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(54) Title: FUSION PROTEINS AND USES THEREOF

(57) Abstract: The present disclosure relates generally to fusion proteins comprising a truncated transforming growth factor beta receptor 2 (TGFBRII) and one or more intracellular co-stimulatory domains.

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FUSION PROTEINS AND USES THEREOF

Related Applications

[0001] This application claims priority from Australian Provisional Patent Application No. 2022903406 filed on 14 November 2022, the entire content of which is hereby incorporated by reference.

Field

[0002] The present disclosure relates generally to fusion proteins comprising a truncated transforming growth factor beta receptor 2 (TGFBRII) and one or more intracellular co-stimulatory domains.

Background

[0003] Chimeric antigen receptor (CAR)-T cells have performed poorly to date in clinical trials in patients with advanced solid cancers. There are many potential reasons for this, including poor CAR-T cell persistence in the patient, heterogeneous target antigen expression, poor trafficking of CAR-T cells into the tumour and tumour microenvironment (TME)-induced immune suppression. This immune suppression is mediated by many soluble factors, including TGF-β.

[0004] TGF-β is highly expressed in advanced refractory tumours and has a profound effect on T cell effector function (cytotoxicity and cytokine secretion) and T cell proliferation. TGF-β is expressed at higher levels across multiple cancer sub-types, where it inhibits effector T cell anti-tumour responses and is associated with T cell exhaustion. In the context of T cell receptor stimulation, increased levels of TGF-β can also increase FOXP3 expression levels skewing $CD4^+$ T cell polarization to a suppressor phenotype (as mediated by regulatory T cells, Tregs). TGF-β has immune-suppression effects across multiple immune cell types including myeloid cells and B cells. The net effect of these broader interactions in the TME leads to both direct and indirect immune suppression. Conversely, TGF-β is also a key T cell homeostatic cytokine. In the absence of response to TGF–β, T cells undergo increased proliferation leading to enlarged lymphoid organs and immune

related toxicity in mouse models (Oh *et al.*, 2013, *Journal of Immunology*, 191(8): 3973-3979).

[0005] Given the variable functions of TGF-β, it is perhaps unsurprising that the inhibition of TGF-β results in unpredictable outcomes. In a study by Kim *et al.* (2021, *Journal of Hematology & Oncology*, 14(1): 55), patient responses to the systemic inhibition of TGF-β included toxic side effects and objective responses. Moreover, TGF-β has also been a target for human CAR-T cells (Hartley and Abken, 2019, *Clinical Translation Immunology*, 8(6): e1065), including by expressing a dominant negative TGF–βRII to abrogate TGF-β signalling and improve tumor control *in vivo* (Kloss *et al.*, 2018, *Molecular Therapy*, 26(7): 1855-1866). However, while the results from a phase I clinical trial in prostate cancer patients showed promising reductions in PSA, significant toxicity was observed (Narayan *et al.*, 2022, *Nature Medicine*, 28: 724-734). Accordingly, there is a need to develop compositions and methods for targeting TGF-β signalling for use in cell therapies, without inducing toxic side effects.

Summary

[0006] In one aspect, the present disclosure provides a fusion protein comprising:

a. a truncated transforming growth factor beta receptor 2 (TGFBRII) comprising at least residues 1 to 187 of TGFBRII (SEQ ID NO: 1), wherein the truncated TGFBRII lacks a kinase domain; and

b. one or more intracellular co-stimulatory domains.

[0007] In another aspect, there is provided a nucleic acid molecule encoding the fusion protein described herein.

[0008] In another aspect, there is provided a vector comprising the nucleic acid molecule described herein.

[0009] In another aspect, there is provided a cell (*e.g.*, a population of cells) comprising the nucleic acid molecule or vector described herein.

[0010] In another aspect, there is provided a pharmaceutical composition comprising the cell (*e.g.*, a population of cells) described herein.

[0011] In another aspect, there is provided a method for the treatment of a subject having cancer, the method comprising administering to the subject an effective amount of the cell (*e.g.*, a population of cells) or the pharmaceutical composition described herein.

[0012] In another aspect, there is provided the cell (*e.g.*, a population of cells) or the pharmaceutical composition described herein for use in a method for the treatment of cancer.

[0013] In another aspect, there is provided a use of the cell (*e.g.*, a population of cells) or the pharmaceutical composition described herein in the manufacture of a medicament for the treatment of cancer.

[0014] In another aspect, there is provided a method of making a cell (*e.g.*, a population of cells), comprising introducing the nucleic acid molecule, or the vector described herein into a cell.

Brief Description of the Drawings

[0015] Embodiments of the disclosure are described herein, by way of non-limiting example only, with reference to the accompanying drawings.

[0016] **Figure 1** shows that TGFBRII.41BB CAR-T cells exhibit superior cytotoxicity and cytokine production in the presence of TGF-β. (**A**) A schematic representation of the TGFBRII.BB switch receptor gene construct for insertion in retrovirus, which includes LeY-CAR (CD28/CD3z) and the TGF-β receptor II extracellular domain fused with the intracellular domain of 4-1BB. (**B**) A graphical representation of CAR (left panel) and TGFBRII.41BB (right panel) expression on CAR T cells assessed by flow cytometry using Flag-tag and Myc-tag, respectively. (**C**) A graphical representation of luciferase activity (y-axis) of HEK293 cells transfected with TGFBRII.41BB and a NF-κB-driven luciferase reporter following treatment with TGF-β (ng/mL; x-axis). Data shown as mean ± SEM of triplicate cultures. (**D**) A series of graphical representations of percentage killing (%; y-axis) of DU-145 (LeY^+ prostate tumour cell line) after 16 hours co-culture with TGFBRII.41BB CAR-T cells or conventional CAR-T cells with or without TGF- β (x-axis), measured by ^{51}Cr release assays. Data shown as mean ± SEM of triplicate cultures, n = 3 healthy donors. (**E**) A series of graphical representations of IFN-γ production (pg/mL; y-axis) after 16 hours co-culture of DU-145 cells with TGFBRII.41BB CAR-T cells or conventional CAR-T cells

with TGF-β (10 ng/mL; x-axis), measured by AlphaLISA assay. Data shown as mean ± SEM of triplicate cultures, n = 3 healthy donors. (**F**) A series of graphical representations of TNF-α production (pg/mL; y-axis) after 16 hours co-culture of DU-145 cells with TGFBRII.41BB CAR-T cells or conventional CAR-T cells with TGF-β (10 ng/mL; x-axis), measured by AlphaLISA assay. Data shown as mean ± SEM of triplicate cultures, n = 3 healthy donors.

[0017] **Figure 2** shows that TGFBRII.41BB enhances proliferation of CAR-T cells in the presence of TGF-β through specific intracellular signalling. (**A**) A graphical representation of proliferation of TGFBRII.41BB CAR-T cells and conventional CAR-T cells labelled with cell trace violet (CTV) at the 7 day time point when cultured in the presence of TGF-β and CAR stimulation by anti-idiotype antibody. (**B**) A graphical representation of the percentage of divided of TGFBRII.41BB CAR-T cells and conventional CAR-T cells (%; y-axis) in baseline media (*i.e.*, without TGF-β) or in the presence of 20 ng/mL TGF-β (x-axis). (**C**) A graphical representation of the proliferation index of TGFBRII.41BB CAR-T cells and conventional CAR-T cells (%; y-axis), in baseline media (*i.e.*, without TGF-β) or in the presence of 20 ng/mL TGF-β (x-axis). (**D**) A graphical representation of histogram overlays of TGFBRII.41BB CAR-T cells and conventional CAR-T cells stained by MitoTracker Deep Red FM. (**E**) A graphical representation of relative mitochondrial mass (%; y-axis) of TGFBRII.41BB CAR-T cells and conventional CAR-T cells calculated based on the MFI of MitoTracker after 7 day culture with same conditions as described above in (A). (**F**) A graphical representation of expression levels of phosphorylated p38 MAPK (% MFI; y-axis) of TGFBRII.41BB CAR-T cells and conventional CAR-T cells, in baseline media (*i.e.*, without TGF-β) or in the presence of 20 ng/mL TGF-β (x-axis). (**G**) A graphical representation of the expression of phosphorylated SMAD2 (x-axis), in baseline media (*i.e.*, without TGF-β) or in the presence of 20 ng/mL TGF-β (y-axis). Representative data from a triplicate assay. Two-tailed unpaired Mann-Whitney test. ns, $p > 0.05$; * $p < 0.05$; **$p < 0.01$.

[0018] **Figure 3** shows the distinct transcriptional profile of TGFBRII.41BB CAR-T cells as compared to with conventional CAR-T cells in the presence of TGF-β. (**A**) A heatmap depicting the top 100 differentially expressed genes (normalized expression) between activated conventional vs activated switch CAR-T cells when with TGF-β. Each row represents one gene. (**B**) A graphical representation of log-fold change and FDR

(Benjamini-Hochberg adjusted p-value < 0.05). Genes with negative log-fold-change are more highly expressed in conventional CAR-T cell with TGF-β. Differentially expressed genes associated with effector functions and chemotaxis are shown in (**C**) and (**D**) as heatmaps. Heatmaps were constructed by Pearson correlation clustering of genes and Euclidean clustering of samples (n = 3 donors, Benjamini-Hochberg adjusted p-value < 0.05). (**E**) A graphical representations of a gene ontology (GO) analysis of differentially expressed genes with Log2FC < 1 and BH-adjusted p-value < 0.05. GO term on the y-axis plotted by the gene ratio matched in each pathway on x-axis. Each dot represents one pathway. The size of the dot represents the number of counts in matched GO pathway and is coloured by the BH-adjusted p-value.

[0019] **Figure 4** shows that TGFBRII.41BB receptor signalling induced a unique gene signature and reduced the TGF-β-induced immunosuppressive signal in TGFBRII.41BB CAR-T cells. (**A**) A graphical representation of the non-overlapped differentially expressed genes of activated conventional CAR-T cells (with TGF-β versus no TGF-β) and activated TGFBRII.41BB CAR-T cells (with TGF-β versus no TGF-β). (**B**) A heatmap showing the gene expression associated with T cell activating signal from uniquely upregulated genes differentially expressed in TGFBRII.41BB CAR-T cells. (**C**) A graphical representation of log-fold change and FDR (Benjamini-Hochberg adjusted p-value < 0.05). Genes with positive log-fold-change are more highly expressed in TGFBRII.41BB CAR-T cell with TGF-β, with several differential expressed genes from (B) highlighted in the plot. NS, not significant. (**D**) A graphical representation of the non-overlapped differentially expressed genes of activated conventional CAR-T cells (with TGF-β versus no TGF-β) and activated TGFBRII.41BB CAR-T cells (with TGF-β versus no TGF-β) from the genes shown in (C). (**E**) Heatmap showing the gene expression associated with T cell activating signal from unique downregulated genes differentially expressed in conventional CAR-T cells. The heatmap was constructed by Pearson correlation clustering of genes and Euclidean clustering of samples (n=3 donors, Benjamini-Hochberg adjusted p-value < 0.05). (**F**) A graphical representation of log-fold change and FDR. Genes with positive log-fold-change are more highly expressed in conventional CAR-T cell with TGF-β, with differential expressed genes from (E) highlighted in the plot.

[0020] **Figure 5** shows that TGFBRII.41BB CAR-T cells significantly enhanced tumour control *in vivo*. (**A**) A schematic representation of the *in vivo* experimental design. (**B**) A graphical representation of DU-145 growth kinetics by tumor size (mm^2; y-axis) and time (day; x-axis) for tumors measured every two days. *** $p < 0.001$, $n = 6$. (**C**) A graphical representation of survival (%; y-axis) and time (day; x-axis) for mice treated with TGFBRII.41BB CAR-T cells or conventional CAR-T cells. Significance for Kaplan-Meier survival analysis was determined by log-rank Mantel-Cox test. (**D**) A graphical representation of the percentage of human CAR-T cells in the peripheral blood (CD3+ cells/mL blood) at 17 days post-treatment with TGFBRII.41BB CAR-T cells or conventional CAR-T cells (x-axis). (**E**) A graphical representation of the level of TGF-β1 (pg/mL tumor; y-axis) at 17 days post-treatment with TGFBRII.41BB CAR-T cells or conventional CAR-T cells (x-axis) evaluated by AlphaLISA assay. Data shown are the mean ± SEM of 3 mice per group. (**F**) A graphical representation of the level of IFN-γ (pg/mL tumor; y-axis) at 17 days post-treatment with TGFBRII.41BB CAR-T cells or conventional CAR-T cells (x-axis) evaluated by AlphaLISA assay. Data shown as mean ± SEM of 3 mice per group.

[0021] **Figure 6** shows endogenous TGF-β type I and type II receptor expression in CAR-T cells. A heatmap showing the normalized gene expression of TGFBR1, TGFBR2, CD33 and CD3E (relative to CD3E) in conventional and TGFBRII.41BB CAR-T cells (n = 3 donors). The expression of selected genes were pre-processed (library size correction) and then compared to a housekeeping gene of T cells CD3E. CD33 is a myeloid marker absent from T cells (barely detectable) and was chosen as negative control. Con = conventional CAR-T cells, Switch = TGFBRII.41BB CAR-T cells, B271, 295 and 296 are healthy donor numbers.

[0022] **Figure 7** shows that TGF-β altered the gene expression profile of conventional CAR-T cells. (**A**) A schematic representation of the group comparison between TGF-β treated and untreated conventional CAR-T cells. (**B**) A heatmap showing top 100 differentially expressed genes (normalized expression) comparing the expression pattern between activated conventional CAR-T cells with TGF-β versus the activation-only CAR-T cells (n = 3 donors, Benjamini-Hochberg (BH) adjusted p-value<0.05). Each row represents one gene. The heatmap was constructed by Pearson correlation clustering of genes and

Euclidean clustering of samples. (**C**) A heatmap showing the gene expression associated with T cell activation, differentiation and effector function (n=3 donors, BH-adjusted p-value < 0.05). The heatmap was constructed by Pearson correlation clustering of genes and Euclidean clustering of samples. (**D**) A graphical representation of gene expression of TGF-β treated and untreated conventional CAR-T cells shown as log_2-fold change (x-axis) and -log_{10}(p-value) (y-axis). Genes with negative log_2-fold-change are more highly expressed in conventional CAR-T cell without TGF-β. Several differential expressed genes from (C) are highlighted in the plot. (**E**) A graphical representation of gene expression of TGF-β treated and untreated conventional CAR-T cells shown as log_2-fold change (x-axis) and -log_{10}(p-value) (y-axis). Genes with negative log_2-fold-change are more highly expressed in conventional CAR-T cell without TGF-β. Genes associated with T cell proliferation, Th1 differentiation and cell survival are highlighted. NS, not significant.

[0023] **Figure 8** shows that TGF-β induced different responses in conventional CAR-T cells and TGFBRII.41BB CAR-T cells. (**A**) A schematic representation of the group comparison between activated TGFBRII.41BB CAR-T cells and activated conventional CAR-T cells. (**B**) A heatmap depicting the top 100 differentially expressed genes (normalized expression) to compare the expression pattern between activated conventional vs activated switch CAR-T cells, both were treated with TGF-β (n = 3 donors, BH-adjusted p-value < 0.05). Each row represents one gene. The heatmap was constructed by Pearson correlation clustering of genes and Euclidean clustering of samples.

