provides chimeric antigen receptors (CARs) that bind to an antigen of interest. The CAR can bind to a tumor antigen or a pathogen antigen.

[0035] In certain embodiments, the CAR binds to a tumor antigen. Any tumor antigen (antigenic peptide) can be used in the tumor-related embodiments described herein. Sources of antigen include, but are not limited to, cancer proteins. The antigen can be expressed as a peptide or as an intact protein or portion thereof. The intact protein or a portion thereof can be native or mutagenized. Non-limiting examples of tumor antigens include carbonic anhydrase IX (CAIX), carcinoembryonic antigen (CEA), CD8, CD7, CD10, CD19, CD20, CD22, CD30, CD33, CLL1, CD34, CD38, CD41, CD44, CD49f, CD56, CD74, CD133, CD138, CD123, CD44V6, an antigen of a cytomegalovirus (CMV) infected cell (e.g., a cell surface antigen), epithelial glycoprotein-2 (EGP-2), epithelial glycoprotein-40 (EGP-40), epithelial cell adhesion molecule (EpCAM), receptor tyrosine-protein kinases erb-B2,3,4 (erb-B2,3,4), folate-binding protein (FBP), fetal acetylcholine receptor (AChR), folate receptor-α, Ganglioside G2 (GD2), Ganglioside G3 (GD3), human Epidermal Growth Factor Receptor 2 (HER-2), human telomerase reverse transcriptase (hTERT), Interleukin-13 receptor subunit alpha-2 (IL-13Rα2), κ-light chain, kinase insert domain receptor (KDR), Lewis Y (LeY), L1 cell adhesion molecule (L1CAM), melanoma antigen family A, 1 (MAGE-A1), Mucin 16 (MUC16), Mucin 1 (MUC1), Mesothelin (MSLN), ERBB2, MAGEA3, p53, MART1, GP100, Proteinase3 (PR1), Tyrosinase, Survivin, hTERT, EphA2, NKG2D ligands, cancer-testis antigen NY-ESO-1, oncofetal antigen (h5T4), prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA), ROR1, tumor-associated glycoprotein 72 (TAG-72), vascular endothelial growth factor R2 (VEGF-R2), and Wilms tumor protein (WT-1), BCMA, NKCS1, EGF1R, EGFR-VIII, CD99, CD70, ADGRE2, CCR1, LILRB2, PRAME CCR4, CD5, CD3, TRBC1, TRBC2, TIM-3, Integrin B7, ICAM-1, CD70, Tim3, CLEC12A and ERBB.

[0036] In certain embodiments, the CAR binds to a pathogen antigen, e.g., for use in treating and/or preventing a pathogen infection or other infectious disease, for example, in an immunocompromised subject. Non-limiting examples of pathogen include viruses, bacteria, fungi, parasite and protozoa capable of causing disease.

[0037] Non-limiting examples of viruses include, Retroviridae (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HDTV-III, LAVE or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (e.g. polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g. strains that cause gastroenteritis); Togaviridae (e.g. equine encephalitis viruses, rubella viruses); Flaviridae (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); Coronoviridae (e.g. coronaviruses); Rhabdoviridae (e.g. vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g. ebola viruses); Paramyxoviridae (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g. influenza viruses); Bungaviridae (e.g. Hantaan viruses, bunga viruses, phleboviruses and Naira viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (e.g. reoviruses, orbiviurses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvovirida (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g. African swine fever virus); and unclassified viruses (e.g. the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

[0038] Non-limiting examples of bacteria include *Pasteurella, Staphylococci, Streptococcus, Escherichia coli, Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to, *Helicobacter pyloris, Borelia burgdorferi, Legionella pneumophilia, Mycobacteria* sps (e.g., *M. tuberculosis, M. avium, M. intracellulare, M. kansaii, M. gordonae*), *Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes* (Group A *Streptococcus*), *Streptococcus agalactiae* (Group B *Streptococcus*), *Streptococcus* (*viridans* group), *Streptococcusfaecalis, Streptococcus bovis, Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae, Bacillus antracis, Corynebacterium diphtheriae, corynebacterium* sp., *Erysipelothrix rhusiopathiae, Clostridium perfringens, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasteurella multocida, Bacteroides* sp., *Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue, Leptospira, Rickettsia*, and *Actinomyces israelli.*

[0039] In certain embodiments, the pathogen antigen is a viral antigen present in Cytomegalovirus (CMV), a viral antigen present in Epstein Barr Virus (EBV), a viral antigen present in Human Immunodeficiency Virus (HIV), or a viral antigen present in influenza virus.

[0040] The intracellular domain transmits the T cell activation signal. The intracellular domain can increase CAR T cell cytokine production and facilitate T cell replication. The intracellular domain reduces CAR T cell exhaustion, increases T cell antitumor activity, and enhances survival of CAR T cells in patients. Exemplary intracellular domains, also call co-stimulatory domains, include CD28, ICOS, CD27, 4-1BB, OX40, CD40L, and CD3-ζ.

[0041] Typically, the antigen-specific extracellular domain is linked to the intracellular domain of the CAR by a transmembrane domain, e.g., derived from a CD4, CD8α, CD28, IgG or CD3-ζ transmembrane domain. The trans-

membrane 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. 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. Suitable transmembrane domains, costimulatory domains, and spacers are known in the art.

[0042] In an aspect, synthetic DNA sequences within the HDRT could incorporate synthetic receptors, cytokine signaling, and short hairpin (sh)RNA. One example is to make use of natural ligand—receptor pairs (e.g., modified interleukin (IL)-13 sequences) and natural ligand-binding domains of receptors (e.g., NKG2D and CD27) to target receptors to disease. Another example is incorporate sequences that encode cytokine receptor signaling important for T cell maintenance and expansion (e.g., IL-2 receptor beta chain (IL-2Rb) and a STAT3-binding motif). In addition, sh(RNA) could also be expressed from the synthetic DNA sequence that helps provide control over the edited T cell behavior.

[0043] In an aspect, the synthetic DNA sequence comprises a coding sequence for a fluorescent protein such as mCherry, mKate, GFP, BFP, RFP, CFP, YFP, mCyan, mOrange, tdTomato, mBanana, mPlum, mRaspberry, mStrawberry, and mTangerine.

[0044] In order to insert the synthetic DNA sequence into the genome of the unmodified T cells, a Cas9 RNP and a non-viral double-stranded HDR template including the synthetic DNA sequence are introduced into the unmodified T cells to provide genome-edited T cells.

[0045] As used herein, "introducing" means refers to the translocation of the Cas9 ribonucleoprotein and a non-viral double-stranded HDR 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, and the like.

[0046] 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 intracerebral injection.

[0047] 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.

[0048] 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.

[0049] 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).

[0050] 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.

[0051] 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.

[0052] 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.

[0053] A "Cas9" polypeptide is a polypeptide that functions as a nuclease when complexed to a guide RNA, e.g., an sgRNA or modified sgRNA. That is, Cas9 is an RNA-

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

[0054] 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: 5); Cas9 polypeptide derived from *Streptococcus thermophilus*, e.g., a polypeptide having the sequence of the Swiss-Prot accession G3ECR1 (SEQ ID NO: 6); a Cas9 polypeptide derived from a bacterial species within the genus *Streptococcus*; a Cas9 polypeptide derived from a bacterial species in the genus *Neisseria* (e.g., GenBank accession number YP_003082577; WP_015815286.1 (SEQ ID NO: 7)); a Cas9 polypeptide derived from a bacterial species within the genus *Treponema* (e.g., GenBank accession number EMB41078 (SEQ ID NO: 8)); 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.

[0055] 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.

[0056] Functional Cas9 mutants are described, for example, in US20170081650 and US20170152508, incorporated herein by reference for its disclosure of Cas9 mutants.

[0057] 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).

[0058] 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 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.

[0059] 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-targeting Cas9 ribonucleoprotein results in a reduction or elimination of expression of functional TRAC gene product (e.g., knock-out of expression of functional TRAC gene product).

[0060] 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 exon 1 of TRAC comprises SEQ ID NO: 9.

[0061] In addition to the Cas9 RNP, a non-viral double-stranded HDR template comprising the synthetic DNA sequence is introduced into the T cells. In prior art methods, viral vectors such as adeno-associated virus vectors have been used to provide the synthetic DNA template. Even when combined with Cas9 RNP gene editing, the use of AAV vectors (a) are expensive; (b) could integrate viral genomes into the human genome; (c) trigger an immune response within the patient to viral components; (d) may result in highly variable transgene expression; and (d) take extended periods of time (e.g., months to years) to manufacture.

[0062] In an aspect, the non-viral double-stranded HDR template comprises the synthetic DNA sequence flanked by homology arms for insertion of the synthetic DNA sequence into the T cell expressed gene by the Cas9 RNP. The homology arms have 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. For example, when the T cell expressed gene comprises exon 1 of TRAC, the homology arms can comprise:

[0063] In an aspect, the non-viral double-stranded HDR template sequentially comprises a left homology arm—a splice acceptor site—a self-cleaving peptide sequence (e.g., a T2A coding sequence)—a CAR gene—a polyA terminator—a right homology arm.

[0064] The splice acceptor site assists in the splicing of the synthetic DNA sequence into the transcript generated from the native T cell expressed gene.

[0065] The 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 the 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.

[0066] The polyA terminator, e.g., a bovine growth hormone polyA. The 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.

[0067] In an aspect, the non-viral double-stranded HDR template is produced by amplifying a sequence from a bacterial plasmid, e.g., SEQ ID NO: 1. Amplification can be done using a Q5® Hot Start Polymerase (NEB).

[0068] Also included herein is the plasmid of SEQ ID NO: 1. In an aspect, the double-stranded HDR template has an OD260/OD280 of 1.8 to 2.1, and/or an OD260/OD230 of 2.0 to 2.3.

[0069] In an aspect, the double-stranded HDR template has a concentration of 2000 to 10000 ng/μl.

[0070] After introducing the Cas9 RNP and a non-viral double-stranded HDR template into the unmodified T cells, a population of genome-edited T cells is produced.

[0071] In an aspect, the genome-edited T cells are deficient in expression of the T-cell expressed gene product, while expressing a gene product of the synthetic DNA sequence. The endogenous promoter of the T-cell expressed gene can drive expression of a gene product within the synthetic DNA sequence.

[0072] 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. Zeno-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.

[0073] 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.

[0074] In an aspect, after culturing, the modified T cells can aggregate to form a cluster of cells. Cells which exhibit a higher degree of aggregation typically recover at higher rates than cells that do not aggregate. The aggregation could help cell-cell interaction through paracrine or juxtacrine signaling that assists in recovery. In an aspect, the method further comprises imaging the population of CAR T cells and determining the degree of aggregation of the CAR T cells, and optionally selecting a population of aggregated CAR T cells.

[0075] In an aspect, culturing is done in round bottom culture wells at 20% of standard culture volume for the wells. It was unexpectedly found that by using round bottom culture wells rather than flat, for example, improved recovery was observed.

[0076] In the methods described herein, more than 4, 5, 6, 7, 8, 9 or 10% of the population of unmodified T cells has the synthetic target gene inserted into their genomes.

[0077] In the cultured population of genome-edited T cells, in the cultured population of genome-edited T cells, an endogenous promoter of the T cell expressed gene drives expression of the synthetic DNA sequence, or the synthetic DNA sequence can include a promoter that drives expression of the synthetic DNA sequence. Exemplary promoters include CAGGS and EF1alpha.

[0078] In an aspect, the CAR T cells produced by the methods described herein have activity against an antigen on a solid tumor in vitro or in vivo.

[0079] In an aspect, described herein is a non-viral produced CAR T cell with a genome having a CAR sequence specifically integrated into a T cell expressed gene, wherein the T cell is enriched for the CD62L and/or CD45RA markers indicative of naïve and stem cell memory phenotypes compared to viral-produced control CAR T cells. Also included is a non-viral produced CAR T cell with a genome having a CAR sequence specifically integrated into a T cell expressed gene, wherein the T cell has reduced expression of TIM3 and/or LAG3 markers of T cell exhaustion compared to viral-produced control CAR T cells. The CD62L and/or CD45RA markers can be enriched more than 2-fold compared to the CD62L and/or CD45RA markers compared to viral-produced control CAR T cells. In another aspect, the TIM3 and/or LAG3 markers are reduced more than 2-fold compared to the TIM3 and/or LAG3 markers compared to viral-produced control CAR T cells.