[0024] **Figure 9** shows that TGF-β induced enhanced activation and T effector gene expression in TGFBRII.41BB CAR-T cells but not in conventional CAR-T cells. (**A**) A heatmap showing the gene expression associated with T cell activation, effector function and immunosuppression (n = 3 donors, BH-adjusted p-value < 0.05). The heatmap was constructed by Pearson correlation clustering of genes and Euclidean clustering of samples. (**B**) A graphical representation of gene expression of TGF-β treated TGFBRII.41BB CAR-T cells and conventional CAR-T cells shown as log_2-fold change (x-axis) and -log_{10}(p-value) (y-axis). Genes with negative log_2-fold-change are more highly expressed in conventional CAR-T cell with TGF-β. Several differentially expressed genes from (A) are highlighted in the plot. Genes which did not reach significance but correlated with T cell function were also labelled in the grey region. (**C**) An enlarged version of (B) showing genes associated

with T cell proliferation, memory formation and cell survival were highlighted in the box. NS, not significant.

[0025] **Figure 10** shows that TGFBRII.II receptor signalling induced a unique gene signature in TGFBRII.41BB CAR-T cells. (**A**) A graphical representation of the non-overlapped differentially expressed genes of activated conventional CAR-T cells (with TGF-β versus no TGF-β) and activated TGFBRII.41BB CAR-T cells (with TGF-β versus no TGF-β). (**B**) A heatmap showing the gene expression associated with T cell activating signal from uniquely upregulated genes differentially expressed in switch CAR-T cells (n=3 donors, BH adjusted p-value < 0.05). The heatmap was constructed by Pearson correlation clustering of genes and Euclidean clustering of samples. (**C**) A graphical representation of gene expression of TGF-β treated TGFBRII.41BB CAR-T cells and untreated TGFBRII.41BB CAR-T cells shown as $\log_2$-fold change (x-axis) and $-\log_{10}$(p-value) (y-axis). Genes with positive $\log_2$-fold-change are more highly expressed in TGFBRII.41BB CAR-T cells with TGF-β. Several differentially expressed genes from (B) are highlighted in the plot. NS, not significant.

[0026] **Figure 11** shows that TGFBRII.41BB receptor reduced the TGF-β-induced immunosuppressive signal in TGFBRII.41BB CAR-T cells. (**A**) A graphical representation of the non-overlapped differentially expressed genes of activated conventional CAR-T cells (with TGF-β versus no TGF-β) and activated TGFBRII.41BB CAR-T cells (with TGF-β versus no TGF-β). (**B**) A heatmap showing the gene expression associated with T cell activating signal from unique downregulated genes differentially expressed in conventional CAR-T cells (n=3 donors, BH-adjusted p-value < 0.05). The heatmap was constructed by Pearson correlation clustering of genes and Euclidean clustering of samples. (**C**) A graphical representation of gene expression of TGF-β treated conventional CAR-T cells and untreated conventional CAR-T cells shown as $\log_2$-fold change (x-axis) and $-\log_{10}$(p-value) (y-axis). Genes with positive $\log_2$-fold-change are more highly expressed in conventional CAR-T cells with TGF-β. Several differentially expressed genes from (B) are highlighted in the plot. NS, not significant.

[0027] **Figure 12** shows the structure of the fusion proteins relative to wild-type TGFBRII. (**A**) A schematic representation of wild-type TGFBRII. (**B**) A schematic representation of the fusion proteins described herein, which comprise truncated TGFBRII

(*i.e.*, comprising residues 1-199, 1-220 and 1-243 of SEQ ID NO: 1). (**C**) A schematic representation of the fusion proteins described herein, which comprise intracellular co-stimulatory domains 4-1BB, OX40, ICOS or CD40.

[0028] **Figure 13** shows the *in vitro* function of CAR-T cells engineered with fusion protein receptors comprising different intracellular domains (ICD). (**A**) A graphical representation of percentage killing (%lysis, y-axis) of CAR-T cells (with different ICD, x-axis) against the LeY^+ prostate cancer cell line DU-145 after 16 hours co-culture, measured by ^{51}Cr release assays. Data shown as the mean ± SEM of triplicate cultures. (**B**) A graphical representation of TNF-α-producing (%, y-axis) CAR-T cells (with different ICD, x-axis) when stimulated with LeY anti-idiotype antibody and TGF-β, measured by AlphaLISA assay. Data shown as mean ± SEM of triplicate cultures and is representative of one donor.

[0029] **Figure 14** shows that fusion proteins can form homodimers. **(A)** A graphical representation of the fusion proteins tested, where wild-type TGFBRII receptor was fused with eGFP ($R2^{WT\text{-}eGFP}$) and the TGF-β binding motif with varying intracellular non-kinase regions (SIS: residues 1 to 199 of TGFBRII, SEQ ID NO: 1; MIS: residues 1 to 220 of TGFBRII, SEQ ID NO: 1; and LIS: residues 1 to 243 of TGFBRII, SEQ ID NO: 1) fused with either eGFP or mCherry. **(B)** A graphical representation of a FACS-based FRET assay showing both homodimerization of two SIS receptors (circles and solid line) and heterodimerisation of a SIS receptor and a wild-type TGFBRII receptor (crosses and dotted line). **(C)** A graphical representation of a FACS-based FRET assay showing both homodimerization of two MIS receptors (circles and solid line) and heterodimerisation of a MIS receptor and a wild-type TGFBRII receptor (crosses and dotted line). **(D)** A graphical representation of a FACS-based FRET assay showing both homodimerization of two LIS receptors (circles and solid line) and heterodimerisation of a LIS receptor and a wild-type TGFBRII receptor (crosses and dotted line).

[0030] **Figure 15** shows that TGFBRII.41BB CAR-T cells significantly enhanced tumor control *in vivo* in a breast cancer model. (**A**) A graphical representation of MDA-MB468 growth kinetics by tumor size (mm^2; y-axis) and time (day; x-axis) for tumors measured every two days. *** $p < 0.001$, $n = 8$. (**B**) A graphical representation of survival (%; y-axis) and time (day; x-axis) for mice treated with TGFBRII.41BB CAR-T cells or

conventional CAR-T cells. Significance for Kaplan-Meier survival analysis was determined by log-rank Mantel-Cox test.

Brief Description of the Sequences

[0031] Nucleic acid and amino acid sequences are referred to by a sequence identifier number (SEQ ID), with reference to the accompanying sequence listing.

[0032] **SEQ ID NO: 1** shows the amino acid sequence of full length human transforming growth factor beta receptor 2 (TGFBRII).

[0033] **SEQ ID NO: 2** shows the amino acid sequence of the TGFBRII extracellular domains, including signal peptide.

[0034] **SEQ ID NO: 3** shows the DNA sequence encoding the TGFBRII extracellular domains of SEQ ID NO: 2.

[0035] **SEQ ID NO: 4** shows the amino acid sequence of the TGFBRII transmembrane domain.

[0036] **SEQ ID NO: 5** shows the DNA sequence encoding the TGFBRII transmembrane domain of SEQ ID NO: 4.

[0037] **SEQ ID NO: 6** shows the amino acid sequence of the TGFBRII intracellular non-kinase region.

[0038] **SEQ ID NO: 7** shows the DNA sequence encoding the TGFBRII intracellular non-kinase region of SEQ ID NO: 6.

[0039] **SEQ ID NO: 8** shows the amino acid sequence of a truncated human TGFBRII comprising residues 1 to 199 of SEQ ID NO: 1.

[0040] **SEQ ID NO: 9** shows the amino acid sequence of a truncated human TGFBRII comprising residues 1 to 220 of SEQ ID NO: 1.

[0041] **SEQ ID NO: 10** shows the amino acid sequence of a truncated human TGFBRII comprising residues 1 to 243 of SEQ ID NO: 1.

[0042] **SEQ ID NO: 11** shows the amino acid sequence of fusion protein comprising the truncated human TGFBRII of SEQ ID NO: 8 and the 4-1BB intracellular co-stimulatory domain (*i.e.*, TGFBRII199.41BB).

[0043] **SEQ ID NO: 12** shows the amino acid sequence of fusion protein comprising the truncated human TGFBRII of SEQ ID NO: 9 and the 4-1BB intracellular co-stimulatory domain (*i.e.*, TGFBRII220.41BB).

[0044] **SEQ ID NO: 13** shows the amino acid sequence of fusion protein comprising the truncated human TGFBRII of SEQ ID NO: 10 and the 4-1BB intracellular co-stimulatory domain (*i.e.*, TGFBRII243.41BB).

[0045] **SEQ ID NO: 14** shows the amino acid sequence of fusion protein comprising the truncated human TGFBRII of SEQ ID NO: 8 and the OX40 intracellular co-stimulatory domain (*i.e.*, TGFBRII199.OX40).

[0046] **SEQ ID NO: 15** shows the amino acid sequence of fusion protein comprising the truncated human TGFBRII of SEQ ID NO: 8 and the ICOS intracellular co-stimulatory domain (*i.e.*, TGFBRII199.ICOS).

[0047] **SEQ ID NO: 16** shows the amino acid sequence of fusion protein comprising the truncated human TGFBRII of SEQ ID NO: 8 and the CD40 intracellular co-stimulatory domain (*i.e.*, TGFBRII199.CD40).

[0048] **SEQ ID NO: 17** shows the DNA sequence encoding the fusion protein of SEQ ID NO: 11.

[0049] **SEQ ID NO: 18** shows the DNA sequence encoding the fusion protein of SEQ ID NO: 12.

[0050] **SEQ ID NO: 19** shows the DNA sequence encoding the fusion protein of SEQ ID NO: 13.

[0051] **SEQ ID NO: 20** shows the DNA sequence encoding the fusion protein of SEQ ID NO: 14.

[0052] **SEQ ID NO: 21** shows the DNA sequence encoding the fusion protein of SEQ ID NO: 15.

[0053] **SEQ ID NO: 22** shows the DNA sequence encoding the fusion protein of SEQ ID NO: 16.

[0054] **SEQ ID NO: 23** shows the amino acid sequence of the 4-1BB intracellular co-stimulatory domain.

[0055] **SEQ ID NO: 24** shows the DNA sequence encoding the 4-1BB intracellular co-stimulatory domain.

[0056] **SEQ ID NO: 25** shows the amino acid sequence of the OX40 intracellular co-stimulatory domain.

[0057] **SEQ ID NO: 26** shows the DNA sequence encoding the OX40 intracellular co-stimulatory domain.

[0058] **SEQ ID NO: 27** shows the amino acid sequence of the ICOS intracellular co-stimulatory domain.

[0059] **SEQ ID NO: 28** shows the DNA sequence encoding the ICOS intracellular co-stimulatory domain.

[0060] **SEQ ID NO: 29** shows the amino acid sequence of the CD40 intracellular co-stimulatory domain.

[0061] **SEQ ID NO: 30** shows the DNA sequence encoding the CD40 intracellular co-stimulatory domain.

[0062] **SEQ ID NO: 31** shows the DNA sequence of the pSAMEN vector comprising nucleic acid sequences encoding TGFBRII199.41BB.

[0063] **SEQ ID NO: 32** shows the DNA sequence of a pSAMEN vector comprising nucleic acid sequences encoding LeY-CD34BEND10-TGFBRII199.41BB.

[0064] **SEQ ID NO: 33** shows the DNA sequence of a pSAMEN vector comprising nucleic acid sequences encoding LeY-CD34BEND10-TGFBRII220.41BB.

[0065] **SEQ ID NO: 34** shows the DNA sequence of a pSAMEN vector comprising nucleic acid sequences encoding LeY-CD34BEND10-TGFBRII243.41BB.

[0066] **SEQ ID NO: 35** shows the DNA sequence of a pSAMEN vector comprising nucleic acid sequences encoding LeY-CD34BEND10-TGFBRII199.OX40.

[0067] **SEQ ID NO: 36** shows the DNA sequence of a pSAMEN vector comprising nucleic acid sequences encoding LeY-CD34BEND10-TGFBRII.ICOS.

[0068] **SEQ ID NO: 37** shows the DNA sequence of a pSAMEN vector comprising nucleic acid sequences encoding LeY-CD34BEND10-TGFBRII199.CD40.

Detailed Description

[0069] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. All patents, patent applications, published applications and publications, databases, websites and other published materials referred to throughout the entire disclosure, unless noted otherwise, are incorporated by reference in their entirety. In the event that there is a plurality of definitions for terms, those in this section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference to the identifier evidences the availability and public dissemination of such information.

[0070] The articles "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a single cell, as well as two or more cells (*e.g.*, a population of cells); reference to "an effector protein" includes a single effector protein, as well as two or more effector proteins; and so forth.

[0071] In the context of this specification, the term "about" is understood to refer to a range of numbers that a person of skill in the art would consider equivalent to the recited value in the context of achieving the same function or result. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 10%. Therefore, about 50% means in the range of 45%-55%. Numerical ranges recited herein by endpoints include all numbers and fractions subsumed within that range (*e.g.*, 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.90, 4, and 5). It is also to be understood that all numbers and fractions thereof are presumed to be modified by the term "about".

[0072] Throughout this specification and the claims that follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements.

[0073] The term "optionally" is used herein to mean that the subsequent described feature may or may not be present or that the subsequently described event or circumstance may or may not occur. Hence the specification will be understood to include and encompass embodiments in which the feature is present and embodiments in which the feature is not present, and embodiment in which the event or circumstance occurs as well as embodiments in which it does not.

[0074] As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

[0075] Amino acids may be referred to herein by either the commonly known three letter symbols or by the single letter symbols recommended by the IUPAC-IUB Biochemical

Nomenclature Commission. Similarly, nucleotides may be referred to by their commonly accepted single letter codes.

[0076] All sequence database identifiers (*e.g.*, GenBank ID, EMBL-Bank ID, DNA Data Bank of Japan (DDBJ) ID, *etc.*) provided herein were current at the filing date.

[0077] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0078] The present disclosure is predicated, in part, on the surprising finding that fusion proteins comprising a truncated TGFRBII and one or more intracellular co-stimulatory domains are capable of improving the function of cell therapies (*e.g.*, CAR T cell therapies) by converting immunosuppressive TGF-β signalling to an activation signal, which increases cytotoxic function, cytokine secretion and proliferation of immune effector cells (*e.g.*, T cells, NK cells, macrophages). These functional features are associated with clinical response and reduced negative side effects, which underscores the need for translation to the clinic.

[0079] Accordingly, in an aspect disclosed herein, there is provided a fusion protein comprising:

a. a truncated transforming growth factor beta receptor 2 (TGFBRII) comprising at least residues 1 to 187 of TGFBRII (SEQ ID NO: 1), wherein the truncated TGFBRII lacks a kinase domain; and
b. one or more intracellular co-stimulatory domains.

Fusion proteins

[0080] The terms “protein”, “peptide” and “polypeptide” are used interchangeably herein to refer to a polymer of amino acid residues linked together by peptide (amide) bonds. The terms refer to a protein, peptide, or polypeptide of any size, structure or function.

[0081] As used herein, "fusion protein" refers to a recombinant protein comprising two or more polypeptides, which are not normally associated in nature, but the respective amino and carboxy termini may be joined together by direct or indirect binding to form one

contiguous polypeptide. In some instances, the term "fusion protein" is used interchangeably with "switch receptor" when referring to the fusion protein as described herein.

[0082] As used herein, "transforming growth factor beta receptor 2" or "TGFBRII" means the receptor for transforming growth factor beta (TGF-β). TGF-β is an immunosuppressive soluble factor that is highly expressed in advanced tumors (Mariathasan *et al.*, 2018, *Nature*, 554(7693): 544-548) and inhibits effector T cell anti-tumor responses (*e.g.*, cytotoxicity and cytokine secretion) and T cell proliferation. The complete human TGFBRII sequence is shown in SEQ ID NO: 1.

[0083] The term "truncated TGFBRII" as used herein refers to a TGFBRII comprising at least residues 1 to 187 of TGFBRII (SEQ ID NO: 1), wherein the truncated TGFBRII lacks a kinase domain (*i.e.*, residues 244-567 of SEQ ID NO: 1). Accordingly, the truncated TGFBRII may comprise at least residues 1 to 187 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 188 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 189 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 190 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 191 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 192 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 193 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 194 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 195 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 196 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 197 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 198 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 199 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 200 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 201 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 202 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 203 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 204 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 205 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 206 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 207 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 208 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 209 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 210 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 211 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 212 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 213 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 214 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 215 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 216 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 217 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 218 of TGFBRII (SEQ ID NO: 1), at

least residues 1 to 219 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 220 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 221 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 222 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 223 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 224 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 225 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 226 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 227 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 228 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 229 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 230 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 231 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 232 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 233 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 234 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 235 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 236 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 237 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 238 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 239 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 240 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 241 of TGFBRII (SEQ ID NO: 1), at least residues 1 to 242 of TGFBRII (SEQ ID NO: 1), or at least residues 1 to 243 of TGFBRII (SEQ ID NO: 1).

[0084] In an embodiment, the truncated TGFBRII comprises at least residues 1 to 199 of TGFBRII (SEQ ID NO: 1).

[0085] In an embodiment, the truncated TGFBRII comprises at least residues 1 to 220 of TGFRBII (SEQ ID NO: 1).

[0086] In an embodiment, the truncated TGFBRII comprises at least residues 1 to 243 of TGFRBII (SEQ ID NO: 1).

[0087] In an embodiment, the truncated TGFBRII comprises, consists or consists essentially of any one the amino acid sequences of SEQ ID NOs: 8-10, or an amino acid sequence having at least 90% sequence identity thereto. Accordingly, the sequence may be at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of the amino acid sequences of SEQ ID NOs: 8-10. Methods for the determination of amino acid sequence identity would be known to persons skilled in the art, illustrative examples of which include

computer programs that employ algorithms such as protein BLAST (Altschul *et al.*, 1997, *Nucleic Acids Research*, 25: 3389-3402).

[0088] In an embodiment, the fusion protein comprises one or more intracellular co-stimulatory domains. In an embodiment, the fusion protein comprise one intracellular co-stimulatory domain. In another embodiment, the fusion protein comprises two intracellular co-stimulatory domains.

[0089] Examples of intracellular co-stimulatory domains include an MHC class I molecule, TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signalling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, 4-1BB (CD137), B7-H3, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, ITGB7, NKG2D, NKG2C, TN1-R2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83, or a functional variant thereof.

[0090] In an embodiment, the intracellular co-stimulatory domain is a functional signalling domain of a protein selected from the group consisting of 4-1BB, OX40, ICOS and CD40.

[0091] In an embodiment, the intracellular co-stimulatory domain comprises, consists or consists essentially of any one the amino acid sequences of SEQ ID NOs: 23, 25, 27 and 29, or an amino acid sequence having at least 90% sequence identity thereto. Accordingly, the sequence may be at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of the amino acid sequences of SEQ ID NOs: 23, 25, 27 and 29.

[0092] In an embodiment, the fusion protein comprises, consists, or consists essentially of any one of the amino acid sequences of SEQ ID NOs: 11-16, or an amino acid sequence having at least 90% sequence identity thereto. Accordingly, the sequence may be at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of the amino acid sequences of SEQ ID NOs: 11-16.

Nucleic acid molecules, vectors and delivery vehicles

[0093] In another aspect disclosed herein, there is provided a nucleic acid molecule encoding the fusion protein described herein.

[0094] As used herein the terms “polynucleotide”, “nucleic acid” or “nucleic acid molecule” mean a single- or double-stranded polymer of deoxyribonucleotide, ribonucleotide bases or known analogues or natural nucleotides, or mixtures thereof, and can include molecules comprising coding and non-coding sequences of a gene, sense and antisense sequences and complements, exons, introns, genomic DNA, cDNA, pre-mRNA, mRNA, rRNA, siRNA, miRNA, tRNA, ribozymes, recombinant polypeptides, isolated and purified naturally occurring DNA or RNA sequences, synthetic RNA and DNA sequences, nucleic acid probes, primers and fragments.

[0095] The term “nucleotide” as used herein refers to the nucleotides adenosine, guanosine, cytidine, thymidine and uridine, each of which comprise a nucleotide base attached to a ribose ring. A person skilled in the art will appreciate that the terms "adenine / adenosine", "uracil / uridine", "guanine / guanosine", "cytosine / cytidine" and "thymidine / thymine" (C) may be used interchangeably herein with the single letters A, U, G, T and T, respectively, which refer the nucleotide base comprised by the nucleotides.

[0096] The terms "non-naturally occurring", "engineered" or "recombinant" may be interchangeably used herein to refer to nucleotides or nucleic acid molecules that are distinguished from their naturally occurring counterparts. For example, the nucleic acid molecule of the present disclosure may be recombinant, synthetic, or comprise mixtures of naturally and non-naturally occurring nucleotides. Non-naturally occurring nucleotides or nucleotide analogs may be modified at the ribose, phosphate and/or base moiety.

[0097] As used herein, the terms “encode”, “encoding” and the like refer to the capacity of a nucleic acid molecule to provide for another nucleic acid or a polypeptide. For example, a nucleic acid molecule is said to "encode" a polypeptide if it can be transcribed and/or translated to produce the polypeptide or if it can be processed into a form that can be transcribed and/or translated to produce the polypeptide. Such a nucleic acid molecule may include a coding sequence or both a coding sequence and a non-coding sequence. Thus, the terms "encode," "encoding" and the like include an RNA product resulting from transcription of a DNA molecule, a protein resulting from translation of an RNA molecule, a protein resulting from transcription of a DNA molecule to form an RNA product and the subsequent translation of the RNA product, or a protein resulting from transcription of a DNA molecule to provide an RNA product, processing of the RNA product to provide a processed RNA product (*e.g.*, mRNA) and the subsequent translation of the processed RNA product.

[0098] In an embodiment, the fusion protein is encoded by a codon optimized nucleic acid sequence for expression in particular cells, *e.g.*, eukaryotic cells. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (*e.g.*, about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (*i.e.*, differences in codon usage between organisms) often correlates with the efficiency of translation of mRNA, which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, *e.g.*, the "Codon Usage Database" available at www.kazusa.orjp/codon/. Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available.

[0099] In an embodiment, the nucleic acid molecule comprises one or more non-naturally occurring nucleotide or nucleotide analog such as a nucleotide with phosphorothioate linkage, boranophosphate linkage, a locked nucleic acid (LNA) nucleotides comprising a methylene bridge between the 2' and 4' carbons of the ribose ring, or bridged nucleic acids (BNA). Other examples of modified nucleotides include 2'-0-methyl analogs, 2'-deoxy analogs, 2-thiouridine analogs, N6-methyladenosine analogs, or 2'-fluoro analogs. Further examples of modified bases include, but are not limited to, 2-aminopurine, 5-bromo-uridine, pseudouridine (Ψ), N^1-methylpseudouridine ($me^1\Psi$), S-methoxyuridine(SmoU), inosine, 7-methylguanosine.

[0100] In an embodiment, the nucleic acid molecule is a recombinant nucleic acid molecule.

[00100] The nucleic acid molecules of the present disclosure may be produced using any method in the art, including synthetically or by recombinant techniques such as expression of polynucleotide constructs encoding the components.

[0101] In an embodiment, the nucleic acid molecule comprises the nucleotide sequence of any one of SEQ ID NOs: 17-22, or a nucleotide sequence having at least 90% sequence identity thereto. Accordingly, the sequence may be at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of the nucleotide sequences of SEQ ID NOs: 17-22. Methods for the determination of nucleotide sequence identity would be known to persons skilled in the art, illustrative examples of which include computer programs that employ algorithms such as protein BLAST (Altschul *et al.*, 1997, *Nucleic Acids Research*, 25: 3389-3402).

[0102] In another aspect, there is provided a vector comprising the nucleic acid molecule described herein.

[0103] The vectors can be episomal vectors (*i.e.*, that do not integrate into the genome of a host cell), or can be vectors that integrate into a host cell genome. Vectors may be replication competent or replication-deficient. Exemplary vectors include, but are not limited to, plasmids, cosmids, and viral vectors, such as adeno-associated virus (AAV)

vectors, lentiviral, retroviral, adenoviral, herpesviral, parvoviral and hepatitis viral vectors. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art. Preferably, however, the vector is suitable for use in biotechnology.

[0104] Vectors suitable for use in biotechnology would be known to persons skilled in the art, illustrative examples of which include viral vectors derived from adenovirus, adeno-associated virus (AAV), herpes simplex virus (HSV), retrovirus, lentivirus, self-amplifying single-strand RNA (ssRNA) viruses such as alphavirus (*e.g.*, Semliki Forest virus, Sindbis virus, Venezuelan equine encephalitis, M1), and flavivirus (*e.g.*, Kunjin virus, West Nile virus, Dengue virus), rhabdovirus (*e.g.*, rabies, vesicular stomatitis virus), measles virus, Newcastle Disease virus (NDV) and poxivirus as described by, for example, Lundstrom (2019, *Diseases*, 6: 42).

[0105] In an embodiment, the vector is a plasmid or a viral vector.

[0106] The present disclosure also provides non-viral delivery vehicles of the genome editing systems as described herein, and components thereof. Suitable non-viral delivery vehicles will be known to persons skilled in the art, illustrative examples of which include using lipids, lipid-like materials or polymeric materials, as described by , *e.g.*, Rui *et al.* (2019, *Trends in Biotechnology*, 37(3): 281-293), and nanoparticles/nanocarriers, as described by, *e.g.*, Nguyen *et al.* (2020, *Nature Biotechnology*, 38: 44-49).

[0107] In some embodiments, cells are engineered to express the fusion protein and a chimeric antigen receptor (CAR) or T-cell receptor (TCR). In accordance with such embodiments, the nucleic acid molecules encoding the fusion protein and the CAR or TCR may be comprised in separate vectors or within the same vector, *e.g.*, the vectors shown as SEQ ID NOs: 32-37.

[0108] When multiple nucleic acid molecules are combined within the same vector, the expression of each nucleic acid molecule may be controlled by the same promoter or different promoters according to the optimal stoichiometry of the different components of the fusion protein, CAR or TCR disclosed herein. Thus, in some examples, the polynucleotide encoding the fusion protein will be operably linked to a first promoter and the polynucleotide encoding a CAR operably linked to a second promoter.

[0109] The term "promoter" as used herein refers to an array of nucleic acid control sequences that direct the transcription of the polynucleotide. Suitable promoters would be known to persons skilled in the art, illustrative examples of which include retroviral LTR elements, constitutive promoters such as CMV, HSV1-TK, SV40, EF-1α, or β-actin, inducible promoters, such as those containing Tet-operator elements, and/or tissue specific promoters.

[0110] Where the nucleic acid molecules are not comprised within the same vector, the nucleic acid molecules may be comprised in separate vectors, or one nucleic acid molecule (*e.g.*, encoding the fusion protein) may be comprised in a vector and a second nucleic acid molecule (*e.g.*, encoding a CAR or TCR) is provided to the cell using non-viral delivery vehicles.

[0111] The nucleic acid molecules may comprise other additional regulatory elements or sequences. Suitable regulatory sequences would be known to persons skilled in the art, illustrative examples of which include leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, and enhancer or activator sequences. It is also contemplated herein that the polypeptides comprises elements and sequences associated with protein localization and interactions.

Cells

[0112] In another aspect, the present disclosure provides a cell (*e.g.*, a population of cells) comprising the nucleic acid molecule or vectors described herein.

[0113] Cells according to the present disclosure include any cell into which the nucleic acid molecules or vectors described herein may be introduced and expressed. It is not intended that use of the nucleic acid molecule or vectors disclosed herein be limited by cell type. Accordingly, the cells of the present disclosure include eukaryotic cells, prokaryotic cells, animal cells, plant cells, fungal cells, archaeal cells, eubacterial cells and the like.

[0114] The term "cell" as used herein refers to an individual cell, cell line, cell culture or population of cells that comprise the nucleic acid molecule or vectors described herein, or that is capable of expressing the fusion protein described herein. The term "population of cells" may refer to homogenous cell populations comprising cells that each comprise the

nucleic acid molecule or vectors described herein, or that is capable of expressing the fusion protein described herein, or heterogeneous cell populations that may comprise progeny of a single parental cell. Due to natural, accidental or deliberate mutation, the progeny cells may not necessarily be identical in morphology or in genome to the original parental cell, but may be capable of expressing the fusion protein described herein.

[0115] The cells contemplated herein may be derived from any species, particularly a vertebrate, and even more particularly a mammal. Suitable vertebrates that fall within the scope of the disclosure include, but are not restricted to, any member of the subphylum Chordata including primates (*e.g.*, humans, monkeys and apes, and includes species of monkeys such from the genus *Macaca* (*e.g.*, cynomologus monkeys such as *Macaca fascicularis*, and/or rhesus monkeys (Macaca *mulatta*)) and baboon (*Papio ursinus*), as well as marmosets (species from the genus *Callithrix*), squirrel monkeys (species from the genus *Saimiri*) and tamarins (species from the genus *Saguinus*), as well as species of apes such as chimpanzees (*Pan troglodytes*), rodents (*e.g.*, mice rats, guinea pigs), lagomorphs (*e.g.*, rabbits, hares), bovines (*e.g.*, cattle), ovines (*e.g.*, sheep), caprines (*e.g.*, goats), porcines (*e.g.*, pigs), equines (*e.g.*, horses), canines (*e.g.*, dogs), felines (*e.g.*, cats), avians (*e.g.*, chickens, turkeys, ducks, geese, companion birds such as canaries, budgerigars *etc.*), marine mammals (*e.g.*, dolphins, whales), reptiles (snakes, frogs, lizards *etc.*), and fish. In a preferred embodiment, the cells are derived from a human.

[0116] In an embodiment, the cell is an immune effector cell. Suitable immune effector cells would be known to persons skilled in the art, illustrative examples of which include T cells, B cells, natural killer (NK) cells, natural killer T (NKT) cells, mast cells and bone marrow-derived phagocytes.

[0117] In an embodiment, the immune effector cell is an autologous immune effector cell.

[0118] The term "autologous" as used herein refers to any material derived from the same subject to whom it is later to be administered into the subject in accordance with the methods disclosed herein. Accordingly, in certain embodiments, T cells isolated from the subject may be contacted with the genome editing systems described herein and cultured *ex vivo* for a time and under conditions suitable for the integration of the heterologous

nucleotide sequence, before being reinfused back into the subject in accordance with the method of treatment described herein.

[0119] In another embodiment, the immune effector cell is an allogenic immune effector cell.

[0120] The term "allogenic" as used herein refers to any material derived from a different animal of the same species as the subject to whom the material is administered.

[0121] In an embodiment, the cell is a T cell.

[0122] Suitable T cells would be known to persons skilled in the art, illustrative examples of which include thymocytes, naïve T lymphocytes, immature T lymphocytes, mature T lymphocytes, resting T lymphocytes, activated T lymphocytes or tumour infiltrating lymphocytes (TILs). Illustrative populations of T cells suitable for use in particular embodiments include but are not limited to helper T cells (HTL; $CD4^+$ T cell), a cytotoxic T cell (CTL; $CD8^+$ T cell), $CD4^+CD8^+$ T cell, $CD4^-CD8^-$ T cell, or any other subset of T cells. Other illustrative populations of T cells suitable for use in particular embodiments include but are not limited to T cells expressing one or more of the following markers: CD3, CD4, CD8, CD27, CD28, CD45RA, CD45RO, CD62L, CD127, CD197, and HLA-DR and if desired, can be further isolated by positive or negative selection techniques.

[0123] In an embodiment, the cell is a NK cell.

[0124] Suitable NK cells would be known to persons skilled in the art, illustrative examples of which include $NK^{tolerant}$, $NK^{cytotoxic}$, $NK^{regulatory}$ cells and memory-like NK cells. Illustrative populations of NK cells suitable for use in particular embodiments include but are not limited to NK cells expressing one or more of the following markers: CD27, CD11b, CD56, CD16, and if desired, can be further isolated by positive or negative selection techniques.