[0080] Also include herein are method of treating a subject comprising administering any of the foregoing genome-edited T cells comprising the synthetic DNA sequence to a subject in need of T cell therapy, such as CART cell therapy. CAR T cell therapy, for example, has been approved to treat hematologic malignancies like acute lymphoblastic leukemia, non-Hodgkin large B-cell lymphomas, and have been used to treat chronic lymphocytic leukemia and multiple myeloma. Herein evidence is presented a method that produces CAR T cells that should not only be active against hematologic malignancies, but could be used to produce CAR T cells with activity against solid tumors. CAR T cells are typically administered by intravenous infusion but could also be administered by subcutaneous, intratumoral, intraperitoneal or intracerebral injection.

[0081] The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Materials and Methods

[0082] Data Reporting. For in vivo experiments, established tumor burden was verified by IVIS luciferase imaging prior to infusion. Mice were arranged according to tumor burden and distributed evenly across conditions. The experiments were not randomized and the investigators were not blinded during experiments and outcome assessment.

[0083] Antibodies. Antibodies used in this study for flow cytometry and fluorescence activated cell sorting are listed in Table 1.

TABLE 1

Antibodies used in flow cytometry and cell experiments

Antigen	Clone	Fluorophore
CAR	anti-id 1A7	APC
CCR7	G043H7	Brilliant Violet ™ 711

TABLE 1-continued

Antibodies used in flow cytometry and cell experiments

Antigen	Clone	Fluorophore
CCR7	G043H7	Brilliant Violet ™ 650
CD19	HIB19	APC-Fire ™ 750
CD3	OKT3	AlexaFluor ® 488
CD3	OKT3	Brilliant Violet ™ 785
CD3	OKT3	PE-Dazzle ™ 594
CD3	OKT3	AlexaFluor ® 488
CD4	OKT4	Brilliant Violet ™ 711
CD4	OKT4	PE-Cyanine5
CD45RA	HI100	PE-Cyanine7
CD62L	DREG56	Brilliant Violet ™ 605
CD62L	DREG56	PE
CD69	FN50	PE-Dazzle ™ 594
CD8	SK1	PerCP-eFluor710
CD95	DX2	AlexaFluor ® 700
GD2	14G2a	APC
Human CD45	HI30	Pacific Blue
IgG2a	RMG2a-62	APC
LAG3	3DS223H	PE
Mouse CD45.1	A20	PE-Cyanine7
PD1	EH12.2H7	AlexaFluor ® 488
TCR α/β	IP26	BV421
TCR α/β	IP26	AlexaFluor ® 488
TIM3	F38-2E2	Brilliant Violet ™ 510
Mouse Lyt2	53-6.7	AlexaFluor ® 488
GhostRed ™780	Viability	—

[0084] Guide RNAs. All guide RNAs used in this study are listed in Table 2.

TABLE 2

gRNA sequences

gRNA	Sequence	SEQ ID NO:
TRAC	5' CAGGGTTCTGGATATCTGT 3'	9

[0085] The full sequence of the cr RNA is 5' CAGGGTTCTGGATATCTGTGTTTTAGAGCTATGCT3' (SEQ ID NO: 10). The tracr portion of the guide RNA is a proprietary 67mer tracr RNA available from IDT.

[0086] Primers. All primers used in this study are listed in Table 3.

TABLE 3

Oligo	Sequence	SEQ ID NO:
TRAC Donor FWD primer	CCTTTTTCCCATGCCTGCCTTT	11
TRAC Donor REV primer	TAAGGCCGAGACCACCAATCAG	12
TRAC sequencing FWD primer	ACACTCTTTCCCTACACGACGCTCTT CCGATCT	13
TRAC sequencing REV primer	GTGACTGGAGTTCAGACGTGTGCTCT TCCGATCT	14
TRAC genomic integration FWD primer	ATCTTGTGCGCATGTGAGGGGC	15
TRAC genomic integration REV primer	GCAAGCCAGGACTCCACCAACC	16

[0087] Cell lines. CHLA-20 human neuroblastoma were a gift from Dr. Maria Otto. These cells were maintained in Dulbecco's Modified Eagle Medium high glucose (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% Penicillin-Streptomycin. AkaLuc-GFP CHLA-20 cells were a gift from the J. Thomson lab (UW-Madison). Phoenix™ cells (ATCC) for viral preparation were maintained in DMEM (high glucose) supplemented with 10% Fetal Bovine Serum (Gibco), and selected using 1 μg/mL diphtheria toxin and 300 μg/mL hygromycin prior to use. Selection for transgene positive cells was confirmed by flow cytometry for Lyt2 expression (Biolegend) (>70%+). 3T3 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% Penicillin-Streptomycin (Gibco). Cell authentication was performed using short tandem repeat analysis (Idexx BioAnalytics, Westbrook, Me.) and per ATCC guidelines using morphology, growth curves, and Mycoplasma testing within 6 months of use using the e-Myco mycoplasma PCR detection kit (iNtRON Biotechnology Inc, Boca Raton, Fla.). Cell lines were maintained in culture at 37° C. in 5% CO_2, and used after 3-5 passages in culture after thawing.

[0088] Plasmid constructs. NV-AAVS1-CAR: An NV-AAVS1-CAR donor plasmid (SEQ ID NO: 1) was designed using a pAAV-CAGGS-GFP backbone (Addgene) and a 2 kb CAR gBlock (IDT), which was inserted into the backbone using restriction cloning. NV-TRAC-CAR: A 2 kb region surrounding the TRAC locus was amplified by PCR from human genomic DNA and cloned into a pCR blunt II TOPO™ backbone (Thermo Fisher Scientific). The CAR transgene was then cloned into the TOPO™ TRAC vector using Gibson Assembly (NEB). Plasmid sequence was verified by Sanger sequencing. TRAC-H2B-mCherry, NV-TRAC-41bb-CAR: These constructs were ordered as synthesized genes in a pUC57 vector (GenScript). All plasmids were grown in NEB®5-alpha competent *E. coli* (NEB) and purified using the PureYield™ MidiPrep system (Promega).

[0089] Double-stranded DNA HDRT production. Plasmid constructs were used as PCR templates for NV (nonviral) products. In brief, NV-CAR and NV-mCh plasmids were MidiPrepped using the PureYield MidiPrep system (Promega). PCR amplicons were generated from plasmid templates using Q5® Hot Start Polymerase (NEB), and pooled into 100 μl reactions for Solid Phase Reversible Immobili-

zation (SPRI) cleanup (1×) using AMPure® XP beads according to the manufacturer's instructions (Beckman Coulter). Each 100 μl starting product was eluted into 5 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 yield. PCR products from round 1 cleanup were pooled and subjected to a second round of SPRI cleanup (1×) to increase total concentration; round 2 elution volume was 20% of round 1 input volume. Template concentration and purity was quantified using NanoDrop™ 2000 and Qubit™ dsDNA BR Assays (Thermo Fisher Scientific), and templates were diluted in water to an exact concentration of 2 μg/μl.

[0090] SpyCas9 RNP preparation. RNPs were produced by complexing a two-component gRNA to SpyCas9. 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 1 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 0.8 μl) was added to the complexed gRNA at a 1:1 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.

[0091] Isolation of human primary T cells. 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, then transferred to sterile 50 mL conical tubes. Primary human T cells were isolated using negative selection per the manufacturer's instructions (RosetteSep™ Human T Cell Enrichment Cocktail, STEMCELL Technologies). T cells were counted using a Countess™ II FL Automated Cell Counter with 0.4% Trypan Blue viability stain (Thermo Fisher). T cells were cultured at a 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.

[0092] T cell culture. Bulk T cells were cultured in ImmunoCult™-XF T cell Expansion Medium at an approximate density of 1 million cells/mL. In brief, T cells were stimulated with ImmunoCult™ Human CD3/CD28/CD2 T cell Activator (STEMCELL) for 2 days prior to electroporation. On day 3, (24 hours post-electroporation), NV T and NV-mCh T cells were transferred to 1 mL of fresh culture medium (with 500 U/mL IL-2, without activator) and allowed to expand. T cells were passaged, counted, and adjusted to 1 million/mL in fresh medium +IL-2 on days 5 and 7 after isolation. RV-CAR T cells were spinoculated with RV-CAR construct on day 3 and passaged on day 5 with the NV-CAR and NV-mCh T cells. Prior to electroporation or spinoculation, the medium was supplemented with 200 U/mL IL-2; post gene editing, medium was supplemented with 500 U/mL IL-2 (Peprotech).

[0093] T cell electroporation. 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). 1 million cells per replicate were aliquoted into 1.5 mL tubes. During RNP complexation step (see RNP production), T cell aliquots were centrifuged for 10 min at 90 g. During the spin step, 2 μl HDR template (total 4 μg) per condition were aliquoted to PCR tubes, followed by RNPs (2.8 μl per well; pipette should be set to a higher volume to ensure complete expulsion of the highly viscous solution). Templates and RNPs were incubated at room temperature for at least 30 seconds. After cell centrifugation, supernatants were aspirated, and cells were resuspended in 20 μl P3 buffer, then transferred to PCR tubes containing RNP. 24 μl total volume per sample was transferred directly into wells of the 16 well Nucleocuvette™ strips. Typically, no more than 8 reactions were completed at a time to minimize the amount of time T cells spend in P3 buffer. T cells were electroporated with a Lonza 4D Nucleofector™ with X Unit using pulse code EH115. Immediately after nucleofection, 80 μl of pre-warmed media with 500 U/mL IL-2 and 25 μl/mL ImmunoCult™ CD3/CD28/CD2 activator was added to each cuvette well. Cuvettes rested at 37° C. in the cell culture incubator for 15 minutes. After 15 minutes, cells were moved to 200 μl total volume of media+IL-2+activator (see Primary T cell culture above) in a round bottom 96 well plate.

[0094] Retrovirus production. CAR retrovirus was manufactured using Phoenix™(ATCC). In brief, pSFG.iCasp9.2A.14G2A-CD28-OX40-CD3z plasmid (was MidiPrepped using the PureYield™ MidiPrep system (Promega). One day prior to transfection, selected Phoenix™ cells were plated on 0.01% Poly-L-Lysine coated 15 cm dishes at a density of 76,000 cells/cm^2, or ~65% confluency. On transfection day, media was replaced 1 hour prior to transfection of 10 μg pSFG.iCasp9.2A.14G2A-CD28-OX40-CD3z plasmid/plate using iMFectin according to the manufacturer's instructions (GenDEPOT). Media was replaced 18-24 hours later with 10 mL of 50 mM HEPES buffered DMEM +10% FBS (Gibco). 48 hours later, media was collected, stored at 4° C., and replaced. A second aliquot of media was collected 24 hours later. A second aliquot of media was collected 24 hours later; media aliquots were pooled and centrifuged for 10 min at 2000 g to pellet contaminating cells, and supernatants were transferred to a clean conical tube. ⅓ volume Retro-X™ concentrator (Takara/Clonetech) was added, and supernatants were refrigerated at 4° C. for 12-18 hours, and then concentrated according to the manufacturer's instructions. Viruses were tested on 3T3 cells prior to use. Yields from one 15 cm dish were used for 5 replicate conditions, where each replicate consisted of a well with 160,000 T cells per transduction. Viruses were either used immediately for T cell spinoculation, or stored at −80° C. in single use aliquots. Retroviral transduction. T cells for RV infection were cultured similarly to NV T and NV-mCh T cells, with two exceptions: 1) T cells were passaged and resuspended without Immunocult™ CD2/CD28/CD3 activator on day 2 post-isolation, then spinoculated on Day 3. RV-CAR T cells were returned to the regular passaging schedule on day 5 post-isolation. (See FIG. **1***b*). Prior to spinoculation, non-treated cell culture 24 well plates were coated with Retronectin® according to the manufacturer's instructions (Takara/Clontech). On day 3 post-isolation, T cells were centrifuged at 200 g for 3 minutes, counted, and resuspended to a concentration of 200,000 cells/mL, then stored in the incubator

until plates were prepared. Virus was added to Retronectin®-coated plates in a volume of 400 μl virus+ImmunoCult™ medium and centrifuged at 2000 g for 2 hours at 32C. 160,000 T cells in 800 μl were added to each well and spinoculated at 2000 g for 60 minutes at 32° C., without brakes. T cells were then transferred to the incubator and left undisturbed for two days.