[0125] The term “isolated” as used herein refers to a cell, which is substantially or essentially free from components that normally accompany or interact with it as found in its naturally occurring environment, *e.g.*, whole blood. Methods for the isolation of immune effector cells from whole blood would be known to persons skilled in the art, illustrative

examples of which include the isolation of T cells from whole blood using the Ficoll-Paque method.

[0126] The cell (*e.g.*, a population of cells) may be provided with the nucleic acid molecules or vectors described herein using any suitable method known in the art. Such methods include transfection, transduction, viral transduction, microinjection, lipofection, nucleofection, nanoparticle bombardment, transformation, conjugation and the like. The skilled person would readily understand and adapt any such method taking consideration of whether the nucleic acid molecules are provided as polynucleotides or vectors. The term "recombinant cell" as used herein refers to a cell which comprises the vectors described herein. The term "recombinant cell" includes the specific cell and the progeny of the cell.

[0127] In an embodiment, the cell has been engineered to express a chimeric antigen receptor (CAR) or a T-cell receptor (TCR).

[0128] The terms “chimeric antigen receptor” or “CAR” as used herein mean a recombinant polypeptide comprising at least an antigen-binding domain that is linked, *via* hinge and transmembrane domains, to an intracellular signalling domain.

[0129] The antigen-binding domain is a functional portion of the CAR that is responsible for transmitting information within the cell to regulate cellular activity *via* defined signaling pathways. In an embodiment, the antigen-binding domain may comprise an antibody or antibody fragment thereof.

[0130] The term "antibody" is used herein in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, multi-specific antibodies (*e.g.*, bispecific antibodies), and single variable domain antibodies so long as they exhibit the desired biological activity. The term “antibody” includes immunoglobulin molecules comprising four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, as well as multimers thereof (*e.g.*, IgM). Each heavy chain comprises a heavy chain variable region (which may be abbreviated as HCVR or V_H) and a heavy chain constant region. The heavy chain constant region comprises three domains, C_{H1}, C_{H2} and C_{H3}. Each light chain comprises a light chain variable region (which may be

abbreviated as LCVR or V_L) and a light chain constant region. The light chain constant region comprises one domain (C_{L1}). The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. In different embodiments disclosed herein, the FRs of an antibody (or antigen-binding portion thereof) may be identical to the human germline sequences, or may be naturally or artificially modified. An amino acid consensus sequence may be defined based on a side-by-side analysis of two or more CDRs. Included within the scope of the term "antibody" is an antibody of any class, such as IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant region of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant regions that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

[0131] An "antigen-binding fragment" may be provided by means of arrangement of one or more CDRs on non-antibody protein scaffolds. "Protein scaffold" as used herein includes but is not limited to an immunoglobulin (Ig) scaffold, for example an IgG scaffold, which may be a four chain or two chain antibody, or which may comprise only the Fc region of an antibody, or which may comprise one or more constant regions from an antibody, which constant regions may be of human or primate origin, or which may be an artificial chimera of human and primate constant regions. The protein scaffold may be an Ig scaffold, for example an IgG, or IgA scaffold. The IgG scaffold may comprise some or all the domains of an antibody (*i.e.*, CH1, CH2, CH3, V_H, V_L). The antigen binding protein may comprise an IgG scaffold selected from IgG1, IgG2, IgG3, IgG4 or IgG4PE. For example, the scaffold may be IgG1. The scaffold may consist of, or comprise, the Fc region of an antibody, or is a part thereof. Non-limiting examples of antigen-binding fragments include: (i) Fab fragments; (ii) F(ab')2 fragments; (iii) Fd fragments; (iv) Fv fragments; (v) single-chain Fv (scFv) molecules; (vi) dAb fragments; and (vii) minimal recognition units consisting of the

amino acid residues that mimic the hypervariable region of an antibody (*e.g.*, an isolated complementarity determining region (CDR) such as a CDR3 peptide), or a constrained FR3-CDR3-FR4 peptide. Other engineered molecules, such as domain-specific antibodies, single domain antibodies, domain-deleted antibodies, chimeric antibodies, CDR-grafted antibodies, diabodies, triabodies, tetrabodies, minibodies, nanobodies (*e.g.*, monovalent nanobodies, bivalent nanobodies, *etc.*), small modular immunopharmaceuticals (SMIPs), and shark variable IgNAR domains, are also encompassed within the expression "antigen-binding fragment," as used herein. An antigen-binding fragment of an antibody will typically comprise at least one variable domain. The variable domain may be of any size or amino acid composition and will generally comprise at least one CDR which is adjacent to or in frame with one or more framework sequences. In antigen-binding fragments having a V_H domain associated with a V_L domain, the V_H and V_L domains may be situated relative to one another in any suitable arrangement. For example, the variable region may be dimeric and contain V_H-V_H, V_H-V_L or V_L-V_L dimers. Alternatively, the antigen-binding fragment of an antibody may contain a monomeric V_H or V_L domain. In certain embodiments, an antigen-binding fragment of an antibody may contain at least one variable domain covalently linked to at least one constant domain. Non-limiting, exemplary configurations of variable and constant domains that may be found within an antigen-binding fragment of an antibody of the present invention include: (i) V_H-C_{H1}; (ii) V_H-C_{H2}; (iii) V_H-C_{H3}; (iv) V_H-C_{H1}-C_H2; (v) V_H-C_{H1}-C_{H2}-C_{H3}, (vi) V_H-C_{H2}-C_{H3}; (vii) V_H-C_L; (viii) V_L-C_H1; (ix) V_L-C_{H2}, (x) V_L-C_{H3}; (xi) V_L-C_{H1}-C_{H2}; (xii) V_L-C_{H1}-C_{H2}-C_{H3}; (xiii) V_L-C_{H2}-C_{H3}; and (xiv) V_L-C_L. In any configuration of variable and constant domains, including any of the exemplary configurations listed above, the variable and constant domains may be either directly linked to one another or may be linked by a full or partial hinge or linker region. A hinge region may consist of at least 2 (*e.g.*, 5, 10, 15, 20, 40, 60 or more) amino acids which result in a flexible or semi-flexible linkage between adjacent variable and/or constant domains in a single polypeptide molecule. Moreover, an antigen-binding fragment of an antibody may comprise a homo-dimer or hetero-dimer (or other multimer) of any of the variable and constant domain configurations listed above in non-covalent association with one another and/or with one or more monomeric V_H or V_L domain (*e.g.*, by disulfide bond(s)). As with full antibody molecules, antigen-binding fragments may be monospecific or multi-specific (*e.g.*, bispecific). A multi-specific antigen-binding fragment of an antibody will typically comprise at least two

different variable domains, wherein each variable domain is capable of specifically binding to a separate antigen or to a different epitope on the same antigen. Any multi-specific antigen-binding molecule format may be adapted for use in the context of an antigen-binding fragment of an antibody using routine techniques available in the art.

[0132] In an embodiment, the antigen-binding domain comprises an antibody fragment. For example, the antigen-binding domain may comprise a scFv consisting of a VL and VH sequence of a monoclonal antibody (mAb) specific for a tumor cell surface molecule (*i.e.*, tumor antigen).

[0133] In an embodiment, the CAR comprises an antigen binding domain that binds specifically to an antigen selected from the group consisting of CD7, CD19, CD20, CD22, CD30, ROR1, mesothelin, CD33, CD38, CD123 (IL3RA), CD133, CD138, CD171, BCMA (CD269), GPC2, GPC3, FGFR4, c-Met, PSCA, PSMA, Glycolipid F77, Her2, EGFR, EGFRvIII, GD-2, NY-ESO-1 TCR, MAGE A3 TCR, Claudin 6, Claudin 18.2, Lewis Y (LeY), GRP-78, EphA2, CEA, CEACAM5, ROR1, FAP and combinations thereof.

[0134] The terms "T-cell receptor" or "TCR" as used herein mean a recombinant or naturally-occurring heterodimeric polypeptide comprising an alpha polypeptide chain (*i.e.*, alpha chain, α chain) and a beta polypeptide chain (*i.e.*, beta chain, β chain), which is capable of binding to a peptide antigen bound to MHC.

[0135] In an embodiment, the TCR binds specifically to an antigen selected from the group consisting of 707- AP, AFP, ART-4, BAGE, Bcr-abl, CAMEL, CAP-1, CASP-8, CDC27m, CDK4/m, CEA, CT, Cyp-B, DAM, EGFRvIII, ELF2M, ETV6-AML1, G250, GAGE, GnT-V, Gp100, HAGE, HER-2/neu, HLA-A, HPV, HSP70-2M, HST-2, hTERT, hTRT, iCE, KIAA0205, LAGE (L antigen), LDLR/FUT, MAGE, MART-1/Melan-A, MC1 R, Myosin/m, MUC1, MUM-1, MUM -2, MUM -3, NA88-A, NY-ESO-1, P15, p190 minor, Pml/RARa, PRAME, PSA, PSMA, RAGE, RU1, RU2, SAGE, SART-1, SART-3, SSX1, SSX2, SSX3, SSX4, TEL/AML1, TPI/m, TRP-1, TRP-2, TRP-2/INT2 and WT1.

[0136] In another aspect, the present disclosure provides a method of making a cell (*e.g.*, a population of cells), comprising introducing the nucleic acid molecule or vector described herein into a cell.

[0137] The nucleic acid molecule or vector may be introduced into the cell using any method known in the art, illustrative examples of which include transfection, transduction, viral transduction, microinjection, lipofection, nucleofection, nanoparticle bombardment, transformation, conjugation and the like.

[0138] In an embodiment, the method further comprises introducing a nucleic acid molecule or vector encoding a CAR or TCR into the cell.

Pharmaceutical compositions

[0139] In another aspect disclosed herein, there is provided a pharmaceutical composition comprising the cell, or population of cells described herein.

[0140] The term "pharmaceutical composition" as used herein refers to a composition that is in a form that allows the biological activity of the active ingredient (*i.e.*, a cell expressing the fusion protein described herein) to be effective, and that does not contain additional ingredients that have unacceptable toxicity to the subject to which the composition is to be administered.

[0141] In an embodiment, the pharmaceutical composition comprises a population of the cells in sufficient number to administer a dosage of 10^4 to 10^9 cells/kg body weight per dose. Accordingly, the pharmaceutical composition may comprise a population of the cells in sufficient number to administer a dosage of 10^4, 10^5, 10^6, 10^7, 10^8 or 10^9 cells/kg body weight per dose.

[0142] In an embodiment, the pharmaceutical composition comprises a population of the cells in sufficient number to administer a dosage of 10^5 to 10^6 cells/kg body weight per dose, including all integer values within those ranges.

[0143] In some embodiments, periodic re-administration of the pharmaceutical composition may be required to achieve a desirable therapeutic effect. The exact amounts and rates of administration of the pharmaceutical composition will depend on a number of factors, examples of which are described elsewhere herein, such as the subject's age, body weight, general health, sex and dietary requirements, as well as any drugs or agents used in combination or coincidental with the administration of the composition. Where multiple divided doses are required, these may be administered hourly, daily, weekly, monthly or at

other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation. Alternatively, a continuous infusion strategy can be employed.

[0144] In an embodiment, the pharmaceutical composition is suitable for parenteral administration. In another embodiment, the composition is suitable for intravenous administration.

[0145] The pharmaceutical compositions disclosed herein may be prepared according to conventional methods well known in the pharmaceutical industries, such as those described in Remington's Pharmaceutical Handbook (Mack Publishing Co., NY, USA), comprising a therapeutically effective amount of the composition alone, with one or more pharmaceutically acceptable carriers or diluents.

[0146] The term "pharmaceutically acceptable carrier" as used herein means any suitable carriers, diluents or excipients. These include all aqueous and non-aqueous isotonic sterile injection solutions, which may contain anti-oxidants, buffers and solutes to render the composition isotonic with the blood of the intended recipient, aqueous and non-aqueous sterile suspensions, which may include suspending agents and thickening agents, dispersion media, anti-fungal and anti-bacterial agents, isotonic and absorption agents, and the like.

[0147] In an embodiment, the pharmaceutical composition further comprises one or more immune adjuvants.

[0148] The term "immune adjuvant" as used herein refers to a compound or substance that is capable of enhancing a subject's immune response to the immunogen including, for example, the subject's antibody response to the immunogen. An immune adjuvant may therefore assist to enhance the immune response to an engineered T cell in a subject, compared to the administration of the engineered T cell or in the absence of the immune adjuvant.

[0149] Suitable immune adjuvants will be familiar to persons skilled in the art, illustrative examples of which include an inhibitor of the PDL-1: PD-1 axis, a TLR3 agonist, a 4-1BB agonist, a TLR7 agonist, an inhibitor of TIM-3, and an inhibitor of CTLA-4.

[0150] It is further contemplated herein that the pharmaceutical composition may be co-administered with one or more other agents suitable for the treatment or amelioration of symptoms associated with cancer, such as a solid tumor, illustrative examples of which include surgery, chemotherapy (*e.g.*, anastrozole, bicalutamide, bleomycin sulfate, busulfan, busulfan injection, capecitabine, N4-pentoxycarbonyl-5- deoxy-5-fluorocytidine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, cyclophosphamide, cytarabine, cytosine arabinoside, cytarabine liposome injection, dacarbazine, dactinomycin, daunorubicin hydrochloride, daunorubicin citrate liposome injection, dexamethasone, docetaxel, doxorubicin hydrochloride, etoposide, fludarabine phosphate, 5- fluorouracil, flutamide, tezacitibine, gemcitabine, hydroxyurea, idarubicin, ifosfamide, irinotecan, L-asparaginase, leucovorin calcium, melphalan, 6-mercaptopurine, methotrexate, mitoxantrone, mylotarg, paclitaxel, phoenix (Yttrium90/MX-DTPA), pentostatin, polifeprosan 20 with carmustine implant, tamoxifen citrate, teniposide, 6-thioguanine, thiotepa, tirapazamine, topotecan hydrochloride for injection, vinblastine, vincristine, and vinorelbine), radiation, immunosuppressive agents (*e.g.*, cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506), antibodies, or other immunoablative agents (*e.g.*, CAMPATH), targeted agents, steroids, and peptide vaccines.

[0151] Such combinations may be administered simultaneous with the pharmaceutical composition or concurrently with the pharmaceutical composition.

Methods of treatment and associated therapeutic uses

[0152] It is further contemplated that the cell (*e.g.*, a population of cells) and pharmaceutical compositions described herein may be adapted for the treatment of diseases and disorders that are characterized by immunosuppression mediated by TGF-β. For example, it has been exemplified herein that T cells co-expressing the fusion protein and a CAR (*i.e.*, TGFBRII.41BB CAR-T cells) are more effective for the treatment of immunosuppressive tumors where TGF-β levels were increased relative to conventional CAR T cells. On this basis, it is reasonable to expect that the cell (*e.g.*, a population of cells) and the pharmaceutical compositions described herein may be useful in the treatment of cancer.

[0153] Accordingly, in an aspect, the present disclosure provides a method for the treatment of a subject having cancer, the method comprising administering a therapeutically effective amount of the cell (*e.g.*, a population of cells) or the pharmaceutical composition described herein.

[0154] In another aspect, the present disclosure provides the use of the cell (*e.g.*, a population of cells) or the pharmaceutical composition described herein in the manufacture of a medicament for the treatment of cancer.

[0155] In yet another aspect, the present disclosure provides the cell (*e.g.*, a population of cells) or the pharmaceutical composition described herein for use in the treatment of cancer.

[0156] The therapeutic regimen for the treatment of cancer can be determined by a person skilled in the art and will typically depend on factors including, but not limited to, the type, size, stage and receptor status of the tumor in addition to the age, weight and general health of the subject. Another determinative factor may be the risk of developing recurrent disease. For instance, for a subject identified as being at high risk or higher risk or developing recurrent disease, a more aggressive therapeutic regimen may be prescribed as compared to a subject who is deemed at a low or lower risk of developing recurrent disease. Similarly, for a subject identified as having a more advanced stage of cancer, for example, stage III or IV disease, a more aggressive therapeutic regimen may be prescribed as compared to a subject that has a less advanced stage of cancer.

[0157] The term “cancer” as used herein means any condition associated with aberrant cell proliferation. Such conditions will be known to persons skilled in the art. In an embodiment, the cancer is a primary cancer (*e.g.,* a tumor). In another embodiment, the cancer is a metastatic cancer.

[0158] Examples of various cancers are described elsewhere herein and include breast cancer, colorectal cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer, sarcoma and the like. The terms "cancer" and "tumor" may be used interchangeably herein, *e.g.*, encompassing both solid and diffuse or circulating tumors.

[0159] In an embodiment, the cancer is a TGF-β-expressing cancer.

[0160] TGF-β-expressing cancers would be known to persons skilled in the art, illustrative examples of which include breast cancer, melanoma, carcinoid, cervical cancer, ovarian cancer, pancreatic cancer, colorectal cancer, prostate cancer, endometrial cancer, renal cancer, glioma, skin cancer, head and neck cancer, stomach cancer, liver cancer, testis cancer, lung cancer, thyroid cancer, lymphoma and urothelial cancer.

[0161] In an embodiment, the TGF-β-expressing cancer is prostate cancer.

[0162] In an embodiment, the TGF-β-expressing cancer is breast cancer.

[0163] The term "subject" as used herein refers to any mammal, including livestock and other farm animals (such as cattle, goats, sheep, horses, pigs and chickens), performance animals (such as racehorses), companion animals (such as cats and dogs), laboratory test animals and humans. In an embodiment, the subject is a human. In an embodiment, the subject is an adult. In another embodiment, the subject is a child.

[0164] As used herein, the term "effective amount" typically refers to an amount of the cell, or population of cells, or pharmaceutical composition described herein that is sufficient to affect one or more beneficial or desired therapeutic outcomes (*e.g.*, reduction in tumor size). Said beneficial or desired therapeutic outcomes may be measured using clinical techniques known in the art, illustrative examples of which include the measurement of imaging biomarkers, tumor size (*e.g.*, as measured by anatomical imaging modalities, such as CT or MRI), quantification of the presence of inflammatory mediators (*e.g.*, Interleukin-1, TNF, TGF-β, *etc.*). An "effective amount" can be provided in one or more administrations. The exact amount required may vary depending on factors such as the nature and severity of the cancer to be treated, and the age and general health of the subject.

[0165] The terms "treat", "treating", "treatment" and the like are used interchangeably herein to mean relieving, reducing, alleviating, ameliorating or otherwise inhibiting the severity and/or progression of cancer, or a symptom thereof, in a subject. It is to be understood that the terms "treat", "treating", "treatment" and the like, as used herein, do not imply that a subject is treated until clinical symptoms of cancer have been eliminated or are no longer evident (*e.g.*, elimination of solid tumor mass and associated metastatic lesions, if

any). Said treatment may also reduce the severity of cancer by preventing progression or alleviating the symptoms associated with cancer.

[0166] The terms "prevent", "preventing", "prevention" and the like are used interchangeably herein to mean inhibit, hinder, retard, reduce or otherwise delay the development of cancer and/or progression of cancer, or a symptom thereof, in a subject. In the context of the present disclosure, the term "prevent" and variations thereof does not necessarily imply the complete prevention of the specified event. Rather, the prevention may be to an extent, and/or for a time, sufficient to produce the desired effect. Prevention may be inhibition, retardation, reduction or otherwise hindrance of the event, activity or function. Such preventative effects may be in magnitude and/or be temporal in nature.

[0167] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

[0168] It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the present disclosure without departing from the spirit or scope of the disclosure as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

[0169] The present disclosure will now be further described in greater detail by reference to the following specific examples, which should not be construed as in any way limiting the scope of the disclosure.

Examples

General methods

Constructs

[0170] The anti-LeY scFv-CD3ζ-CD28 CAR (LeY CAR) construct was used in this study as described by Williford *et al.* (2019, *Scientific Advances*, 5(12): p.eaay1357). The DNA fragment containing a T2A self-cleaving peptide, the human TGF-β receptor 2

extracellular and transmembrane domains and the 4-1BB intracellular domain fusion protein (*e.g.*, SEQ ID NOs: 17-19) was synthesized by Genscript (Piscataway, NJ, USA). The DNA fragment was then cloned into the pSAMEN retroviral vector containing the LeY CAR (*e.g.*, SEQ ID NOs: 32-34).

Luciferase reporter assay

[0171] HEK293 cells were transduced with retrovirus to express the TGFBRII.41BB fusion protein. Transduced cells were sorted and subsequently transfected with pGL4.73[hRluc/SV40] (E6911, Promega, Madison, WI, USA) and pGL4.32[luc2P/NF-κB-RE/Hygro] (E8491, Promega) by Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Transfected cells were then treated with either plain media or media containing 100 ng/ml TGF-β (Peprotech, Cranbury, NJ, USA) overnight. The cells were lysed and assayed by Dual-Luciferase® Reporter Assay System (E1910, Promega) on Cytation 5 (Bioteck, Winooski, VT, USA).

CAR-T cell production

[0172] Retroviral packaging cell line PG13-LeY-CAR and PG13-LeY-TGFBRII.41BB CAR was prepared and used in this study as described by Williford *et al.* (2019, *supra*). Packaging cells were cultured with RPMI 1640 (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS to produce retrovirus-containing media for transduction. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy coats (Australian Red Cross Blood Service) by density gradient centrifugation (Ficoll-Paque, GE Healthcare Life Science). Healthy donor packs were accessed from the ARCBS *via* the Peter Mac HREC approval number 01-14.

Cytotoxicity assay

[0173] Chromium release assay was performed as described by Davenport *et al.* (2015, *Cancer Immunology Research*, 3(5): 483-494). Briefly, target cells (LeY+ OVCAR-3 and HER2+ MB-468) were labeled with 100 μCi Chromium-51 (51Cr, PerkinElmer) for one hour at 37°C and subsequently co-cultured with conventional or TGFBRII.41BB CAR-T cells or empty vector T cells in triplicate wells at an effector:target ratio of 2:1 with or without 10 ng/ml human TGF-β. Wells with targets alone (spontaneous release) and targets

with 10% Triton X 100 (maximum release) were included as controls. After 16 hours, supernatants were collected, and the amount of 51Cr released detected using a gamma counter (Wallac Wizard 1470). The % specific lysis was calculated by [(experimental release – spontaneous release)/(maximum release – spontaneous release)] * 100.

AlphaLISA cytokine assay

[0174] Conventional and TGFBRII.41BB CAR-T cells were co-cultured with the LeY$^+$ cell line OVCAR-3 with 10 ng/ml human TGF-β, and culture supernatant was harvested after 16 hours. The cytokine secretion was assessed by AlphaLISA Detection Kit (PerkinElmer, Waltham, MA, USA) for indicated cytokines according to the manufacturer's instructions. The plates were read on Cytation 5 for cytokine levels as pg/mL.

CAR-T cell proliferation assay

[0175] Conventional and TGFBRII.41BB CAR-T cells were labelled with CellTrace™ Violet (CTV, Molecular Probes, Thermo Fischer Scientific) in PBS at a final concentration of 1.25 μM for 15 min at 37°C. Cells were then washed with growth media. 1x10^5 CAR-T cells were incubated with plate-bound anti-idiotype antibody or OKT3 for five days in media supplemented with IL-2 (100 UI/mL). Proliferating cells were harvested, and CTV dilution of CAR-T cells was assessed by flow cytometry.

Flow cytometry (FACS) analysis

[0176] For all surface staining, cells were first labelled with LIVE/DEAD Near-IR (Thermo Fisher Scientific) in PBS for 10 min at 4°C and washed with FACS buffer (PBS with 2% FCS). Before staining, cells were pre-incubated with human Fc block (BD Biosciences) for 10 min at 4°C. Cells were then stained with antibodies against surface markers in FACS buffer for 30 min at 4°C, washed with FACS buffer and subsequently re-suspended in FACS buffer before acquisition on a Fortessa (Becton Dickinson, New Jersey, USA, BD Biosciences).

[0177] For the staining on phosphorylation markers, the conventional and TGFBRII.41BB CAR-T cells were incubated with or without 100 ng/ml human TGF-β for 0 – 30 minutes as indicated. The pre-warmed fixation buffer (BioLegend, #420801) was

added to the cell culture at the time points indicated. After 15 minutes incubation at 37°C, the cells were washed with FACS buffer and re-suspended in pre-chilled True-Phos Perm Buffer (BioLegend, #425401) and kept at -20°C for 2 hours. After a wash, the cells were stained with the antibodies for the phosphorylation markers according to the manufacturer's instructions.

[0178] The antibodies used in the FACS staining were CD3-BUV395 (BD, #564001), Flag-tag-PerCP/Cy5.5 (BioLegend, #637325), Flag-tag-PE (BioLegend, #637310), Myc-tag-PE(Cell Signaling Technology, #2233S), pERK1/2-PE/Cy7 (BioLegend, #369515), pNFkB-PE (Cell Signaling Technology, #5733S), pSMAD2-AF488(Cell Signaling Technology, #56532S), pMAPKp83-PE (BioLegend, #690203).

Mitochondria staining

[0179] Conventional and TGFBRII.41BB CAR-T cells were labelled with MitoTracker™ Deep Red FM Dye (Thermo Fischer Scientific, #M46753) in growth media at a final concentration of 20 nM for 20 min at 37°C. After a wash with growth media, the cells were processed for subsequent FACS staining.

RNA-seq analysis

[0180] RNA was extracted from 2×10^5 cells using RNeasy mini kit (Qiagen, Netherlands) according to the manufacturer's instructions. The quality of the RNA was assessed by TapeStation (Agilent, California, USA). RNA-seq library was generated using QuantSeq 3' mRNA-Seq Library Prep FWD Kit (Lexogen, Vienna, Austria) as per manufacturer's instruction. The sequencing was run by NextSeq HO 75SE (Illumina, California, USA).

[0181] Raw sequence reads were quality-controlled using FastQC module (version 0.11.8) and undergone trimming using Cutadapt (version 3.4) in the Galaxy platform. The single-end reads were aligned to the human reference genome (hg19) using HISAT2 (version 2.2.1, Galaxy) and quantified using HTSeq. Gene normalization was performed by edge R package (version 3.35.0) with library size adjustment by Trimmed Mean of M-values method (Robinson & Oshlack, 2010, *Genome Biology*, 11(3): R25). Differential expression analysis (DEG) was performed using Limma-Voom workflow (limma version 3.46.0).

Genes with consistent extreme-low count (less than 2 CPM) across 85% samples were discarded to reduce the noise in the downstream analysis.

In vivo analysis

[0182] NSG (NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJl) mice aged between 6-8 weeks were injected s.c. on the right flank with 1×10^6 DU-145 cells in 100 µl of PBS on Day 14. When the tumors were ~20-25 mm^2 in size (Day 1), the mice were irradiated by 0.5 Gy. Mice received three doses of 1×10^7 conventional or TGFBRII.BB CAR-T cells i.v. on Days 1, 2, and 3 with cytokine support (IL-2, 50 000 UI/dose). Tumor size was measured by using calipers and presented as the product of perpendicular diameters. On Day 17, mice were bled for blood sampling to investigate the persistence of $CD3^+$ human T cells in the peripheral blood. Tumors were also collected from a group of three mice per treatment to assess the TGF-β and IFN-γ level of expression in the tumor by AlphaLISA, and the T cell infiltration in the tumor by immunohistochemistry (IHC). Mice were euthanized according to the criteria or at Day 50. All animal experiments were performed in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals. All protocols were approved by the Peter Mac Callum Animal Ethics and Experimentation Committee (Ethics number: E647).

Immunohistochemistry (IHC)

[0183] Formalin fixed paraffin embedded (FFPE) slides were dewaxed in xylene and antigens were retrieved at 125°C for 10 minutes in sodium citrate pH6 buffer (for CD3) or Tris EDTA pH9 buffer (for PD-L1). Endogenous peroxidases were inactivated with 3% H_2O_2 (Merck), and the slides were blocked in PKI blocking buffer (Akoya Biosciences). Primary CD3 antibody (clone SP7, Abcam, # ab16669) or PD-L1 antibody (clone SP142, Abcam, #ab228462) was incubated 30 minutes at room temperature, and the secondary anti-mouse ImmPress (Vector Laboratories, MP-7402) or anti-rabbit ImmPress (Vector Laboratories, MP-7401) was added for 30 minutes at RT, followed by Dako Liquid DAB for 15 minutes. The slides were then counter-stained by Jung Autostainer (Leica) and scanned using the Olympus VS120 microscope at 20× magnification.

Statistical analysis

[0184] Statistical analyses were performed in Prism v9 software (GraphPad). A p-value ≤ 0.05 was considered statically significant. Data analyses were conducted using unpaired Student's t-test to compare two data sets of data. Data presented as mean ± standard error of the mean (SEM). All statistical calculations for RNA-seq were performed in R software (version 4.1.0). Multiple comparison was used in RNA-seq analysis, and a Benjamini-Hochberg adjusted p-value < 0.05 was considered as statistically significant.

Example 1

The TGFBRII.BB fusion protein enhances CAR-T cell cytotoxicity in the presence of TGF-β

[0185] The TGFBRII.41BB fusion protein was designed by fusing a truncated TGFBRII comprising the extracellular and transmembrane domain of TGFBRII with the intracellular domain of 4-1BB. A Myc-tag was added to the N-terminal of the TGFBRII.41BB fusion protein. By using a T_2A self-cleaving peptide, the TGFBRII.41BB fusion protein was engineered to co-express a CAR and TGFBRII.41BB fusion protein in T cells (Figure 1A). Using flow cytometry, the T cells were shown to constitutively co-express the CAR and TGFBRII.41BB fusion protein as indicated by Flag-tag and Myc-Tag (Figure 1B). To confirm the function of the TGFBRII.41BB fusion protein, a NF-κB driven luciferase reporter was introduced to TGFBRII expressing HEK293 cells. Thereafter, signaling derived from the TGFBRII.41BB fusion protein was assessed by treating the reporter HEK293 cells with external TGF-β. The results indicated that luciferase activity significantly increased when the cells were treated with TGF-β, indicating that the TGFBRII.41BB fusion protein was activated by TGF-β and subsequently induced NF-κB signaling (Figure 1C). To assess the effect of the fusion protein on CAR-T cell function, the TGFBRII.41BB fusion protein was assembled with a LeY-CAR, which targets the Lewis Y antigen on tumor cells. LeY-CAR-T cells lysed LeY^+ DU-145 prostate cancer cells. However, when exogenous TGF-β was added to the co-culture of DU-145 with CAR-T cells, the % lysis of DU-145 cells was significantly decreased. In contrast, in the presence of the TGFBRII.41BB LeY-CAR-T cells, TGF-β induced a significant increase in % lysis of DU145 cells, further indicating that the TGFBRII.41BB fusion protein was activated by

TGF-β and subsequently enhanced T cell killing of the tumor cells (Figure 1D). In the presence of TGF-β, the cytokine production by conventional and TGFBRII.41BB LeY-CAR-T cells also differed. After 16-hours co-culture in the presence or absence of TGF-β, the conventional CAR-T cells produced more IFN-γ, whereas the TGFBRII.41BB CAR-T cells produced more TNF-α (Figures 1E and 1F).