[0095] Flow cytometry and fluorescence activated cell sorting. CAR was detected using 1A7 anti-14G2a idiotype antibody (gift from Paul Sondel) conjugated to APC with the Lightning-Link® APC Antibody Labeling kit (Novus Biologicals). T cells were stained in BD Brilliant™ Stain Buffer (BD Biosciences). For panels including TRAC and CD3, cells were permeabilized and fixed using the BD Cytofix/Cytoperm™ Plus kit according to the manufacturer's instructions. Flow cytometry was performed on an Attune™ N×T Flow cytometer, and fluorescence-activated cell sorting was performed on a BD FACS Aria™. All antibodies used in this study are described in Table 1. T cells from Donors 1 and 2 were stained and analyzed on day 9 of manufacture using fresh cells. For donors 3 and 4, only TCR, CAR, and CD62L were measured on day 9 of manufacture. The change in protocol was made due to equipment restrictions related to institutional COVID-19 biosafety precautions, and CD62L was selected for analysis due to the known effects of cryopreservation on expression levels.

[0096] "In-Out PCR". 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. Primer sequences are listed in Table 3. PCR was performed according to the manufacturer's instructions using Q50® Hot Start Polymerase (NEB) using the following program: 98° C. (30 s), 35 cycles of 98° C. (10 s), 62° C. (20 s), 72° C. (2 min), and a final extension at 72° C. (2 min).

[0097] Next Generation Sequencing Indel formation at the TRAC locus was measured using Next Generation Sequencing (Illumina). Genomic PCR was performed according to the manufacturer's instructions using Q5® Hot Start polymerase (NEB); primers are listed in Table 1. Products were purified using SPRI cleanup with AMPure® XP beads (Beckman Coulter), and sequencing indices were added with a second round of PCR using indexing primers (Illumina), followed by a second SPRI cleanup. Samples were pooled and sequenced on an Illumina® MiniSeq according to the manufacturer's instructions. Analysis was performed using CRISPR RGEN.

[0098] Genome-wide, off-target analysis. Genomic DNA from human primary $CD4^+/CD8^+$ T cells was isolated using Gentra® Puregene® Kit (Qiagen) according to the manufacturer's instructions. CHANGE-seq was performed as described in the art. Briefly, purified genomic DNA was tagmented with a custom Tn5-transposome to an average length of 400 bp, followed by gap repair with Kapa HiFi™ HotStart Uracil+ DNA Polymerase (KAPA Biosystems) and Taq DNA ligase (NEB). Gap-repaired tagmented DNA was treated with USER enzyme (NEB) and T4 polynucleotide kinase (NEB). Intramolecular circularization of the DNA was performed with T4 DNA ligase (NEB) and residual linear DNA was degraded by a cocktail of exonucleases containing Plasmid-Safe™ ATP-dependent DNase (Lucigen), Lambda exonuclease (NEB) and Exonuclease I (NEB). In vitro cleavage reactions were performed with 125 ng of exonuclease-treated circularized DNA, 90 nM of SpCas9 protein (NEB), NEB buffer 3.1 (NEB) and 270 nM of sgRNA, in a 50 μL volume. Cleaved products were A-tailed, ligated with a hairpin adaptor (NEB), treated with USER enzyme (NEB) and amplified by PCR with barcoded universal primers NEBNext® Multiplex Oligos for Illumina® (NEB), using Kapa HiFi™ Polymerase (KAPA Biosystems). Libraries were quantified by qPCR (KAPA Biosystems) and sequenced with 151 bp paired-end reads on an Illumina® NextSeq™ instrument. CHANGE-seq data analyses were performed using open-source CHANGE-seq analysis software.

[0099] Cytokine Analysis. Cytokine analysis is performed using a V-PLEX® Proinflammatory Panel 1 Human Kit (MSD, Catalog No K15049D-2) according to the manufacturer's protocol. Measured cytokines include IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. In brief, media was collected from the final day of cell culture before injection into mice and flash frozen and stored at −80° C. For co-culture samples, 250,000 T cells were co-cultured with 50,000 cancer cells in 250 μl ImmunoCult™ XF T cell expansion medium for 24 hours prior to media collection. On the day of the assay, media was thawed and 50 μl of media was used to perform all measurements in duplicate. Figures were produced using GraphPad PRISM 8. Data were normalized by calculating cytokine production per cell based on the total concentration of cells calculated at media collection.

[0100] In Vitro Cytotoxicity Assays. For FIG. **2***b*: 10,000 AkaLUC-GFP CHLA20 cells were seeded in triplicate per condition in a 96 well flat bottom plate. 48 hours later, 50,000 T cells were added to each well. 1μl (0.05 μg) of CF® 594 Annexin V antibody (Biotium) was added to the wells. The plate was centrifuged at 100 g for 1 minute and then placed in the IncuCyte® S3 Live-Cell Analysis System (Sartorius, Catalog No 4647), stored at 37° C., 5% CO_2. Images were taken every 2 hours for 48 hours. Green object count was used to calculate the number of cancer cells in each well. Red object count was used to calculate the number of objects staining positive for Annexin V, an early apoptosis marker. Fluorescent images were analyzed with IncuCyte Base Analysis Software.

[0101] Single cell RNA sequencing: 24 hours prior to assay, 200,000 AkaLUC-CHLA-20 cells were plated in 12 well plates and cultured overnight. One week after electroporation (day 9 post-isolation), T cells were counted and pooled into a single bank for all characterization studies (scRNA-seq, IncuCyte® cytotoxicity assay and in vivo experiments). Media was aspirated from cancer cells, and 1 million T cells in ImmunoCult™M-XF Medium +500 U/mL IL-2 were seeded on the cancer cells, then cultured for 24 hours. A parallel culture of T cells without cancer cells was set up at the same T cell density in a separate 12 well plate. The next day, co-culture cells were trypsinized for donor 1 and washed off the plate with media, and cells were singularized with a 35 μM cell strainer (Corning). For donor 2, co-culture cells were stained for CD45 and CAR, and FACS sorted into $CD45^+$ CAR^+ and $CD45^+CAR^-$ fractions prior to sample submission. Cells were counted with a Countess II FL cell counter using trypan blue exclusion (Thermo Fisher Scientific), and samples were prepared for single cell RNA sequencing with the 10× Genomics 3' kit (v3 chemistry)

according to the manufacturer's instructions. Libraries were sequenced using the Illumina® NovaSeq™ 6000 system. FASTQ files were aligned with Cellranger v3.1.0 to custom reference genomes that included added sequences for the transgene(s) used in each culture condition (e.g. the NV TRAC_CAR HDRT sequence, AkaLuc-GFP, etc.). Downstream analyses were performed using Seurat 3. For each sample, cells either expressing the transgene of interest (CAR or mCherry) were identified, and transgene-negative cells were removed from the dataset.

[0102] Gene set enrichment analysis (GSEA). GSEA was performed using the natural log-fold change values between sample pairs, using only the set of transgene-positive cells in each dataset. GSEA v.4.0.3 (Broad Institute) with the v7.1. Reactome signatures database from MSigDB was used with default parameters (1000 permutations). Data were exported and graphed in Microsoft Excel.

[0103] In vivo human neuroblastoma xenograft mouse model. All animal experiments were approved by the University of Wisconsin-Madison Animal Care and Use Committee (ACUC). Male and female NSG mice (9-25 weeks old) were subcutaneously injected with 10 million AkaLUC-GFP CHLA20 human neuroblastoma cells in the side flank to establish tumors. Six days later (Day 0), established tumors were verified by bioluminescence with the PerkinElmer In Vivo Imaging System (IVIS), and 10 million T cells were injected through the tail vein into each mouse. Mice were followed for weight loss and overall survival. On imaging days, mice were sedated using isoflurane and received intraperitoneal injections of ~120 mg/kg D-luciferin (GoldBio). Fifteen minutes later, mice were imaged via IVIS. Imaging was repeated every 3 to 4 days, starting 1 day before initial T cell injection (Day -1). Mice were injected with 100,000 IU of human IL-2 subcutaneously on day 0, day 4, and with each subsequent IVIS reading. In order to quantify the total flux in the IVIS images, a region of interest (ROI) was drawn around the bottom half of each mouse with the total flux being calculated by Living Image® software (PerkinElmer; Total flux=the radiance (photons/sec) in each pixel summed or integrated over the ROI area (cm^2)×4π). The absolute minimum total flux value was subtracted from each image to minimize background signal. For donors 1, 3, and 4, mice were maintained until tumors reached 20 mm in any dimension by digital caliper as defined by the ACUC.

[0104] Flow cytometric analysis of splenic and tumor-infiltrating T cells. For donor 2, all mice were euthanized on day 25. Tumors and spleens were removed, mechanically dissociated, and passed through a Corning® 35 μm cell strainer. Cell suspensions were centrifuged at 300 g for 10 minutes, and then digested with ACK lysing buffer (Lonza). The cells were then washed and centrifuged at 300 g for 10 minutes, and then resuspended in 10 ml PBS, 10 μl of which was added to 10 ml of ISOTON® diluent and counted on the COULTER COUNTER® Z1 Series Particle Counter (Beckman Coulter). From this count, 1×10^6 cells were added to flow cytometry tubes in staining buffer (PBS with 2% FBS) and stained with antibodies for hCD45, mCD45, scFV 14G2a CAR, and PD-1 (see Table 1 for antibody information). The cells were then washed with PBS, centrifuged at 300 g for 10 minutes, and 0.5 μl of Ghost Dye™ Red 780 viability dye (Tonbo Biosciences) was added for 20 minutes at room temperature. Cells were then washed with staining buffer, spun down, and resuspended in 400 μl of staining buffer. Cells were then run on an Attune™ NXT flow cytometer (Thermo Fisher Scientific). Subsequent analysis was performed using Flowjo™ software (BD). For donors 3 and 4, spleens and tumors were analyzed as mice reached euthanasia criteria and were stained with an extended antibody panel outlined in Table 1.

[0105] Statistical analysis. Unless otherwise specified, all analyses were performed using GraphPad Prism (v.8.0.1), and error bars represent mean +-SD; ns=p>=0.05, * for p<0.05, ** for p<0.01, *** for p<0.001,**** for p<0.0001. For FIG. **2***b*, error bars show SEM. Statistical analyses for cytokine data (FIG. **1***m*, FIG. **2***a*, Extended Data FIG. **1***e*, Extended Data FIG. **2***a*) were performed using a two-tailed Mann-Whitney test in GraphPad Prism. All box plots show median (horizontal line), interquartile range (hinges), and smallest and largest values (whiskers). Statistical significance for differential gene expression was determined with Seurat 3 using the non-parametric Wilcoxon rank sum test. All 11 scRNA-seq samples were integrated and normalized, and 2 replicate samples per donor were combined to calculate differential expression between transgene-positive cells in each sample type. P values were adjusted using Bonferroni correction. p<0.001 was used as the threshold for assigning significant versus non-significant changes in gene expression. Volcano plots were generated in RStudio (v 1.1.456) using the ggplot2 and EnhancedVolcano packages. Statistical significance for FIG. **2***h* was calculated using the Mantel-Cox Test.

Example 1: Design of HDR Donor Template Plasmid

[0106] Described herein is a new method to insert CAR transgenes using Cas9 ribonucleoproteins (RNPs) targeted to a T-cell expressed gene locus such as the human TRAC locus in combination with a donor, specifically a PCR-amplified donor, encoding the CAR transgene (FIG. **1***a*). The TRAC exon is SEQ ID Nos. 13 and 14. For proof-of-principle, a published GD2-targeting CAR sequence was used for construction of the HDR donor template (HDRT). This HDRT is readily generated by PCR on a plasmid containing the CAR sequence. For the plasmid construction, a splice acceptor followed by a self-cleaving peptide, 2A, was cloned upstream of the GD2-CAR, and a transcriptional terminator followed by a poly A sequence was added downstream of the GD2-CAR. To facilitate HDR, homology arms around the Cas9 cut site in targeted gene (e.g., TRAC) was added to both ends of this construct. The resulting novel donor construct within a plasmid is shown in FIG. **5**. The sequence of the TRAC-CAR is SEQ ID NO: 1.