Example 2

The TGFBRII.41BB fusion protein improves CAR-T cell proliferation and altered mitochondrial biogenesis while retaining endogenous TGF-β signaling

[0186] To further investigate the TGFBRII.41BB LeY-CAR-T cells response to TGF-β, a proliferation assay using CAR stimulation (anti-idiotype antibody) was performed in the presence or absence of TGF-β. In the presence of TGF-β significantly more TGFBRII.41BB LeY-CAR-T cells divided compared to conventional CAR-T cells divided (Figure 2A-B). Further, proliferating TGFBRII.41BB CAR-T cells had more cell divisions in the presence of TGF-β than conventional CAR-T cells (Figure 2C). As the 4-1BB signaling is reported to be involved in the mitochondrial metabolism (see, *e.g.*, Menk *et al.*, 2018, *Journal of Experimental Medicine*, 215(4): 1091-1100), we used MitoTracker to assess the effect of TGF-β on mitochondrial capacity in both conventional and TGFBRII.41BB CAR-T cells. These result suggest that when the CAR-T cells were treated with TGF-β, mitochondrial mass was significantly increased in TGFBRII.41BB CAR-T cells but not conventional CAR-T cells (Figure 2D-E).

[0187] To further identify the signaling downstream of the TGFBRII.41BB fusion protein upon TGF-β activation, flow cytometry was performed to analyze phosphorylation of the key molecules downstream of 4-1BB signaling. Phosphorylation of MAPK, but not NF-κB or ERK, was significantly increased upon the treatment of TGF-β in TGFBRII.41BB CAR-T cells (Figure 2F). Because the TGFBRII.41BB fusion protein was constitutively expressed on the CAR-T cells, competition of the TGFBRII.41BB fusion protein for TGF-β binding with the endogenous TGF-β receptor was assessed. To do this, endogenous TGF-β signaling was assessed by SMAD2 phosphorylation using flow cytometry. Compared with conventional CAR-T cells, treating TGFBRII.41BB CAR-T cells with exogenous TGF-β, slightly decreased the MFI of phosphorylated SMAD2 but the response of endogenous TGF-

β signaling was maintained (Figure 2G). Taken together, these data demonstrate that the TGFBRII.41BB fusion protein improved CAR-T cell proliferation and increased mitochondrial mass in the presence of TGF-β, while retaining endogenous TGF-β signaling. Accordingly, endogenous TGF-β signaling was not abolished by the TGFBRII.41BB fusion protein, rather that endogenous TGF-β signaling is ongoing in these CAR-T cells, which is critical for the homeostasis of the TGFBRII.41BB CAR-T cells.

Example 3

TGF-β induces a different response in TGFBRII.41BB CAR-T cells

[0188] To further investigate the dynamics between endogenous TGF-β signaling and the TGFBRII.41BB fusion protein signaling, bulk RNA-seq was performed using TGF-β treated conventional and TGFBRII.41BB CAR-T cells. The expression (*i.e.*, transcription level) of endogenous TGF-β receptors was assessed by using the normalized RNA-seq data (Figure 6). Besides the functional response, it was also confirmed the conventional CAR-T cell response to TGF- β at the transcriptional level, where TGF-β significantly repressed the expression of genes associated with T cell activation, migration, and cytotoxicity (Figure 7).

[0189] The gene expression profile of TGFBRII.41BB CAR-T cells versus conventional CAR-T cells in the presence of TGF-β. 243 DEGs were identified including 104 upregulated genes and 139 downregulated genes (Figures 3A and 8). Genes associated with T cell activation and cytotoxicity were highly expressed in TGFBRII.41BB CAR-T cells treated with TGF-β, including *IFNG, SLAMF7*, *EOMES*, *PRF and GZMA* (Figure 3B). Similarly, other genes associated with T cell effector function showed a trend to increased expression, such as *GZMB* and *TBX21* (Figure 3B). Interestingly, genes associated with immune memory formation, proliferation, and survival (*e.g.*, *BCL2, LEF1* and *MYC*) showed a minor upregulation in TGFBRII.41BB CAR-T cells (Figure 9C). In contrast, conventional CAR-T cells exhibited high-level expression of genes associated with T cell dysfunction (*TOX2* and *NDFIP1*). In line with this result, immunosuppressive pathway genes also showed a trend to enrichment in the conventional CAR-T cells including SMAD family genes (*SMAD4* and *SMAD2)*, and *PDCD1* (Figure 3B).

[0190] TGFBRII.41BB CAR-T cells also showed a distinct profile of T cell chemotaxis, including *CCR1*, *CCR5*, *CCL5*, *IL16*, *ITGB7* and *ITGA1* (Figure 3D). The chemokine

receptor gene, *CXCR3*, known to drive the migration and trafficking of cytotoxic T cells to solid tumors, also displayed significant upregulation in switch CAR-T cells. In contrast, conventional CAR-T cells were characterized by high expression of *CXCL13*, *CCL20* and *CXCR4* (Figure 3D). GO analysis was also performed to further investigate the molecular pathways involved in the TGF-β induced transformation of TGFBRII.41BB CAR-T cells. These data demonstrated that TGFBRII.41BB CAR-T cells were characterized by gene sets associated with the T cell activation and cytolysis (Figure 3E). Pathways associated with T cell migration and trafficking (*e.g.*, chemotaxis, chemokine- and integrin-mediated signaling pathway) were also upregulated by TGF-β in TGFBRII.41BB CAR-T cells versus the conventional counterparts.

[0191] Collectively, these data demonstrate that TGF-β provokes a different transcriptional response in TGFBRII.41BB CAR-T cells versus conventional CAR-T cells. TGFBRII.41BB CAR-T cells were also resistant to TGF-β induced immunosuppressive signals, with enhanced activation signals and acquired an altered chemotaxis profile.

Example 4

TGFBRII.41BB fusion protein converts inhibitory signals to unique activation signals

[0192] To further characterize the mechanism whereby the TGFBRII.41BB fusion protein drives activation in TGFBRII.41BB CAR-T cells, the differentially expressed genes of TGFBRII.41BB CAR-T cells versus conventional CAR-T cells were compared following CAR stimulation in the presence or absence of TGF-β. There were 115 differentially expressed genes, wherein 58 genes were also found in the conventional CAR-T cells and 75 genes were uniquely upregulated only in TGFBRII.41BB CAR-T cells (Figures 4A and 10). All the 58 upregulated genes in conventional CAR-T cells were also found in TGFBRII.41BB CAR-T cells, indicating the TGFBRII.41BB fusion protein did not completely compromise the endogenous TGF-β signaling, consistent with the *in vitro* data presented above (Figure 2E). Among the unique upregulated genes, TGFBRII.41BB CAR-T cells increased expression of genes associated with T cell activation (*TNFSF4*, *CAMK4* and *NFATC2),* chemotaxis (*EPHA1* and *ITGB7*) and immune synapse formation (*PAK1* and *MYH9*) (Figures 4B and 4C). Moreover, TGFBRII.41BB CAR-T cells were also characterized by increased *SLC3A2* and *SLC7A5*, which encode the amino-acid transporters

required for proliferation and effector differentiation in T cells (see, *e.g.*, Sinclair *et al.*, 2013, *Nature Immunology*, 14(5): 500-508).

[0193] To further investigate whether the TGFBRII.41BB fusion protein could tip the net balance from a TGF-β mediated inhibitory signal to activation signaling in TGFBRII.41BB CAR-T cells, the uniquely downregulated differentially expressed genes in the conventional CAR-T cells were explored. There were 101 differentially expressed genes, wherein 32 genes were shared between conventional and TGFBRII.41BB CAR-T cells, and 43 genes were uniquely downregulated in conventional CAR-T cells (Figures 4D and 11). Accordingly, conventional CAR-T cells were characterized by unique downregulation of genes associated with T cell activation and expansion, including *CD40LG*, *DPP4*, and *CORO1A* (Figure 4E). The most prominent change was found with *SELL*, a central regulator of T cell trafficking to the lymph nodes (LogFC = -1.37, BH-adjusted p-value = 0.012) and *CCL4*, which has been reported as an essential chemokine to recruit $CD103^+$ dendritic cell trafficking to the solid tumor (Williford *et al.*, 2019, *supra*), also displayed a moderate decrease (Figure 4F).

Example 5

TGFBRII.41BB CAR-T cells have enhanced anti-tumor activity *in vivo* in a prostate cancer model

[0194] In the DU-145 prostate tumor model, established DU-145 tumors develop resistance to CAR-T cell therapy and also have high level of TGF-β *in vivo*. NSG mice were engrafted with DU-145 tumor cells and treated with three doses of CAR-T cells, supported with human IL-2 (Figure 5A). The TGFBRII.41BB CAR-T cells showed significantly improved tumor control up to day 30 post-infusion and better mouse survival, whereas conventional CAR-T cells showed no significant difference compared with the control treated mice (Figures 5B and 5C). To explore the *in vivo* mechanism further, the persistence of the CAR-T cells was assessed. There was no significant difference in the % of conventional and TGFBRII.41BB CAR-T cells in the peripheral blood 17 days after infusion (Figure 5D). Tumors were also taken on day 17, demonstrating that the TGF-β level was slightly reduced, whereas the IFN-γ level was increased in TGFBRII.41BB CAR-T cell treated tumors (Figures 5E and 5F).

Example 6

Fusion proteins comprising different intracellular domains enhance CAR-T cell cytotoxicity in the presence of TGF-β

[0195] Additional fusion proteins were produced comprising a range of different intracellular domains, specifically TGFBRII.OX40 (SEQ ID NO: 14), TGFBRII.ICOS (SEQ ID NO: 15) and TGFBRII.CD40 (SEQ ID NO: 16). CAR-T cells were engineered to express each of these fusion proteins, using the method described in Example 1.

[0196] To assess the cytotoxicity of these CAR-T cells, TGFBRII.ICOS, TGFBRII.CD40, TGFBRII.OX40 and TGFBRII.41BB CAR-T cells were co-cultured with the prostate cancer cell line, DU-145, in the presence of exogenous TGF-β. As shown in Figure 13, CAR-T cells expressing these fusion proteins significantly increased percentage lysis of tumor cells, indicating that the fusion proteins were activated by TGF-β and subsequently enhanced CAR-T cell-mediated killing of the tumor cells (Figure 13A). In the presence of TGF-β, the cytokine production by conventional and fusion protein-expressing CAR-T cells also differed, where fusion protein-expressing CAR-T cells significantly increased production of TNF-α (Figure 13B).

Example 7

Homodimer formation by fusion proteins

[0197] Fusion proteins with different length of intracellular non-kinase region (SIS: residues 1 to 199 of TGFBRII, SEQ ID NO: 1; MIS: residues 1 to 220 of TGFBRII, SEQ ID NO: 1; and LIS: residues 1 to 243 of TGFBRII, SEQ ID NO: 1) were tagged with either eGFP or mCherry and co-transfected in pairs into HEK293 cells to assess their homodimerization. Heterodimerization was examined by co-transfection of $R2^{WT\text{-}eGFP}$ (wild-type TGFBRII tagged with eGFP) along with fusion proteins tagged mCherry. The protein interaction was detected by the concomitant decrease in GFP signal and increase in FRET signal. Only SIS receptor (residues 1 to 199 of TGFBRII, SEQ ID NO: 1) showed significantly enhanced homodimerization, whereas MIS and LIS receptors showed equivalent level of heterodimerzation and homodimerization (Figure 14). These data demonstrate that the structure (length of the intracellular non-kinase region) is pivotal to the homodimerization of the fusion protein, and successful homodimerization of the SIS

construct results in signal transduction through the dimerized fusion protein receptors (*e.g.*, TGFBRII.41BB).

Example 8

TGFBRII.41BB CAR-T cells have enhanced anti-tumor activity *in vivo* in a breast cancer model

[0198] In the MDA-MB468 breast cancer model, established MDA-MB468 tumors develop resistance to CAR-T cell therapy due to high level of TGF-β *in vivo*. NSG mice were engrafted with MDA-MB-468 tumor cells and treated with three doses of CAR-T cells, supported with human IL-2. TGFBRII.41BB CAR-T cells showed significantly improved tumor control up to day 30 post-infusion and better survival relative to treatment with conventional CAR-T cells, which showed no significant difference compared with the control treated mice (Figure 15).

Conclusion

[0199] CAR-T cells have performed poorly to date in clinical trials treating patients with solid tumors. A key factor in this negative outcome is the immune suppression induced by soluble factors within the tumor microenvironment including TGF-β. TGF-β has a profound direct effect on T cell effector function, proliferation and homeostasis, and also on $CD4^+$ T cell and myeloid cell functional polarization to a suppressor phenotype. In addition, TGF-β inhibits CAR-T cell effector function, proliferation and persistence. This inhibitory effect is more profound in CAR-T cells expressing the 2^{nd} generation CAR with CD3z-41BB than the CD3z-CD28 signaling domains (see, *e.g.*, Koehler *et al.*, 2007, *Cancer Research*, 67(5): 2265-2273). The pleiotropic effects of TGF-β on immune cells types indicate this is a key target for inhibiting immune suppression in the solid tumor microenvironment.

[0200] As described herein, this issue has been addressed by engineering a novel fusion protein comprising a truncated TGFRBII and one or more intracellular co-stimulatory domains. In the context of exogenous TGF-β, the TGFBRII.41BB fusion protein described herein transduced an activation signal *via* the 4-1BB intracellular co-stimulatory domain. This led to increased proliferation, cytotoxicity and cytokine secretion *in vitro* and also improved control over established prostate and breast tumors *in vivo*. The TGFBRII.41BB

fusion protein induced a unique transcriptional profile in the LeY-CAR-T cells, which was distinct from that generated by the endogenous TGF-βRII. The TGFBRII.41BB CAR-T cells described herein express both the TGFBRII.41BB fusion protein and the endogenous TGF-β receptor. The data presented herein demonstrate that the TGFBRII.41BB fusion protein is dominant, and actively signals in the context of a target antigen expressing tumor cell, *i.e.*, the tumor microenvironment. However, the signaling from the TGFBRII.41BB fusion protein did not interfere with that of the endogenous TGF-β receptor, which should be sufficient to avoid the serious toxicity that has previously been associated with approaches for inhibiting the immunosuppressive function of TGF-β.

[0201] Similarly, the TGFBRII.ICOS, TGFBRII.CD40 and TGFBRII.OX40 fusion proteins transduced an activation signal *via* their respective intracellular domains. This led to increased proliferation, cytotoxicity and cytokine secretion *in vitro*. Moreover, signaling from the TGFBRII.ICOS, TGFBRII.CD40 or TGFBRII.OX40 fusion proteins did not interfere with that of the endogenous TGF-β receptor, which should be sufficient to avoid the serious toxicity that has previously been associated with approaches for inhibiting the immunosuppressive function of TGF-β.

[0202] Taken together, these data enable the design, selection and use of novel fusion proteins comprising a truncated TGFRBII and one or more intracellular co-stimulatory domains. In the context of CAR-T cells, the co-expression of such fusion proteins with a CAR significantly improved function in the presence of exogenous TGF-β, and controlled TGF-β expressing prostate tumors. Importantly, the signaling mediated by the fusion protein is distinct from that of the endogenous TGF-β receptor, which results in increased cytotoxic function, cytokine secretion and proliferation of immune effector cells (*e.g.*, T cells, NK cells, macrophages). These functional features are associated with clinical response and reduced negative side effects, which underscores the need for translation to the clinic.

[0203] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or

indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A fusion protein comprising:
   a. a truncated transforming growth factor beta receptor 2 (TGFBRII) comprising at least residues 1 to 187 of TGFBRII (SEQ ID NO: 1), wherein the truncated TGFBRII lacks a kinase domain; and
   b. one or more intracellular co-stimulatory domains.

2. The fusion protein of claim 1, wherein the truncated TGFBRII comprises at least residues 1 to 199 of TGFBRII (SEQ ID NO: 1).

3. The fusion protein of claim 1 or claim 2, wherein the truncated TGFBRII comprises at least residues 1 to 220 of TGFRBII (SEQ ID NO: 1).

4. The fusion protein of any one of claims 1 to 3, wherein the truncated TGFBRII comprises at least residues 1 to 243 of TGFRBII (SEQ ID NO: 1).

5. The fusion protein of claim 1, which comprises the truncated TGFBRII of any one of the amino acid sequences of SEQ ID NOs: 8-10, or an amino acid sequence having 90% sequence identity thereto.

6. The fusion protein of any one of claims 1 to 5, wherein the intracellular co-stimulatory domain is a functional signalling domain of a protein selected from the group consisting of an MHC class I molecule, TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signalling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, 4-1BB (CD137), B7-H3, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, ITGB7, NKG2D, NKG2C, TN1-R2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229),

CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83, or a functional variant thereof.

7. The fusion protein of claim 6, wherein the intracellular co-stimulatory domain is a functional signalling domain of a protein selected from the group consisting of OX40, CD40, 4-1BB and ICOS.

8. The fusion protein of claim 1, which comprises, consists, or consists essentially of any one of the amino acid sequences of SEQ ID NOs: 11-16, or an amino acid sequence having 90% sequence identity thereto.

9. A nucleic acid molecule encoding the fusion protein of any one of claims 1 to 8.

10. A vector comprising the nucleic acid molecule of claim 9.

11. The vector of claim 10, which is a plasmid or viral vector.

12. A cell comprising the nucleic acid molecule of claim 9, or the vector of claim 11 or claim 12.

13. The cell of claim 12, which is a human cell.

14. The cell of claim 12 or claim 13, which is an immune effector cell.

15. The cell of claim 14, which is a T cell.

16. The cell of any one of claims 12 to 15, wherein the cell has been engineered to express a chimeric antigen receptor (CAR) or T-cell receptor (TCR).

17. The cell of claim 16, wherein the CAR comprises an antigen binding domain that binds specifically to an antigen selected from the group consisting of CD7, CD19, CD20, CD22, CD30, ROR1, mesothelin, CD33, CD38, CD123 (IL3RA), CD133, CD138, CD171, BCMA (CD269), GPC2, GPC3, FGFR4, c-Met, PSCA, PSMA, Glycolipid F77, Her2, EGFR, EGFRvIII, GD-2, NY-ESO-1 TCR, MAGE A3 TCR, Claudin 6, Claudin 18.2, Lewis Y (LeY), GRP-78, EphA2, CEA, CEACAM5, ROR1, FAP and combinations thereof.

18. A pharmaceutical composition comprising the cell of any one of claims 12 to 17.

20. A method for the treatment of a subject having cancer, the method comprising administering to the subject an effective amount of the cell of any one of claims 12 to 17, or the pharmaceutical composition of claim 18.

21. The cell of any one of claims 12 to 17, or the pharmaceutical composition of claim 18 for use in the treatment of cancer.

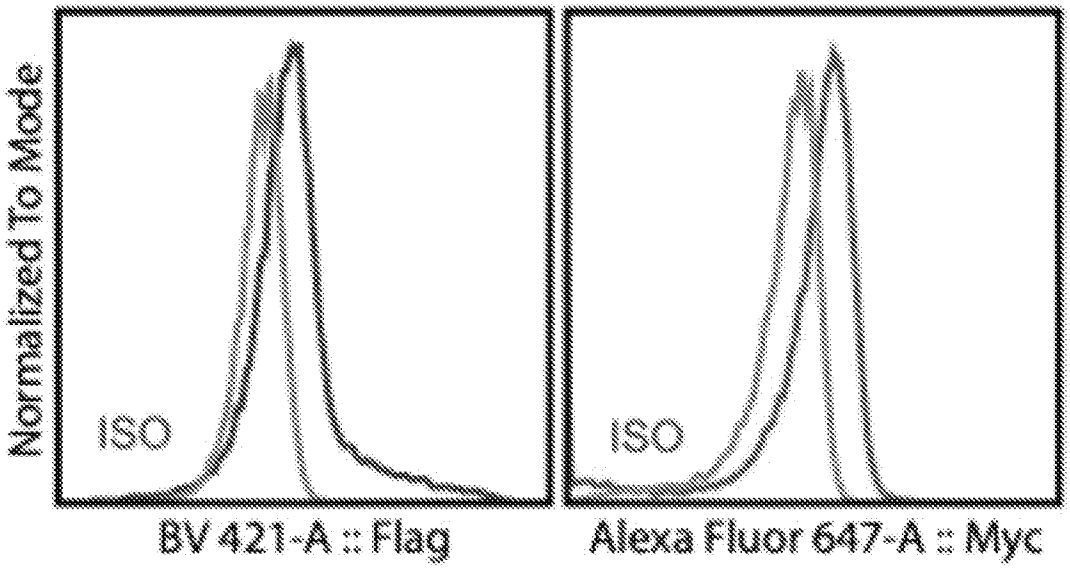
22. Use of the cell of any one of claims 12 to 17, or the pharmaceutical composition of claim 18, in the manufacture of a medicament for the treatment of cancer.

FIGURE 1

A.


scFv
CD8 Hinge
CD28 TM/ICD
CD3z
T2A
TGFBRII
4-1BB ICD
Flag-tag
Myc-tag

B.


Normalized To Mode
ISO
ISO
BV 421-A :: Flag
Alexa Fluor 647-A :: Myc

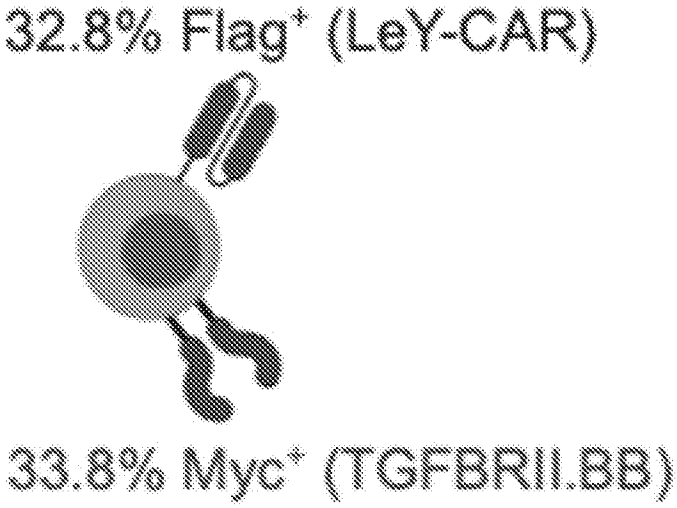

32.8% Flag+ (LeY-CAR)
33.8% Myc+ (TGFBRII.BB)

FIGURE 1 (continued)

C.

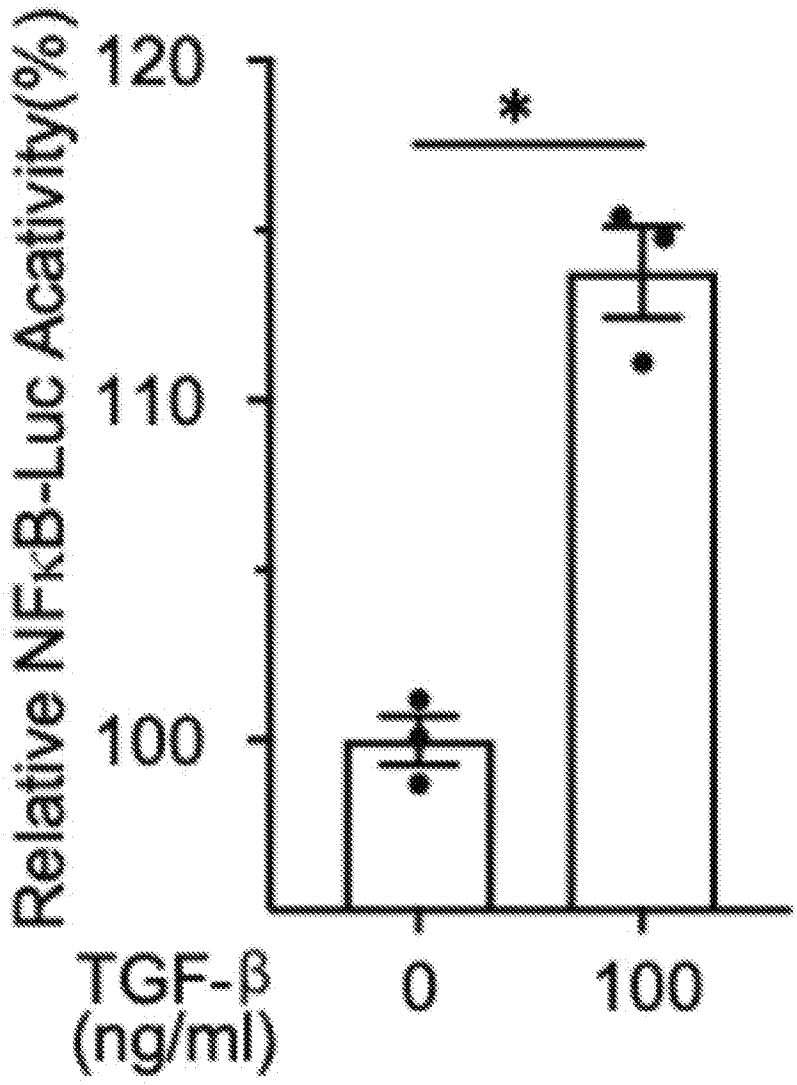


FIGURE 1 (continued)

D.

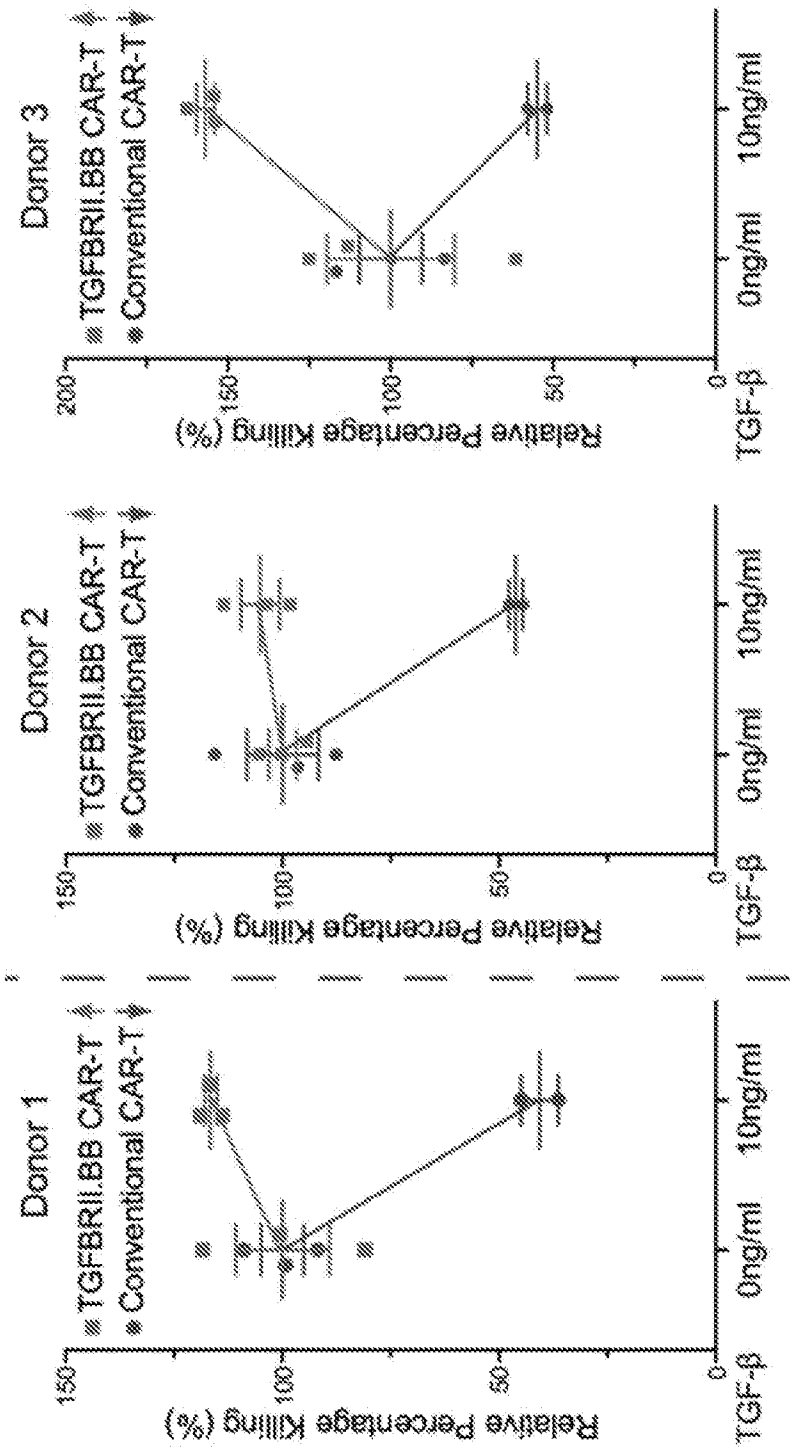


FIGURE 1 (continued)

E.

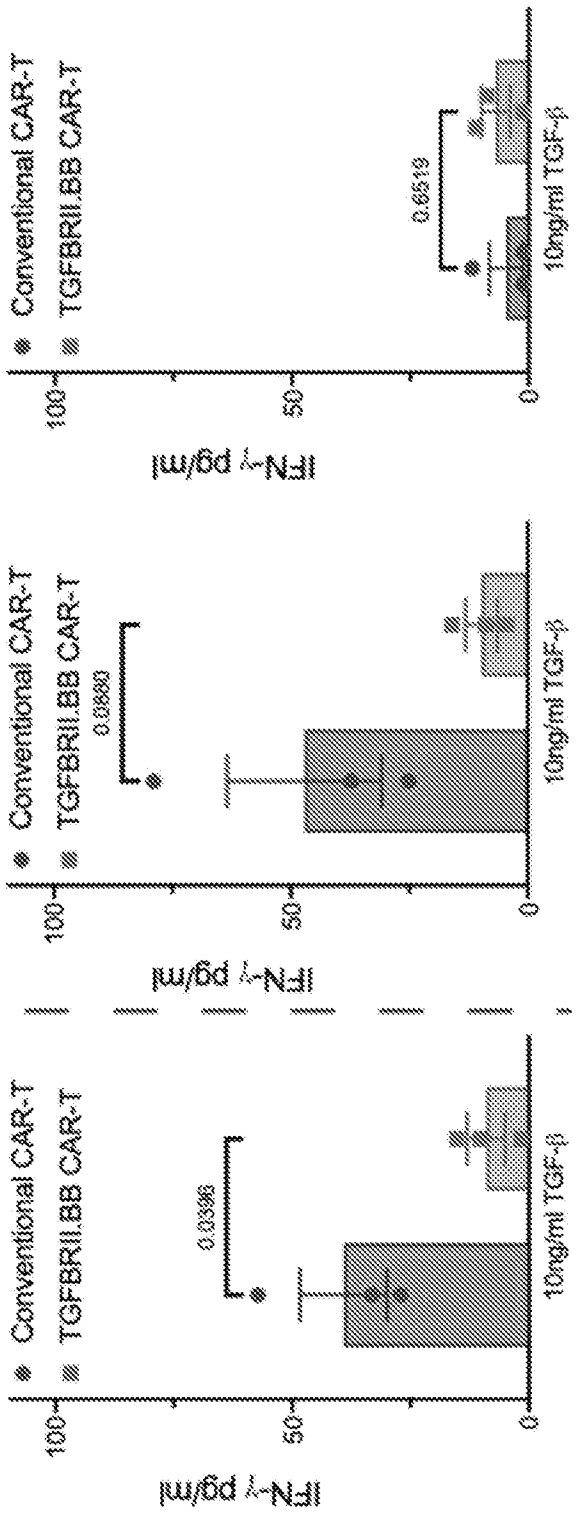


FIGURE 1 (continued)

F.