Example 2: Production of HDR Donor Template (HDRT)

[0107] We next generated double-stranded DNA (dsDNA) HDR templates via PCR amplification off the plasmid and performed a two-step purification process to purify and concentrate the templates. Primary human T cells were electroporated with the HDR templates and Cas9 ribonucleoproteins (RNPs) targeting the human TRAC locus. Cells were subsequently expanded in xeno-free media and assayed on days 7 and 9 post-isolation (FIG. **1***b*). The viability of NV-CAR and RV-CAR T cells was comparably high (>80%) by the end of manufacturing (FIG. **3***a*). Cell proliferation and growth over 9 days was robust for both

groups (FIG. **3***a*). We assessed gene editing at multiple points post-isolation and achieved higher levels of CAR integration when cells were edited at 48 hours after CD3/CD28/CD2 stimulation (FIG. **3***b*). As a control, we include an "NV-mCherry" (NV-mCh) condition in which cells harbor the same disruption of the TRAC locus, but with an insertion of a signaling-inert mCherry fluorescent protein in place of the CAR (FIG. **1***b*). Using these templates, we achieved consistently high genome editing rates across 31 technical replicates over 4 donors, with CAR integration averaging >15% as measured by flow cytometry (FIG. **1***c, d*). The mean fluorescence intensity (MFI) of CAR expression was significantly elevated and showed greater range (~1.6 fold; FIG. **1***d*) in the RV-CAR samples in comparison to the NV-CAR samples indicating decreased CAR expression heterogeneity within the NV-CAR product and consistent with prior findings with CRISPR CAR T cells[4]. Within the NV-CAR samples, the TCR was knocked out in 90% of cells (FIG. **1***c, e*). We also assayed the immunophenotype by cell surface staining and found significantly elevated CD62L expression in both NV-CAR (CAR+TCR−fraction) and NV-mCh (mCh+TCR−fraction) cells relative to RV-CAR cells (CAR+TCR+fraction). The mean fluorescence intensity (MFI) of CD62L increased by ~3 fold in the NV-CAR T cells relative to the RV-CAR T cells, indicating a naïve and/or stem cell memory or central memory phenotype in these populations after manufacturing (FIG. **1***f*).

Example 3: NGS Sequencing and scRNA-seq

[0108] After obtaining high quality HDRT, next-generation sequencing of genomic DNA from the manufactured cell products confirmed high rates of indel formation at the TRAC locus, averaging 93.06% of reads for NV-CAR samples, and mirroring surface protein levels (FIG. **3***c,d*). Proper genomic integration of the CAR was confirmed via "in-out" PCR amplification with primers specific to the TRAC locus and the transgene (FIG. **3***e*). Highly sensitive genome-wide, off-target analysis for our editing strategy was assayed by CHANGE-seq. The top identified modified locus was the intended on-target site (FIG. **1***h, i*) with a rapid drop-off for off-target modifications elsewhere in the genome (Data not shown). The CHANGE-seq specificity ratio of our TRAC editing strategy is above average (0.056; 57th percentile) when compared to all editing strategies previously profiled by CHANGE-seq.

[0109] To further define the phenotypic differences between NV-CAR and RV-CAR T cells, we performed single-cell RNA-sequencing (scRNA-seq) on 29,122 cells from two different donors at the end of the manufacturing process (data not shown). To distinguish edited transgene-positive and transgene-negative cells within each sample, we aligned reads to a custom reference genome containing an added sequence mapping to the CAR or mCherry transgenes. We detected transgene expression in 6,376 across all samples assayed at the end of manufacturing (22% of assayed cells); and, all subsequent transcriptional analyses were carried out on transgene-positive cells only within each sample. UMAP dimensionality reduction of transgene-positive cells showed similar clustering for both NV-CAR and RV-CAR T cells but not NV-mCh T cells, indicating that the presence of CAR signaling alters the phenotype of the cells even prior to antigen stimulation (FIG. **1***j*, FIG. **4** *a-c*). We observed a variety of differentially expressed genes between both NV-CAR and RV-CAR T cells, and NV-CAR and NV-mCh T cells, which were significant for both donors ($p<0.001$ cutoff. Gene set enrichment analysis of the 6,209 differentially expressed genes ($p<0.001$ cutoff) between the CAR-positive T cells from the donor-matched NV-CAR and RV-CAR samples revealed enrichment of T cell activation and innate immune response pathways in the RV-CAR T cells (FIG. **1***k*; Data not shown), indicating that RV-CAR T cells activate broad signaling in response to the retroviral manufacturing process, CAR transgene, or retroviral vector elements. In comparison, none of these pathways were significantly enriched when comparing transgene-positive NV-CAR T cells to NV-mCh T cells (FIG. **1***k*). Transgene-positive RV-CAR T cells exhibited elevated levels of transcripts associated with an exhausted T cell signature (high CTLA4, ENTPD1, LAG3, TIGIT, CD244; FIG. **11**) relative to transgene-positive NV-CAR T cells, but there were minimal significant differences in the exhaustion transcriptional profile between transgene-positive, donor-matched NV-CAR and NV-mCh T cells (Data not shown). Finally, we observed no significant changes in transcript levels for genes at or within 5 kb of off-target sites predicted by CHANGE-seq (Data not shown), indicating that any potential genomic disruptions at these sites did not lead to detectable changes in proximal transcripts.

Example 4: Cytokine Production Levels

[0110] On day 9 of manufacturing, cytokine production levels were measured from the conditioned culture media. Prior to antigen exposure, RV-CAR T cells had higher levels of IFNγ, TNFα, IL-2, IL-4, IL-10, and IL-13, in comparison to both the NV-CAR and NV-mCh T cells (FIG. **1***l*). This result is consistent with the above transcriptional analysis showing hyperactive CAR signaling and recent observations that some RV-CAR T cells display elevated levels of tonic signaling prior to antigen exposure[15]. After a 24 h co-culture between the engineered T cells and GD2+ CHLA20 neuroblastoma, NV-CAR T cells either matched or surpassed the level of cytokine production of the RV-CAR T cells (FIG. **2***a*), indicating that NV-CART cells were capable of mounting a response to their target antigen, and suggesting that the RV-CAR T cells may be more exhausted prior to antigen exposure than the NV-CAR T cells. These trends, both pre-antigen exposure and post-antigen exposure, were also observed for IL-6, IL-1β and IL-12p70, but not for IL-8 (Data not shown)

Example 5: In Vitro Potency of NV-CAR T Cells

[0111] After characterizing cellular phenotypes and gene expression at the end of the manufacturing process, we measured the in vitro potency of NV-CAR T cells against two GD2+ solid tumors: CHLA20 neuroblastoma and M21 melanoma (Data not shown). We observed robust killing using a 5:1 effector:target ratio for both NV-CAR and RV-CAR T cells (FIG. **2***b*, Data not shown). We again performed scRNA-seq on T cells that were co-cultured with CHLA20 neuroblastoma for 24 hours (FIG. **2***c*, data not shown). Gene set enrichment analysis of the 1,588 differentially expressed genes ($p<0.001$ cutoff) between the transgene-positive T cells from the NV-CAR and NV-mCh samples revealed high activation of T cell activation pathways in transgene-positive NV-CAR T cells (FIG. **2***d*), specifically CD28 activation pathways involving the CAR. When comparing the enrichment scores of pathways within

CAR-positive cells between NV-CAR/RV-CAR paired samples, lower differences were observed in T cells post-antigen exposure relative to pre-antigen exposure (FIG. **2***d* vs. FIG. **1***k*; Data not shown). These results, corroborated by elevated cytokine production observed after CHLA20 co-culture (FIG. **2***a*), demonstrate that NV-CAR T cells can properly achieve high levels of activation upon antigen exposure, while avoiding potentially detrimental high tonic-signaling prior to antigen exposure. Tonic signaling is when intracellular signaling from both the TCR and CAR, in the absence of binding to the CAR target antigen, drives T cell phenotypes and differentiation toward effector or exhausted phenotypes given they both share common signaling pathways. Therefore, NV-CAR T cells that lack the TCR and have lower mean protein levels of the CAR could have lower intracellular tonic signaling, in the absence of binding to the CAR target antigen, relative to control viral CAR T cell products.

Example 6: In Vivo Potency of NV-CART Cells

[0112] We assessed CAR T cell potency in vivo in an established human GD2+ neuroblastoma xenograft model. After 9 total days of culture, multiple replicate wells of RV-CAR, NV-CAR, or NV-mCh T cells were pooled for injection into NOD-SCID-$\gamma c^{-/-}$ (NSG) mice. Ten million T cells were delivered via tail vein injection to each NSG mouse with an established luciferase-expressing CHLA20 neuroblastoma tumor identified by bioluminescence (FIG. **2***e*). Tumor sizes were quantified over time by IVIS imaging and digital caliper (FIG. **2***f*). Both CAR-treated cohorts showed robust tumor regression in the first 3 weeks post-infusion (FIG. **2***g*, data not shown). These cohorts also showed significantly improved survival as compared to NV-mCh-treated mice; however, there was no significant difference in survival between NV-CAR and RV-CAR treated mice by day 80 (p-value=0.4099, n.s.). The percentage of CAR+ cells per dose was lower in NV-CAR T cells, which may have contributed to a slight decrease in complete remission rates (5/8 RV-CAR vs. 4/9 NV-CAR) but had no significant impact on overall survival, suggesting enhanced potency of the CAR-positive NV-CAR T cells. None of the control NV-mCh mice showed tumor regression, and all seven mice died of tumor progression by day 60. We also assessed persistence, memory and exhaustion phenotypes in T cells isolated from spleens and tumors. NV-CART cells persisted in both the spleens and tumors of the treated mice, but not for NV-mCh T cell treatments, indicating successful trafficking of NV-CAR T cells to the tumor microenvironment (FIG. **2***h*, Data not shown). Additionally, we observed that cells in the spleen had lower levels of PD-1 and TIM-3 exhaustion markers after NV-CAR treatment relative to the RV-CAR treatment (FIG. **2***i*), suggesting that the higher CAR MFI on RV-CARs (FIG. **1***d*) and detrimental signaling after expansion (FIG. **1***k*) could be contributing to increased propensity for exhaustion in RV-CARs. These findings demonstrate comparable potency of NV-CAR T cells to standard RV-CAR T cells, establishing the potential clinical relevance of NV-CAR T cells.

[0113] Example 7. Amplification of Long Double-stranded Homology-Directed Repair (HDR) Template

[0114] A set of primers was used to amplify a longer double-stranded HDR template from the donor template plasmid of SEQ ID NO: 1.

TABLE 4

Oligo	Sequence	SEQ ID NO:
TRAC Long Donor FWD primer	TCGAGTAAACGGTAGTGCTGGG	17
TRAC Long Donor REV primer	CCTCTCCTGC-CACCTTCTCTTC	18

[0115] This strategy generates a double-stranded HDR template length of 3.4 kb total, and the CAR knockin percentages have been consistently high as shown in FIG. **8**. The leftmost homology arm includes 588 bp of the TRAC locus directly upstream of the cutsite, and the rightmost homology arm includes 499 bases. These homology arms are longer than those from Example 2 which were 383 bp (left) and 391 bp (right). It was unexpected that increasing the length of the homology arms would increase the percentage of CAR knockin about 2-fold compared to the templates with shorter homology arms.

Discussion

[0116] Overall, we describe a rapid 9-day manufacturing of third generation GD2-specific CAR T cells using recombinant SpyCas9 protein and nucleic acids which results in stable, genomically-integrated, durable CAR expression (>80 days in vivo) without the use of any viral vectors. NV-CAR T cells exhibit proper TRAC-specific integration of the CAR transgene and an increased percentage and expression level of CD62L relative to conventional strategies. Robust upregulation of gene transcripts prevalent in cytotoxic transcriptional programs and secretion of pro-inflammatory cytokines like IFNγ and TNFα occur only after target antigen exposure, in contrast to conventional RV-CAR T cells that exhibit detrimental signaling during manufacturing. After injection into a GD2+ human neuroblastoma xenograft model, NV-CAR T cells induce strong regression of solid tumors compared to mock-edited T cells, and at levels comparable to RV-CAR T cells. NV-CAR T cells show reduced propensity to exhaustion at the gene expression and protein levels before antigen exposure, and at the protein level after antigen exposure.

[0117] Relative to conventional T cell manufacturing, our streamlined, nonviral manufacturing process could: 1) reduce the batch-to-batch variability, supply chain challenges, and costs associated with vector production alleviate a number of regulatory considerations (e.g., the need to monitor replication competency of the vector and the levels of xenogeneic components in the clinical cell product, notably plasmid DNA and serum during cell culture that can introduce infectious agents or toxic components); and 3) eliminate the potential for integration of viral elements into the human genome, which can generate a high degree of gene perturbation, up to 10^4-10^5 different insertional sites within a single product. Integration of the vector, in particular, presents risks of insertional oncogenesis, transgene silencing or overexpression, and adverse immune response to the vector, which could result in the rejection of therapeutic cells. While off-target analysis of genome editors is necessary for any clinical translation of our approach, there are now many experimental and computational tools that can readily be used for this purpose and next-generation high-

fidelity Cas9 enzymes could be used to further decrease the potential for any off-target effects. Our fully-defined, non-viral manufacturing method therefore has high potential to enable the rapid and flexible manufacture of highly-defined and highly-potent CART cell products.

Example 7: Analysis of NV-CRISPR CART Cells

[0118] FIG. **6** shows representative images of NV-CRISPR CART cells post-editing. Cells that demonstrate a high degree of aggregation are recovered at a higher rate. PCR donor refers to the HDRT. Aggregation can be indicative of successful genome editing and cell health, as non-aggregated cells are typically less viable with low to no levels of editing.

[0119] 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.

[0120] 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.

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tcactcatta ggcaccccag gctttacact ttatgcttcc ggctcgtatg ttgtgtggaa 180

ttgtgagcgg ataacaattt cacacaggaa acagctatga ccatgattac gccaagctat 240

ttaggtgaca ctatagaata ctcaagctat gcatcaagct tggtaccgag ctcggatcca 300

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<210> SEQ ID NO 2
<211> LENGTH: 58
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CAR genetic construct sense strand

<400> SEQUENCE: 2

tcctaaccct gatcctcttg tcccacagat atccagaacc ctgaccctgc cctgtacc 58

<210> SEQ ID NO 3
<211> LENGTH: 58
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: CAR genetic construct antisense strand

<400> SEQUENCE: 3

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<210> SEQ ID NO 4
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: target sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 4

cagggttctg gatatctgtn gg 22

<210> SEQ ID NO 5
<211> LENGTH: 1368
<212> TYPE: PRT
<213> ORGANISM: Streptococcus pyogenes

<400> SEQUENCE: 5

Met Asp Lys Lys Tyr Ser Ile Gly Leu Asp Ile Gly Thr Asn Ser Val
1 5 10 15

Gly Trp Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe
20 25 30

Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile
35 40 45

Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
50 55 60

Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
65 70 75 80

Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser
85 90 95

Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
100 105 110

His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr
115 120 125

His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp
130 135 140

Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
145 150 155 160

Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
165 170 175

Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr
180 185 190

Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp Ala
195 200 205

Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn
210 215 220

Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn
225 230 235 240

Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe
245 250 255

Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp
260 265 270

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Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp
        275                 280                 285

Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp
    290                 295                 300

Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser
305                 310                 315                 320

Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys
                325                 330                 335

Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe
            340                 345                 350

Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser
        355                 360                 365

Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp
    370                 375                 380

Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg
385                 390                 395                 400

Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu
                405                 410                 415

Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe
            420                 425                 430

Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile
        435                 440                 445

Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp
    450                 455                 460

Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu
465                 470                 475                 480

Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr
                485                 490                 495

Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser
            500                 505                 510

Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys
        515                 520                 525

Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln
    530                 535                 540

Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr
545                 550                 555                 560

Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp
                565                 570                 575

Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly
            580                 585                 590

Thr Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp
        595                 600                 605

Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr
    610                 615                 620

Leu Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala
625                 630                 635                 640

His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr
                645                 650                 655

Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp
            660                 665                 670
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Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe
        675                 680                 685

Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr Phe
    690                 695                 700

Lys Glu Asp Ile Gln Lys Ala Gln Val Ser Gly Gln Gly Asp Ser Leu
705                 710                 715                 720

His Glu His Ile Ala Asn Leu Ala Gly Ser Pro Ala Ile Lys Lys Gly
                725                 730                 735

Ile Leu Gln Thr Val Lys Val Val Asp Glu Leu Val Lys Val Met Gly
            740                 745                 750

Arg His Lys Pro Glu Asn Ile Val Ile Glu Met Ala Arg Glu Asn Gln
        755                 760                 765

Thr Thr Gln Lys Gly Gln Lys Asn Ser Arg Glu Arg Met Lys Arg Ile
    770                 775                 780

Glu Glu Gly Ile Lys Glu Leu Gly Ser Gln Ile Leu Lys Glu His Pro
785                 790                 795                 800

Val Glu Asn Thr Gln Leu Gln Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu
                805                 810                 815

Gln Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg
            820                 825                 830

Leu Ser Asp Tyr Asp Val Asp His Ile Val Pro Gln Ser Phe Leu Lys
        835                 840                 845

Asp Asp Ser Ile Asp Asn Lys Val Leu Thr Arg Ser Asp Lys Asn Arg
    850                 855                 860

Gly Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys
865                 870                 875                 880

Asn Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys
                885                 890                 895

Phe Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp
            900                 905                 910

Lys Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr
        915                 920                 925

Lys His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp
    930                 935                 940

Glu Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser
945                 950                 955                 960

Lys Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg
                965                 970                 975

Glu Ile Asn Asn Tyr His His Ala His Asp Ala Tyr Leu Asn Ala Val
            980                 985                 990

Val Gly Thr Ala Leu Ile Lys Lys  Tyr Pro Lys Leu Glu  Ser Glu Phe
        995                 1000                1005

Val Tyr  Gly Asp Tyr Lys Val  Tyr Asp Val Arg Lys  Met Ile Ala
    1010                1015                1020

Lys Ser  Glu Gln Glu Ile Gly  Lys Ala Thr Ala Lys  Tyr Phe Phe
    1025                1030                1035

Tyr Ser  Asn Ile Met Asn Phe  Phe Lys Thr Glu Ile  Thr Leu Ala
    1040                1045                1050

Asn Gly  Glu Ile Arg Lys Arg  Pro Leu Ile Glu Thr  Asn Gly Glu
    1055                1060                1065

Thr Gly  Glu Ile Val Trp Asp  Lys Gly Arg Asp Phe  Ala Thr Val
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    1070                1075                1080

Arg Lys  Val Leu Ser Met Pro  Gln Val Asn Ile Val  Lys Lys Thr
    1085                1090                1095

Glu Val  Gln Thr Gly Gly Phe  Ser Lys Glu Ser Ile  Leu Pro Lys
    1100                1105                1110

Arg Asn  Ser Asp Lys Leu Ile  Ala Arg Lys Lys Asp  Trp Asp Pro
    1115                1120                1125

Lys Lys  Tyr Gly Gly Phe Asp  Ser Pro Thr Val Ala  Tyr Ser Val
    1130                1135                1140

Leu Val  Val Ala Lys Val Glu  Lys Gly Lys Ser Lys  Lys Leu Lys
    1145                1150                1155

Ser Val  Lys Glu Leu Leu Gly  Ile Thr Ile Met Glu  Arg Ser Ser
    1160                1165                1170

Phe Glu  Lys Asn Pro Ile Asp  Phe Leu Glu Ala Lys  Gly Tyr Lys
    1175                1180                1185

Glu Val  Lys Lys Asp Leu Ile  Ile Lys Leu Pro Lys  Tyr Ser Leu
    1190                1195                1200

Phe Glu  Leu Glu Asn Gly Arg  Lys Arg Met Leu Ala  Ser Ala Gly
    1205                1210                1215

Glu Leu  Gln Lys Gly Asn Glu  Leu Ala Leu Pro Ser  Lys Tyr Val
    1220                1225                1230

Asn Phe  Leu Tyr Leu Ala Ser  His Tyr Glu Lys Leu  Lys Gly Ser
    1235                1240                1245

Pro Glu  Asp Asn Glu Gln Lys  Gln Leu Phe Val Glu  Gln His Lys
    1250                1255                1260

His Tyr  Leu Asp Glu Ile Ile  Glu Gln Ile Ser Glu  Phe Ser Lys
    1265                1270                1275

Arg Val  Ile Leu Ala Asp Ala  Asn Leu Asp Lys Val  Leu Ser Ala
    1280                1285                1290

Tyr Asn  Lys His Arg Asp Lys  Pro Ile Arg Glu Gln  Ala Glu Asn
    1295                1300                1305

Ile Ile  His Leu Phe Thr Leu  Thr Asn Leu Gly Ala  Pro Ala Ala
    1310                1315                1320

Phe Lys  Tyr Phe Asp Thr Thr  Ile Asp Arg Lys Arg  Tyr Thr Ser
    1325                1330                1335

Thr Lys  Glu Val Leu Asp Ala  Thr Leu Ile His Gln  Ser Ile Thr
    1340                1345                1350

Gly Leu  Tyr Glu Thr Arg Ile  Asp Leu Ser Gln Leu  Gly Gly Asp
    1355                1360                1365


<210> SEQ ID NO 6
<211> LENGTH: 1409
<212> TYPE: PRT
<213> ORGANISM: Streptococcus thermophilus

<400> SEQUENCE: 6

Met Leu Phe Asn Lys Cys Ile Ile Ile Ser Ile Asn Leu Asp Phe Ser
1               5                   10                  15

Asn Lys Glu Lys Cys Met Thr Lys Pro Tyr Ser Ile Gly Leu Asp Ile
            20                  25                  30

Gly Thr Asn Ser Val Gly Trp Ala Val Ile Thr Asp Asn Tyr Lys Val
        35                  40                  45
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Pro Ser Lys Lys Met Lys Val Leu Gly Asn Thr Ser Lys Lys Tyr Ile
    50                  55                  60

Lys Lys Asn Leu Leu Gly Val Leu Leu Phe Asp Ser Gly Ile Thr Ala
65                  70                  75                  80

Glu Gly Arg Arg Leu Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg
                85                  90                  95

Arg Asn Arg Ile Leu Tyr Leu Gln Glu Ile Phe Ser Thr Glu Met Ala
            100                 105                 110

Thr Leu Asp Asp Ala Phe Phe Gln Arg Leu Asp Asp Ser Phe Leu Val
        115                 120                 125

Pro Asp Asp Lys Arg Asp Ser Lys Tyr Pro Ile Phe Gly Asn Leu Val
    130                 135                 140

Glu Glu Lys Val Tyr His Asp Glu Phe Pro Thr Ile Tyr His Leu Arg
145                 150                 155                 160

Lys Tyr Leu Ala Asp Ser Thr Lys Lys Ala Asp Leu Arg Leu Val Tyr
                165                 170                 175

Leu Ala Leu Ala His Met Ile Lys Tyr Arg Gly His Phe Leu Ile Glu
            180                 185                 190

Gly Glu Phe Asn Ser Lys Asn Asn Asp Ile Gln Lys Asn Phe Gln Asp
        195                 200                 205

Phe Leu Asp Thr Tyr Asn Ala Ile Phe Glu Ser Asp Leu Ser Leu Glu
    210                 215                 220

Asn Ser Lys Gln Leu Glu Glu Ile Val Lys Asp Lys Ile Ser Lys Leu
225                 230                 235                 240

Glu Lys Lys Asp Arg Ile Leu Lys Leu Phe Pro Gly Glu Lys Asn Ser
                245                 250                 255

Gly Ile Phe Ser Glu Phe Leu Lys Leu Ile Val Gly Asn Gln Ala Asp
            260                 265                 270

Phe Arg Lys Cys Phe Asn Leu Asp Glu Lys Ala Ser Leu His Phe Ser
        275                 280                 285

Lys Glu Ser Tyr Asp Glu Asp Leu Glu Thr Leu Leu Gly Tyr Ile Gly
    290                 295                 300

Asp Asp Tyr Ser Asp Val Phe Leu Lys Ala Lys Lys Leu Tyr Asp Ala
305                 310                 315                 320

Ile Leu Leu Ser Gly Phe Leu Thr Val Thr Asp Asn Glu Thr Glu Ala
                325                 330                 335

Pro Leu Ser Ser Ala Met Ile Lys Arg Tyr Asn Glu His Lys Glu Asp
            340                 345                 350

Leu Ala Leu Leu Lys Glu Tyr Ile Arg Asn Ile Ser Leu Lys Thr Tyr
        355                 360                 365

Asn Glu Val Phe Lys Asp Asp Thr Lys Asn Gly Tyr Ala Gly Tyr Ile
    370                 375                 380

Asp Gly Lys Thr Asn Gln Glu Asp Phe Tyr Val Tyr Leu Lys Asn Leu
385                 390                 395                 400

Leu Ala Glu Phe Glu Gly Ala Asp Tyr Phe Leu Glu Lys Ile Asp Arg
                405                 410                 415

Glu Asp Phe Leu Arg Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro
            420                 425                 430

Tyr Gln Ile His Leu Gln Glu Met Arg Ala Ile Leu Asp Lys Gln Ala
        435                 440                 445

Lys Phe Tyr Pro Phe Leu Ala Lys Asn Lys Glu Arg Ile Glu Lys Ile
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    450                 455                 460

Leu Thr Phe Arg Ile Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn
465                 470                 475                 480

Ser Asp Phe Ala Trp Ser Ile Arg Lys Arg Asn Glu Lys Ile Thr Pro
                485                 490                 495

Trp Asn Phe Glu Asp Val Ile Asp Lys Glu Ser Ser Ala Glu Ala Phe
            500                 505                 510

Ile Asn Arg Met Thr Ser Phe Asp Leu Tyr Leu Pro Glu Glu Lys Val
        515                 520                 525

Leu Pro Lys His Ser Leu Leu Tyr Glu Thr Phe Asn Val Tyr Asn Glu
    530                 535                 540

Leu Thr Lys Val Arg Phe Ile Ala Glu Ser Met Arg Asp Tyr Gln Phe
545                 550                 555                 560

Leu Asp Ser Lys Gln Lys Lys Asp Ile Val Arg Leu Tyr Phe Lys Asp
                565                 570                 575

Lys Arg Lys Val Thr Asp Lys Asp Ile Ile Glu Tyr Leu His Ala Ile
            580                 585                 590

Tyr Gly Tyr Asp Gly Ile Glu Leu Lys Gly Ile Glu Lys Gln Phe Asn
        595                 600                 605

Ser Ser Leu Ser Thr Tyr His Asp Leu Leu Asn Ile Ile Asn Asp Lys
    610                 615                 620

Glu Phe Leu Asp Asp Ser Ser Asn Glu Ala Ile Ile Glu Glu Ile Ile
625                 630                 635                 640

His Thr Leu Thr Ile Phe Glu Asp Arg Glu Met Ile Lys Gln Arg Leu
                645                 650                 655

Ser Lys Phe Glu Asn Ile Phe Asp Lys Ser Val Leu Lys Lys Leu Ser
            660                 665                 670

Arg Arg His Tyr Thr Gly Trp Gly Lys Leu Ser Ala Lys Leu Ile Asn
        675                 680                 685

Gly Ile Arg Asp Glu Lys Ser Gly Asn Thr Ile Leu Asp Tyr Leu Ile
    690                 695                 700

Asp Asp Gly Ile Ser Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp
705                 710                 715                 720

Ala Leu Ser Phe Lys Lys Lys Ile Gln Lys Ala Gln Ile Ile Gly Asp
                725                 730                 735

Glu Asp Lys Gly Asn Ile Lys Glu Val Val Lys Ser Leu Pro Gly Ser
            740                 745                 750

Pro Ala Ile Lys Lys Gly Ile Leu Gln Ser Ile Lys Ile Val Asp Glu
        755                 760                 765

Leu Val Lys Val Met Gly Gly Arg Lys Pro Glu Ser Ile Val Val Glu
    770                 775                 780

Met Ala Arg Glu Asn Gln Tyr Thr Asn Gln Gly Lys Ser Asn Ser Gln
785                 790                 795                 800

Gln Arg Leu Lys Arg Leu Glu Lys Ser Leu Lys Glu Leu Gly Ser Lys
                805                 810                 815

Ile Leu Lys Glu Asn Ile Pro Ala Lys Leu Ser Lys Ile Asp Asn Asn
            820                 825                 830

Ala Leu Gln Asn Asp Arg Leu Tyr Leu Tyr Tyr Leu Gln Asn Gly Lys
        835                 840                 845

Asp Met Tyr Thr Gly Asp Asp Leu Asp Ile Asp Arg Leu Ser Asn Tyr
    850                 855                 860
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Asp Ile Asp His Ile Ile Pro Gln Ala Phe Leu Lys Asp Asn Ser Ile
865                 870                 875                 880

Asp Asn Lys Val Leu Val Ser Ser Ala Ser Asn Arg Gly Lys Ser Asp
                885                 890                 895

Asp Phe Pro Ser Leu Glu Val Val Lys Lys Arg Lys Thr Phe Trp Tyr
            900                 905                 910

Gln Leu Leu Lys Ser Lys Leu Ile Ser Gln Arg Lys Phe Asp Asn Leu
        915                 920                 925

Thr Lys Ala Glu Arg Gly Gly Leu Leu Pro Glu Asp Lys Ala Gly Phe
    930                 935                 940

Ile Gln Arg Gln Leu Val Glu Thr Arg Gln Ile Thr Lys His Val Ala
945                 950                 955                 960

Arg Leu Leu Asp Glu Lys Phe Asn Asn Lys Lys Asp Glu Asn Asn Arg
                965                 970                 975

Ala Val Arg Thr Val Lys Ile Ile Thr Leu Lys Ser Thr Leu Val Ser
            980                 985                 990

Gln Phe Arg Lys Asp Phe Glu Leu  Tyr Lys Val Arg Glu  Ile Asn Asp
        995                 1000                1005

Phe His  His Ala His Asp Ala  Tyr Leu Asn Ala Val  Ile Ala Ser
    1010                1015                1020

Ala Leu  Leu Lys Lys Tyr Pro  Lys Leu Glu Pro Glu  Phe Val Tyr
    1025                1030                1035

Gly Asp  Tyr Pro Lys Tyr Asn  Ser Phe Arg Glu Arg  Lys Ser Ala
    1040                1045                1050

Thr Glu  Lys Val Tyr Phe Tyr  Ser Asn Ile Met Asn  Ile Phe Lys
    1055                1060                1065

Lys Ser  Ile Ser Leu Ala Asp  Gly Arg Val Ile Glu  Arg Pro Leu
    1070                1075                1080

Ile Glu  Val Asn Glu Glu Thr  Gly Glu Ser Val Trp  Asn Lys Glu
    1085                1090                1095

Ser Asp  Leu Ala Thr Val Arg  Arg Val Leu Ser Tyr  Pro Gln Val
    1100                1105                1110

Asn Val  Val Lys Lys Val Glu  Glu Gln Asn His Gly  Leu Asp Arg
    1115                1120                1125

Gly Lys  Pro Lys Gly Leu Phe  Asn Ala Asn Leu Ser  Ser Lys Pro
    1130                1135                1140

Lys Pro  Asn Ser Asn Glu Asn  Leu Val Gly Ala Lys  Glu Tyr Leu
    1145                1150                1155

Asp Pro  Lys Lys Tyr Gly Gly  Tyr Ala Gly Ile Ser  Asn Ser Phe
    1160                1165                1170

Ala Val  Leu Val Lys Gly Thr  Ile Glu Lys Gly Ala  Lys Lys Lys
    1175                1180                1185

Ile Thr  Asn Val Leu Glu Phe  Gln Gly Ile Ser Ile  Leu Asp Arg
    1190                1195                1200

Ile Asn  Tyr Arg Lys Asp Lys  Leu Asn Phe Leu Leu  Glu Lys Gly
    1205                1210                1215

Tyr Lys  Asp Ile Glu Leu Ile  Ile Glu Leu Pro Lys  Tyr Ser Leu
    1220                1225                1230

Phe Glu  Leu Ser Asp Gly Ser  Arg Arg Met Leu Ala  Ser Ile Leu
    1235                1240                1245
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Ser Thr  Asn Asn Lys Arg Gly  Glu Ile His Lys Gly  Asn Gln Ile
    1250                 1255                 1260

Phe Leu  Ser Gln Lys Phe Val  Lys Leu Leu Tyr His  Ala Lys Arg
    1265                 1270                 1275

Ile Ser  Asn Thr Ile Asn Glu  Asn His Arg Lys Tyr  Val Glu Asn
    1280                 1285                 1290

His Lys  Lys Glu Phe Glu Glu  Leu Phe Tyr Tyr Ile  Leu Glu Phe
    1295                 1300                 1305

Asn Glu  Asn Tyr Val Gly Ala  Lys Lys Asn Gly Lys  Leu Leu Asn
    1310                 1315                 1320

Ser Ala  Phe Gln Ser Trp Gln  Asn His Ser Ile Asp  Glu Leu Cys
    1325                 1330                 1335

Ser Ser  Phe Ile Gly Pro Thr  Gly Ser Glu Arg Lys  Gly Leu Phe
    1340                 1345                 1350

Glu Leu  Thr Ser Arg Gly Ser  Ala Ala Asp Phe Glu  Phe Leu Gly
    1355                 1360                 1365

Val Lys  Ile Pro Arg Tyr Arg  Asp Tyr Thr Pro Ser  Ser Leu Leu
    1370                 1375                 1380

Lys Asp  Ala Thr Leu Ile His  Gln Ser Val Thr Gly  Leu Tyr Glu
    1385                 1390                 1395

Thr Arg  Ile Asp Leu Ala Lys  Leu Gly Glu Gly
    1400                 1405


<210> SEQ ID NO 7
<211> LENGTH: 1082
<212> TYPE: PRT
<213> ORGANISM: Neisseria

<400> SEQUENCE: 7

Met Ala Ala Phe Lys Pro Asn Pro Ile Asn Tyr Ile Leu Gly Leu Asp
1               5                   10                  15

Ile Gly Ile Ala Ser Val Gly Trp Ala Met Val Glu Ile Asp Glu Glu
            20                  25                  30

Glu Asn Pro Ile Arg Leu Ile Asp Leu Gly Val Arg Val Phe Glu Arg
        35                  40                  45

Ala Glu Val Pro Lys Thr Gly Asp Ser Leu Ala Met Val Arg Arg Leu
    50                  55                  60

Ala Arg Ser Val Arg Arg Leu Thr Arg Arg Arg Ala His Arg Leu Leu
65                  70                  75                  80

Arg Ala Arg Arg Leu Leu Lys Arg Glu Gly Val Leu Gln Ala Ala Asp
                85                  90                  95

Phe Asp Glu Asn Gly Leu Ile Lys Ser Leu Pro Asn Thr Pro Trp Gln
            100                 105                 110

Leu Arg Ala Ala Ala Leu Asp Arg Lys Leu Thr Pro Leu Glu Trp Ser
        115                 120                 125

Ala Val Leu Leu His Leu Ile Lys His Arg Gly Tyr Leu Ser Gln Arg
    130                 135                 140

Lys Asn Glu Gly Glu Thr Ala Asp Lys Glu Leu Gly Ala Leu Leu Lys
145                 150                 155                 160

Gly Val Ala Asp Asn Ala His Ala Leu Gln Thr Gly Asp Phe Arg Thr
                165                 170                 175

Pro Ala Glu Leu Ala Leu Asn Lys Phe Glu Lys Glu Ser Gly His Ile
            180                 185                 190
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Arg Asn Gln Arg Gly Asp Tyr Ser His Thr Phe Ser Arg Lys Asp Leu
        195                 200                 205

Gln Ala Glu Leu Ile Leu Leu Phe Glu Lys Gln Lys Glu Phe Gly Asn
    210                 215                 220

Pro His Ile Ser Gly Gly Leu Lys Glu Gly Ile Glu Thr Leu Leu Met
225                 230                 235                 240

Thr Gln Arg Pro Ala Leu Ser Gly Asp Ala Val Gln Lys Met Leu Gly
                245                 250                 255

His Cys Thr Phe Glu Pro Ala Glu Pro Lys Ala Ala Lys Asn Thr Tyr
            260                 265                 270

Thr Ala Glu Arg Phe Ile Trp Leu Thr Lys Leu Asn Asn Leu Arg Ile
        275                 280                 285

Leu Glu Gln Gly Ser Glu Arg Pro Leu Thr Asp Thr Glu Arg Ala Thr
    290                 295                 300

Leu Met Asp Glu Pro Tyr Arg Lys Ser Lys Leu Thr Tyr Ala Gln Ala
305                 310                 315                 320

Arg Lys Leu Leu Gly Leu Glu Asp Thr Ala Phe Phe Lys Gly Leu Arg
                325                 330                 335

Tyr Gly Lys Asp Asn Ala Glu Ala Ser Thr Leu Met Glu Met Lys Ala
            340                 345                 350

Tyr His Ala Ile Ser Arg Ala Leu Glu Lys Glu Gly Leu Lys Asp Lys
        355                 360                 365

Lys Ser Pro Leu Asn Leu Ser Pro Glu Leu Gln Asp Glu Ile Gly Thr
    370                 375                 380

Ala Phe Ser Leu Phe Lys Thr Asp Glu Asp Ile Thr Gly Arg Leu Lys
385                 390                 395                 400

Asp Arg Ile Gln Pro Glu Ile Leu Glu Ala Leu Leu Lys His Ile Ser
                405                 410                 415

Phe Asp Lys Phe Val Gln Ile Ser Leu Lys Ala Leu Arg Arg Ile Val
            420                 425                 430

Pro Leu Met Glu Gln Gly Lys Arg Tyr Asp Glu Ala Cys Ala Glu Ile
        435                 440                 445

Tyr Gly Asp His Tyr Gly Lys Lys Asn Thr Glu Glu Lys Ile Tyr Leu
    450                 455                 460

Pro Pro Ile Pro Ala Asp Glu Ile Arg Asn Pro Val Val Leu Arg Ala
465                 470                 475                 480

Leu Ser Gln Ala Arg Lys Val Ile Asn Gly Val Val Arg Arg Tyr Gly
                485                 490                 495

Ser Pro Ala Arg Ile His Ile Glu Thr Ala Arg Glu Val Gly Lys Ser
            500                 505                 510

Phe Lys Asp Arg Lys Glu Ile Glu Lys Arg Gln Glu Glu Asn Arg Lys
        515                 520                 525

Asp Arg Glu Lys Ala Ala Ala Lys Phe Arg Glu Tyr Phe Pro Asn Phe
    530                 535                 540

Val Gly Glu Pro Lys Ser Lys Asp Ile Leu Lys Leu Arg Leu Tyr Glu
545                 550                 555                 560

Gln Gln His Gly Lys Cys Leu Tyr Ser Gly Lys Glu Ile Asn Leu Gly
                565                 570                 575

Arg Leu Asn Glu Lys Gly Tyr Val Glu Ile Asp His Ala Leu Pro Phe
            580                 585                 590
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Ser Arg Thr Trp Asp Asp Ser Phe Asn Asn Lys Val Leu Val Leu Gly
        595                 600                 605

Ser Glu Asn Gln Asn Lys Gly Asn Gln Thr Pro Tyr Glu Tyr Phe Asn
    610                 615                 620

Gly Lys Asp Asn Ser Arg Glu Trp Gln Glu Phe Lys Ala Arg Val Glu
625                 630                 635                 640

Thr Ser Arg Phe Pro Arg Ser Lys Lys Gln Arg Ile Leu Leu Gln Lys
                645                 650                 655

Phe Asp Glu Asp Gly Phe Lys Glu Arg Asn Leu Asn Asp Thr Arg Tyr
            660                 665                 670

Val Asn Arg Phe Leu Cys Gln Phe Val Ala Asp Arg Met Arg Leu Thr
        675                 680                 685

Gly Lys Gly Lys Lys Arg Val Phe Ala Ser Asn Gly Gln Ile Thr Asn
    690                 695                 700

Leu Leu Arg Gly Phe Trp Gly Leu Arg Lys Val Arg Ala Glu Asn Asp
705                 710                 715                 720

Arg His His Ala Leu Asp Ala Val Val Val Ala Cys Ser Thr Val Ala
                725                 730                 735

Met Gln Gln Lys Ile Thr Arg Phe Val Arg Tyr Lys Glu Met Asn Ala
            740                 745                 750

Phe Asp Gly Lys Thr Ile Asp Lys Glu Thr Gly Glu Val Leu His Gln
        755                 760                 765

Lys Thr His Phe Pro Gln Pro Trp Glu Phe Phe Ala Gln Glu Val Met
    770                 775                 780

Ile Arg Val Phe Gly Lys Pro Asp Gly Lys Pro Glu Phe Glu Glu Ala
785                 790                 795                 800

Asp Thr Pro Glu Lys Leu Arg Thr Leu Leu Ala Glu Lys Leu Ser Ser
                805                 810                 815

Arg Pro Glu Ala Val His Glu Tyr Val Thr Pro Leu Phe Val Ser Arg
            820                 825                 830

Ala Pro Asn Arg Lys Met Ser Gly Gln Gly His Met Glu Thr Val Lys
        835                 840                 845

Ser Ala Lys Arg Leu Asp Glu Gly Val Ser Val Leu Arg Val Pro Leu
    850                 855                 860

Thr Gln Leu Lys Leu Lys Asp Leu Glu Lys Met Val Asn Arg Glu Arg
865                 870                 875                 880

Glu Pro Lys Leu Tyr Glu Ala Leu Lys Ala Arg Leu Glu Ala His Lys
                885                 890                 895

Asp Asp Pro Ala Lys Ala Phe Ala Glu Pro Phe Tyr Lys Tyr Asp Lys
            900                 905                 910

Ala Gly Asn Arg Thr Gln Gln Val Lys Ala Val Arg Val Glu Gln Val
        915                 920                 925

Gln Lys Thr Gly Val Trp Val Arg Asn His Asn Gly Ile Ala Asp Asn
    930                 935                 940

Ala Thr Met Val Arg Val Asp Val Phe Glu Lys Gly Asp Lys Tyr Tyr
945                 950                 955                 960

Leu Val Pro Ile Tyr Ser Trp Gln Val Ala Lys Gly Ile Leu Pro Asp
                965                 970                 975

Arg Ala Val Val Gln Gly Lys Asp Glu Glu Asp Trp Gln Leu Ile Asp
            980                 985                 990

Asp Ser Phe Asn Phe Lys Phe Ser  Leu His Pro Asn Asp  Leu Val Glu
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        995                 1000                1005

Val Ile  Thr Lys Lys Ala Arg  Met Phe Gly Tyr Phe  Ala Ser Cys
    1010                1015                1020

His Arg  Gly Thr Gly Asn Ile  Asn Ile Arg Ile His  Asp Leu Asp
    1025                1030                1035

His Lys  Ile Gly Lys Asn Gly  Ile Leu Glu Gly Ile  Gly Val Lys
    1040                1045                1050

Thr Ala  Leu Ser Phe Gln Lys  Tyr Gln Ile Asp Glu  Leu Gly Lys
    1055                1060                1065

Glu Ile  Arg Pro Cys Arg Leu  Lys Lys Arg Pro Pro  Val Arg
    1070                1075                1080


<210> SEQ ID NO 8
<211> LENGTH: 1395
<212> TYPE: PRT
<213> ORGANISM: Treponema

<400> SEQUENCE: 8

Met Lys Lys Glu Ile Lys Asp Tyr Phe Leu Gly Leu Asp Val Gly Thr
1               5                   10                  15

Gly Ser Val Gly Trp Ala Val Thr Asp Thr Asp Tyr Lys Leu Leu Lys
            20                  25                  30

Ala Asn Arg Lys Asp Leu Trp Gly Met Arg Cys Phe Glu Thr Ala Glu
        35                  40                  45

Thr Ala Glu Val Arg Arg Leu His Arg Gly Ala Arg Arg Arg Ile Glu
    50                  55                  60

Arg Arg Lys Lys Arg Ile Lys Leu Leu Gln Glu Leu Phe Ser Gln Glu
65                  70                  75                  80

Ile Ala Lys Thr Asp Glu Gly Phe Phe Gln Arg Met Lys Glu Ser Pro
                85                  90                  95

Phe Tyr Ala Glu Asp Lys Thr Ile Leu Gln Glu Asn Thr Leu Phe Asn
            100                 105                 110

Asp Lys Asp Phe Ala Asp Lys Thr Tyr His Lys Ala Tyr Pro Thr Ile
        115                 120                 125

Asn His Leu Ile Lys Ala Trp Ile Glu Asn Lys Val Lys Pro Asp Pro
    130                 135                 140

Arg Leu Leu Tyr Leu Ala Cys His Asn Ile Ile Lys Lys Arg Gly His
145                 150                 155                 160

Phe Leu Phe Glu Gly Asp Phe Asp Ser Glu Asn Gln Phe Asp Thr Ser
                165                 170                 175

Ile Gln Ala Leu Phe Glu Tyr Leu Arg Glu Asp Met Glu Val Asp Ile
            180                 185                 190

Asp Ala Asp Ser Gln Lys Val Lys Glu Ile Leu Lys Asp Ser Ser Leu
        195                 200                 205

Lys Asn Ser Glu Lys Gln Ser Arg Leu Asn Lys Ile Leu Gly Leu Lys
    210                 215                 220

Pro Ser Asp Lys Gln Lys Lys Ala Ile Thr Asn Leu Ile Ser Gly Asn
225                 230                 235                 240

Lys Ile Asn Phe Ala Asp Leu Tyr Asp Asn Pro Asp Leu Lys Asp Ala
                245                 250                 255

Glu Lys Asn Ser Ile Ser Phe Ser Lys Asp Asp Phe Asp Ala Leu Ser
            260                 265                 270
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Asp Asp Leu Ala Ser Ile Leu Gly Asp Ser Phe Glu Leu Leu Leu Lys
        275                 280                 285

Ala Lys Ala Val Tyr Asn Cys Ser Val Leu Ser Lys Val Ile Gly Asp
    290                 295                 300

Glu Gln Tyr Leu Ser Phe Ala Lys Val Lys Ile Tyr Glu Lys His Lys
305                 310                 315                 320

Thr Asp Leu Thr Lys Leu Lys Asn Val Ile Lys Lys His Phe Pro Lys
                325                 330                 335

Asp Tyr Lys Lys Val Phe Gly Tyr Asn Lys Asn Glu Lys Asn Asn Asn
            340                 345                 350

Asn Tyr Ser Gly Tyr Val Gly Val Cys Lys Thr Lys Ser Lys Lys Leu
        355                 360                 365

Ile Ile Asn Asn Ser Val Asn Gln Glu Asp Phe Tyr Lys Phe Leu Lys
    370                 375                 380

Thr Ile Leu Ser Ala Lys Ser Glu Ile Lys Glu Val Asn Asp Ile Leu
385                 390                 395                 400

Thr Glu Ile Glu Thr Gly Thr Phe Leu Pro Lys Gln Ile Ser Lys Ser
                405                 410                 415

Asn Ala Glu Ile Pro Tyr Gln Leu Arg Lys Met Glu Leu Glu Lys Ile
            420                 425                 430

Leu Ser Asn Ala Glu Lys His Phe Ser Phe Leu Lys Gln Lys Asp Glu
        435                 440                 445

Lys Gly Leu Ser His Ser Glu Lys Ile Ile Met Leu Leu Thr Phe Lys
    450                 455                 460

Ile Pro Tyr Tyr Ile Gly Pro Ile Asn Asp Asn His Lys Lys Phe Phe
465                 470                 475                 480

Pro Asp Arg Cys Trp Val Val Lys Lys Glu Lys Ser Pro Ser Gly Lys
                485                 490                 495

Thr Thr Pro Trp Asn Phe Phe Asp His Ile Asp Lys Glu Lys Thr Ala
            500                 505                 510

Glu Ala Phe Ile Thr Ser Arg Thr Asn Phe Cys Thr Tyr Leu Val Gly
        515                 520                 525

Glu Ser Val Leu Pro Lys Ser Ser Leu Leu Tyr Ser Glu Tyr Thr Val
    530                 535                 540

Leu Asn Glu Ile Asn Asn Leu Gln Ile Ile Ile Asp Gly Lys Asn Ile
545                 550                 555                 560

Cys Asp Ile Lys Leu Lys Gln Lys Ile Tyr Glu Asp Leu Phe Lys Lys
                565                 570                 575

Tyr Lys Lys Ile Thr Gln Lys Gln Ile Ser Thr Phe Ile Lys His Glu
            580                 585                 590

Gly Ile Cys Asn Lys Thr Asp Glu Val Ile Ile Leu Gly Ile Asp Lys
        595                 600                 605

Glu Cys Thr Ser Ser Leu Lys Ser Tyr Ile Glu Leu Lys Asn Ile Phe
    610                 615                 620

Gly Lys Gln Val Asp Glu Ile Ser Thr Lys Asn Met Leu Glu Glu Ile
625                 630                 635                 640

Ile Arg Trp Ala Thr Ile Tyr Asp Glu Gly Glu Gly Lys Thr Ile Leu
                645                 650                 655

Lys Thr Lys Ile Lys Ala Glu Tyr Gly Lys Tyr Cys Ser Asp Glu Gln
            660                 665                 670

Ile Lys Lys Ile Leu Asn Leu Lys Phe Ser Gly Trp Gly Arg Leu Ser
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        675                 680                 685

Arg Lys Phe Leu Glu Thr Val Thr Ser Glu Met Pro Gly Phe Ser Glu
    690                 695                 700

Pro Val Asn Ile Ile Thr Ala Met Arg Glu Thr Gln Asn Asn Leu Met
705                 710                 715                 720

Glu Leu Leu Ser Ser Glu Phe Thr Phe Thr Glu Asn Ile Lys Lys Ile
                725                 730                 735

Asn Ser Gly Phe Glu Asp Ala Glu Lys Gln Phe Ser Tyr Asp Gly Leu
            740                 745                 750

Val Lys Pro Leu Phe Leu Ser Pro Ser Val Lys Lys Met Leu Trp Gln
        755                 760                 765

Thr Leu Lys Leu Val Lys Glu Ile Ser His Ile Thr Gln Ala Pro Pro
    770                 775                 780

Lys Lys Ile Phe Ile Glu Met Ala Lys Gly Ala Glu Leu Glu Pro Ala
785                 790                 795                 800

Arg Thr Lys Thr Arg Leu Lys Ile Leu Gln Asp Leu Tyr Asn Asn Cys
                805                 810                 815

Lys Asn Asp Ala Asp Ala Phe Ser Ser Glu Ile Lys Asp Leu Ser Gly
            820                 825                 830

Lys Ile Glu Asn Glu Asp Asn Leu Arg Leu Arg Ser Asp Lys Leu Tyr
        835                 840                 845

Leu Tyr Tyr Thr Gln Leu Gly Lys Cys Met Tyr Cys Gly Lys Pro Ile
    850                 855                 860

Glu Ile Gly His Val Phe Asp Thr Ser Asn Tyr Asp Ile Asp His Ile
865                 870                 875                 880

Tyr Pro Gln Ser Lys Ile Lys Asp Asp Ser Ile Ser Asn Arg Val Leu
                885                 890                 895

Val Cys Ser Ser Cys Asn Lys Asn Lys Glu Asp Lys Tyr Pro Leu Lys
            900                 905                 910

Ser Glu Ile Gln Ser Lys Gln Arg Gly Phe Trp Asn Phe Leu Gln Arg
        915                 920                 925

Asn Asn Phe Ile Ser Leu Glu Lys Leu Asn Arg Leu Thr Arg Ala Thr
    930                 935                 940

Pro Ile Ser Asp Asp Glu Thr Ala Lys Phe Ile Ala Arg Gln Leu Val
945                 950                 955                 960

Glu Thr Arg Gln Ala Thr Lys Val Ala Ala Lys Val Leu Glu Lys Met
                965                 970                 975

Phe Pro Glu Thr Lys Ile Val Tyr Ser Lys Ala Glu Thr Val Ser Met
            980                 985                 990

Phe Arg Asn Lys Phe Asp Ile Val  Lys Cys Arg Glu Ile  Asn Asp Phe
        995                 1000                1005

His His  Ala His Asp Ala Tyr  Leu Asn Ile Val Val  Gly Asn Val
    1010                1015                1020

Tyr Asn  Thr Lys Phe Thr Asn  Asn Pro Trp Asn Phe  Ile Lys Glu
    1025                1030                1035

Lys Arg  Asp Asn Pro Lys Ile  Ala Asp Thr Tyr Asn  Tyr Tyr Lys
    1040                1045                1050

Val Phe  Asp Tyr Asp Val Lys  Arg Asn Asn Ile Thr  Ala Trp Glu
    1055                1060                1065

Lys Gly  Lys Thr Ile Ile Thr  Val Lys Asp Met Leu  Lys Arg Asn
    1070                1075                1080
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Thr Pro  Ile Tyr Thr Arg Gln  Ala Ala Cys Lys Lys  Gly Glu Leu
    1085                1090                1095

Phe Asn  Gln Thr Ile Met Lys  Lys Gly Leu Gly Gln  His Pro Leu
    1100                1105                1110

Lys Lys  Glu Gly Pro Phe Ser  Asn Ile Ser Lys Tyr  Gly Gly Tyr
    1115                1120                1125

Asn Lys  Val Ser Ala Ala Tyr  Tyr Thr Leu Ile Glu  Tyr Glu Glu
    1130                1135                1140

Lys Gly  Asn Lys Ile Arg Ser  Leu Glu Thr Ile Pro  Leu Tyr Leu
    1145                1150                1155

Val Lys  Asp Ile Gln Lys Asp  Gln Asp Val Leu Lys  Ser Tyr Leu
    1160                1165                1170

Thr Asp  Leu Leu Gly Lys Lys  Glu Phe Lys Ile Leu  Val Pro Lys
    1175                1180                1185

Ile Lys  Ile Asn Ser Leu Leu  Lys Ile Asn Gly Phe  Pro Cys His
    1190                1195                1200

Ile Thr  Gly Lys Thr Asn Asp  Ser Phe Leu Leu Arg  Pro Ala Val
    1205                1210                1215

Gln Phe  Cys Cys Ser Asn Asn  Glu Val Leu Tyr Phe  Lys Lys Ile
    1220                1225                1230

Ile Arg  Phe Ser Glu Ile Arg  Ser Gln Arg Glu Lys  Ile Gly Lys
    1235                1240                1245

Thr Ile  Ser Pro Tyr Glu Asp  Leu Ser Phe Arg Ser  Tyr Ile Lys
    1250                1255                1260

Glu Asn  Leu Trp Lys Lys Thr  Lys Asn Asp Glu Ile  Gly Glu Lys
    1265                1270                1275

Glu Phe  Tyr Asp Leu Leu Gln  Lys Lys Asn Leu Glu  Ile Tyr Asp
    1280                1285                1290

Met Leu  Leu Thr Lys His Lys  Asp Thr Ile Tyr Lys  Lys Arg Pro
    1295                1300                1305

Asn Ser  Ala Thr Ile Asp Ile  Leu Val Lys Gly Lys  Glu Lys Phe
    1310                1315                1320

Lys Ser  Leu Ile Ile Glu Asn  Gln Phe Glu Val Ile  Leu Glu Ile
    1325                1330                1335

Leu Lys  Leu Phe Ser Ala Thr  Arg Asn Val Ser Asp  Leu Gln His
    1340                1345                1350

Ile Gly  Gly Ser Lys Tyr Ser  Gly Val Ala Lys Ile  Gly Asn Lys
    1355                1360                1365

Ile Ser  Ser Leu Asp Asn Cys  Ile Leu Ile Tyr Gln  Ser Ile Thr
    1370                1375                1380

Gly Ile  Phe Glu Lys Arg Ile  Asp Leu Leu Lys Val
    1385                1390                1395


<210> SEQ ID NO 9
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TRAC gRNA

<400> SEQUENCE: 9

cagggttctg gatatctgt                                    19
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<210> SEQ ID NO 10
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: cr RNA

<400> SEQUENCE: 10

cagggttctg gatatctgtg ttttagagct atgct 35

<210> SEQ ID NO 11
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 11

cctttttccc atgcctgcct tt 22

<210> SEQ ID NO 12
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 12

taaggccgag accaccaatc ag 22

<210> SEQ ID NO 13
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 13

acactctttc cctacacgac gctcttccga tct 33

<210> SEQ ID NO 14
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 14

gtgactggag ttcagacgtg tgctcttccg atct 34

<210> SEQ ID NO 15
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 15

atcttgtgcg catgtgaggg gc 22

<210> SEQ ID NO 16
<211> LENGTH: 22
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 16

gcaagccagg actccaccaa cc 22

<210> SEQ ID NO 17
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TRAC Long Donor FWD primer

<400> SEQUENCE: 17

tcgagtaaac ggtagtgctg gg 22

<210> SEQ ID NO 18
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: TRAC Long Donor REV primer

<400> SEQUENCE: 18

cctctcctgc caccttctct tc 22

1. An ex vivo, non-viral 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 a non-viral double-stranded homology-directed repair (HDR) template comprising the transgene flanked by homology arms that are complementary to sequences on both sides of a cleavage site in the T cell expressed gene,

introducing into a population of unmodified T cells a Cas9 ribonucleoprotein (RNP) and the double-stranded 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,

wherein the non-viral double-stranded HDR template contains the transgene sequence flanked by homology arms that are complementary to sequences on both sides of the 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 CAR T 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 one or more antigen-specific extracellular domains coupled to an intracellular domain by a transmembrane domain.

2. The method of claim **1**, wherein the homology arms have a length of 400 to 1000 base pairs.

3. The method of claim **1**, wherein the homology arms have a length of 450 to 750 base pairs.

4. The method of claim **1**, wherein the antigen-specific extracellular domain of the CAR is from antigen recognition molecule that recognizes a cell surface molecule on malignant cells such as hematologic malignancies or solid tumors.

5. The method of claim **1**, wherein the T cell expressed gene is TRAC, TRBC, AAVS1, TET2, FAS, BID, CTLA4, PDCD1, CBLB, PTPN6, CIITA or B2M genes.

6. The method of claim **1**, wherein the CART cell kills target-antigen-positive human cancer cells in vitro in a co-culture assay, in an in vivo animal model, or both.

7. The method of claim **1**, wherein the CAR T cells have activity against an antigen on a solid tumor in vitro or in vivo.

8. The method of claim **1**, further comprising imaging the population of CAR T cells and determining the degree of aggregation of the CAR T cells, and optionally selecting a population of aggregated CAR T cells.

9. The method of claim **1**, further comprising, prior to introducing the double-stranded HDR template, determining the concentration and purity of the double-stranded HDR template, wherein the double-stranded HDR template has an OD260/OD280 of 1.8 to 2.1, and/or an OD260/OD230 of 2.0 to 2.3, and diluting the double-stranded HDR template to a concentration of 2000 to 10000 ng/μ1.

10. The method of claim **1**, wherein the intracellular domain comprises a CD28, ICOS, CD27, 4-1BB, OX40,

CD40L, or CD3-ζ intracellular domain and the transmembrane domain comprises a CD4, CD8α, CD28, or CD3-ζ transmembrane domain.

11. The method of claim **1**, wherein the HDR template comprises a coding sequence for a fluorescent protein, a synthetic receptor, a gene for a cytokine signaling protein, or a short hairpin (sh)RNA.

12. The method of claim **1**, wherein the non-viral double-stranded HDR template sequentially comprises a left homology arm—a splice acceptor site—a self-cleaving peptide sequence—CAR gene—a polyA terminator—a right homology arm.

13. The method of claim **12**, wherein the self-cleaving peptide sequence is a T2A coding sequence.

14. The method of claim **1**, wherein the double-stranded HDR template is produced by amplifying a sequence from SEQ ID NO: 1.

15. The method of claim **14**, wherein the forward primer comprises SEQ ID NO 17 and the reverse primer comprises SEQ ID NO: 18.

16. The method of claim **1**, wherein the guide RNA targets the 5' end of the first exon of TRAC.

17. The method of claim **16**, wherein the guide RNA comprises SEQ ID NOs: 2 and 3.

18. The method of claim **1**, wherein culturing is done in round bottom culture wells at 20% of standard culture volume for the wells.

19. The method of claim **1**, wherein the unmodified T cells are autologous T cells isolated from a patient in need of cancer treatment, or T cells from an allogeneic healthy donor.

20. The method of claim **1**, wherein more than 4% of the population of unmodified T cells has the CAR transgene inserted into their genomes and expressed on the cell surface.

21. A non-viral produced CAR T cell with a genome having a CAR sequence specifically integrated into a T cell expressed gene, wherein the T cell is enriched for the CD62L and/or CD45RA markers indicative of naïve and stem cell memory phenotypes compared to retroviral-produced control CAR T cells.

22. The non-viral produced CART cell of claim **21**, wherein the CD62L and/or CD45RA markers are enriched more than 2-fold compared to the CD62L and/or CD45RA markers compared to retroviral-produced control CAR T cells.

23. A non-viral produced CAR T cell with a genome having a CAR sequence specifically integrated into a T cell expressed gene, wherein the T cell has reduced expression of TIM3 and/or LAG3 markers of T cell exhaustion compared to retroviral-produced control CAR T cells.

24. The non-viral produced CAR T cell of claim **23**, wherein the TIM3 and/or LAG3 markers are reduced more than 2-fold compared to the TIM3 and/or LAG3 markers compared to retroviral-produced control CAR T cells.

25. A plasmid of SEQ ID NO: 1.

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