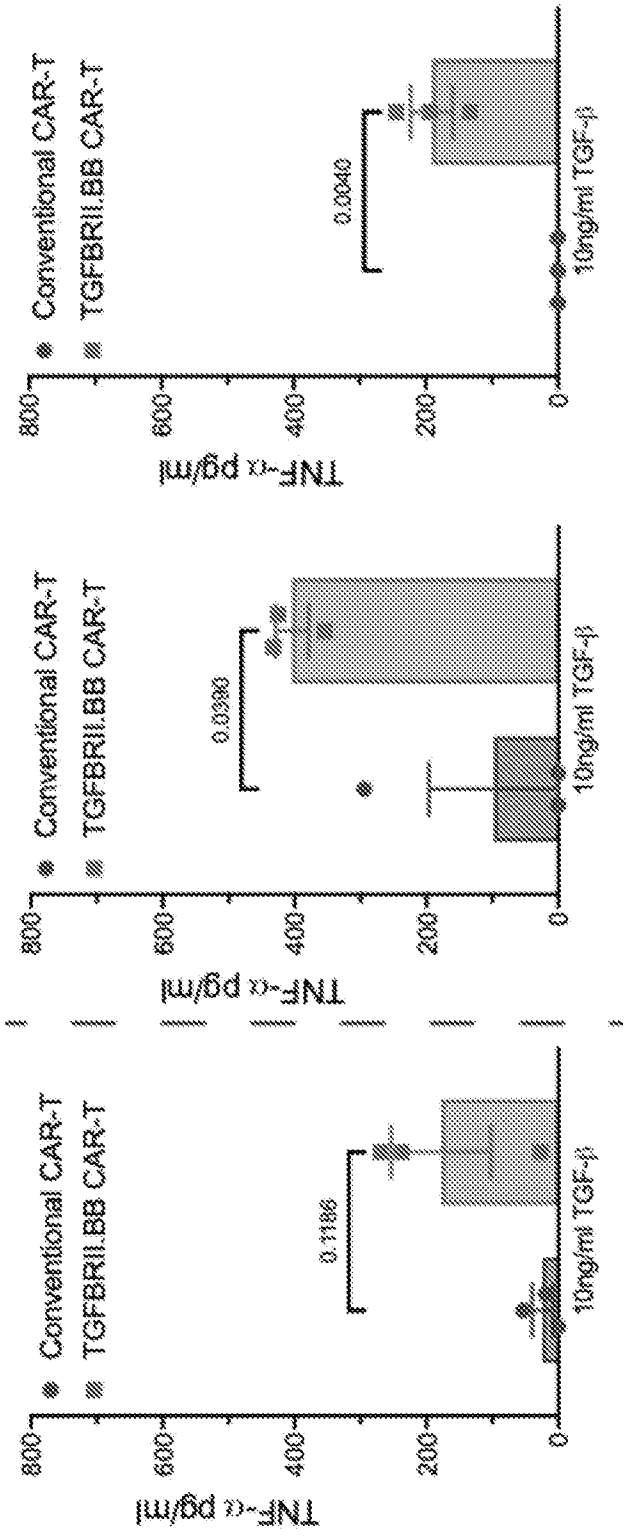


FIGURE 2

A.

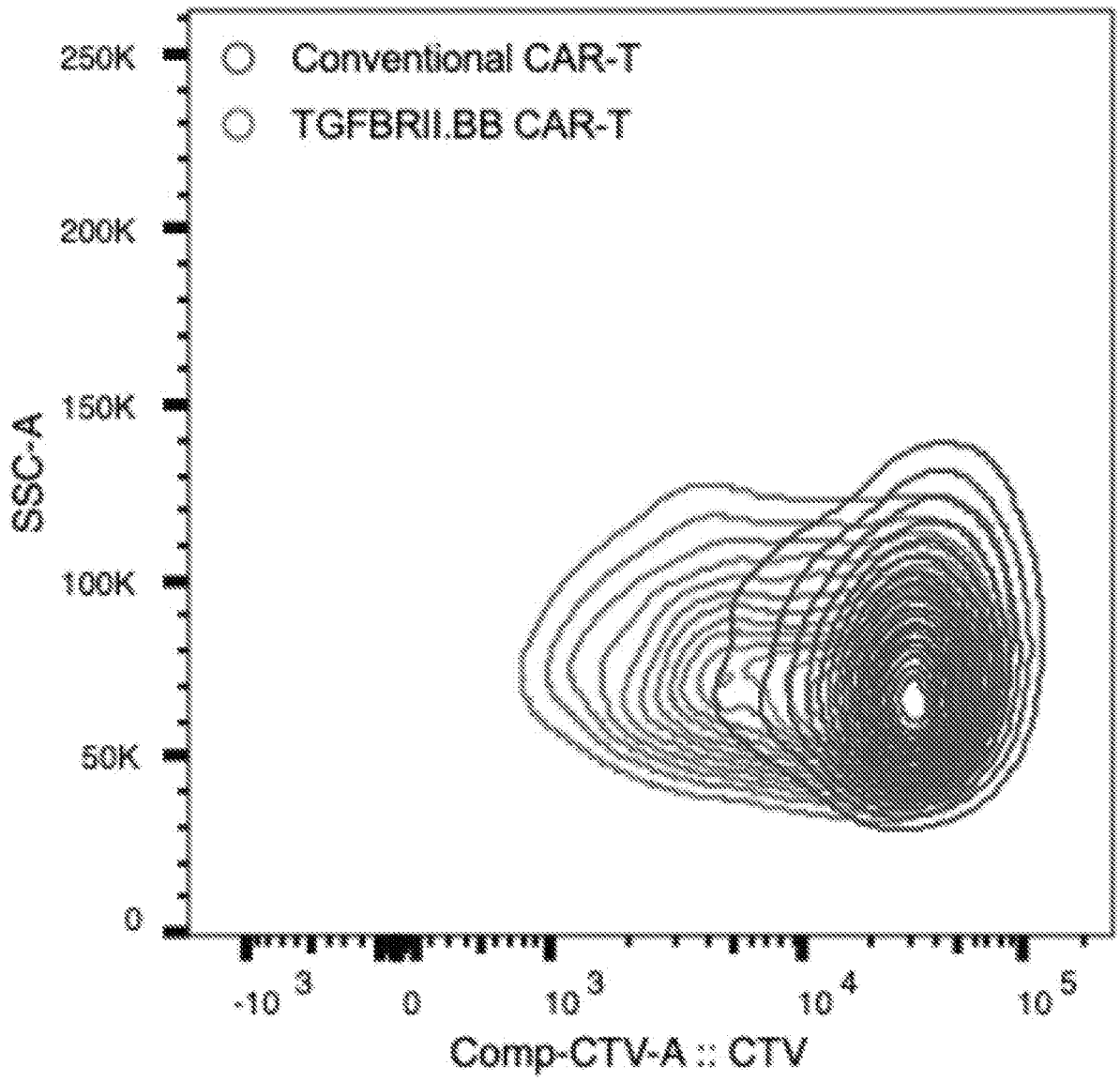
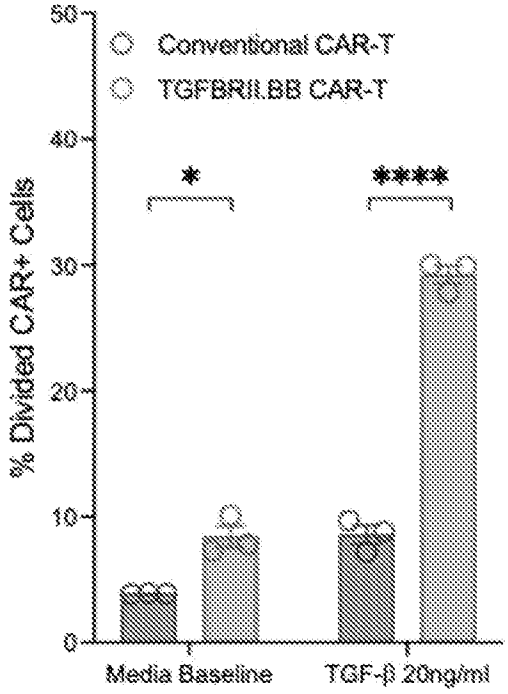

Conventional CAR-T
TGFBRII.BB CAR-T
250K
200K
150K
100K
50K
0
SSC-A
-10 3
0
10 3
10 4
10 5
Comp-CTV-A :: CTV

FIGURE 2 (continued)

B.



C.

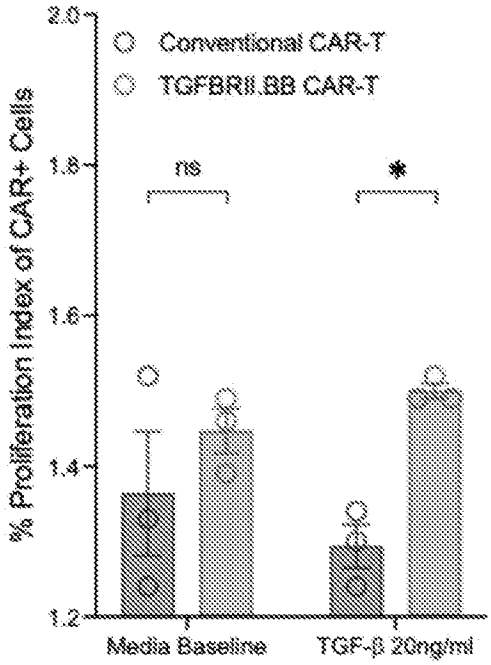


FIGURE 2 (continued)

D.

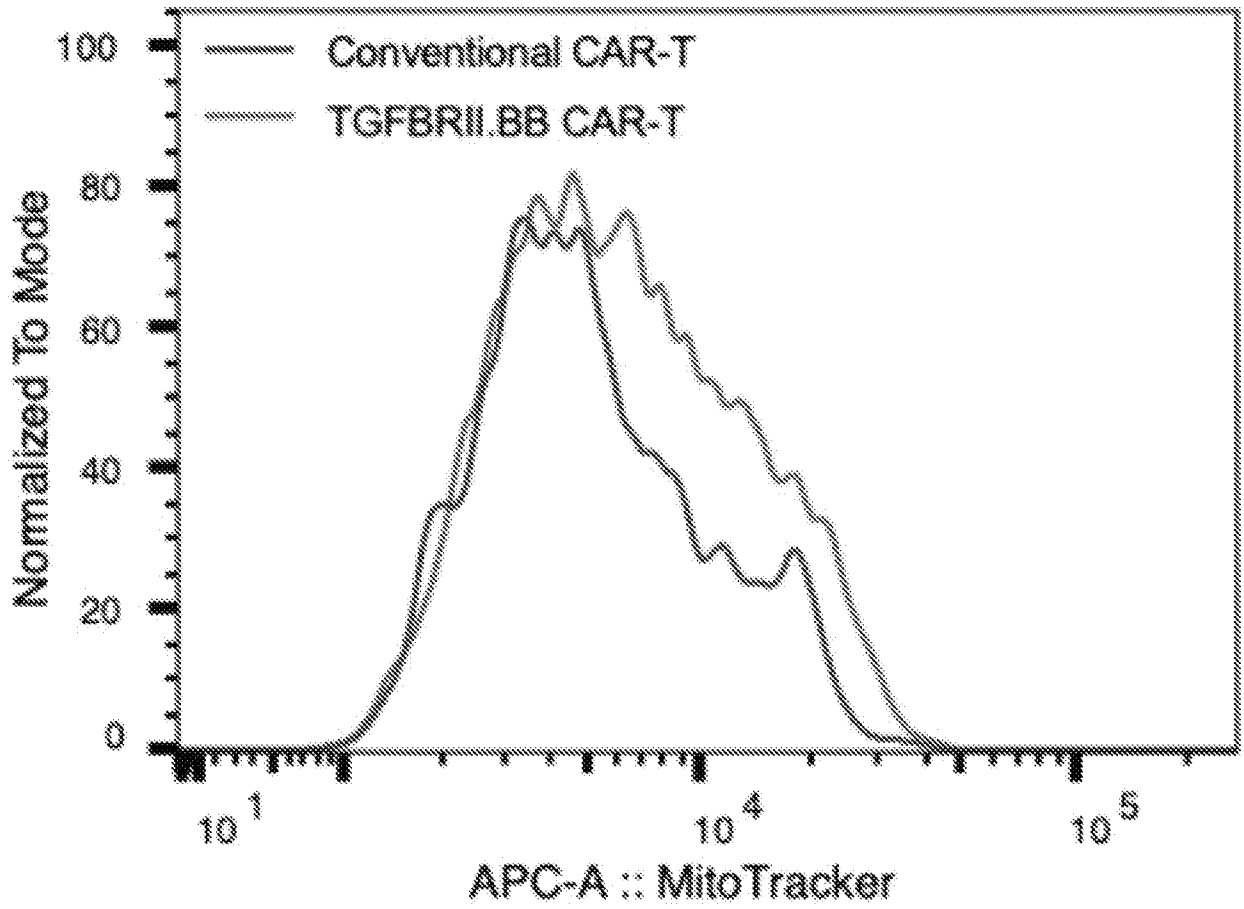
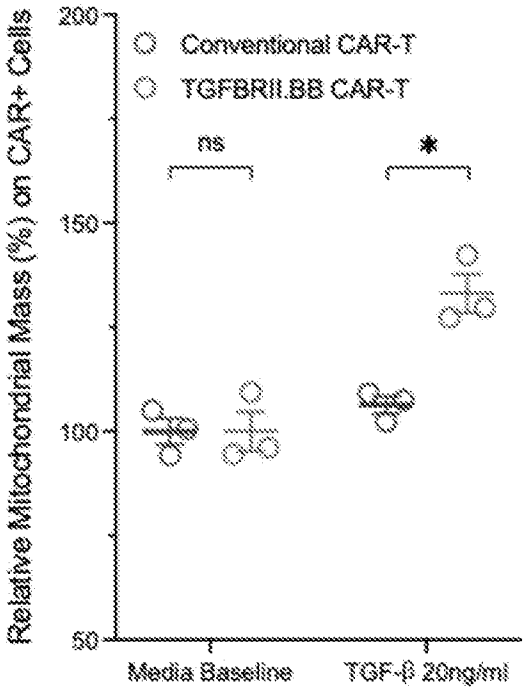


FIGURE 2 (continued)

E.



F.

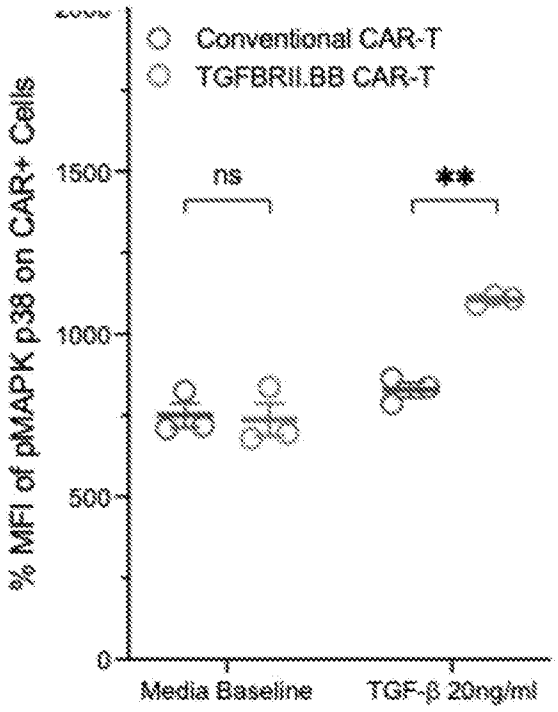


FIGURE 2 (continued)

G.

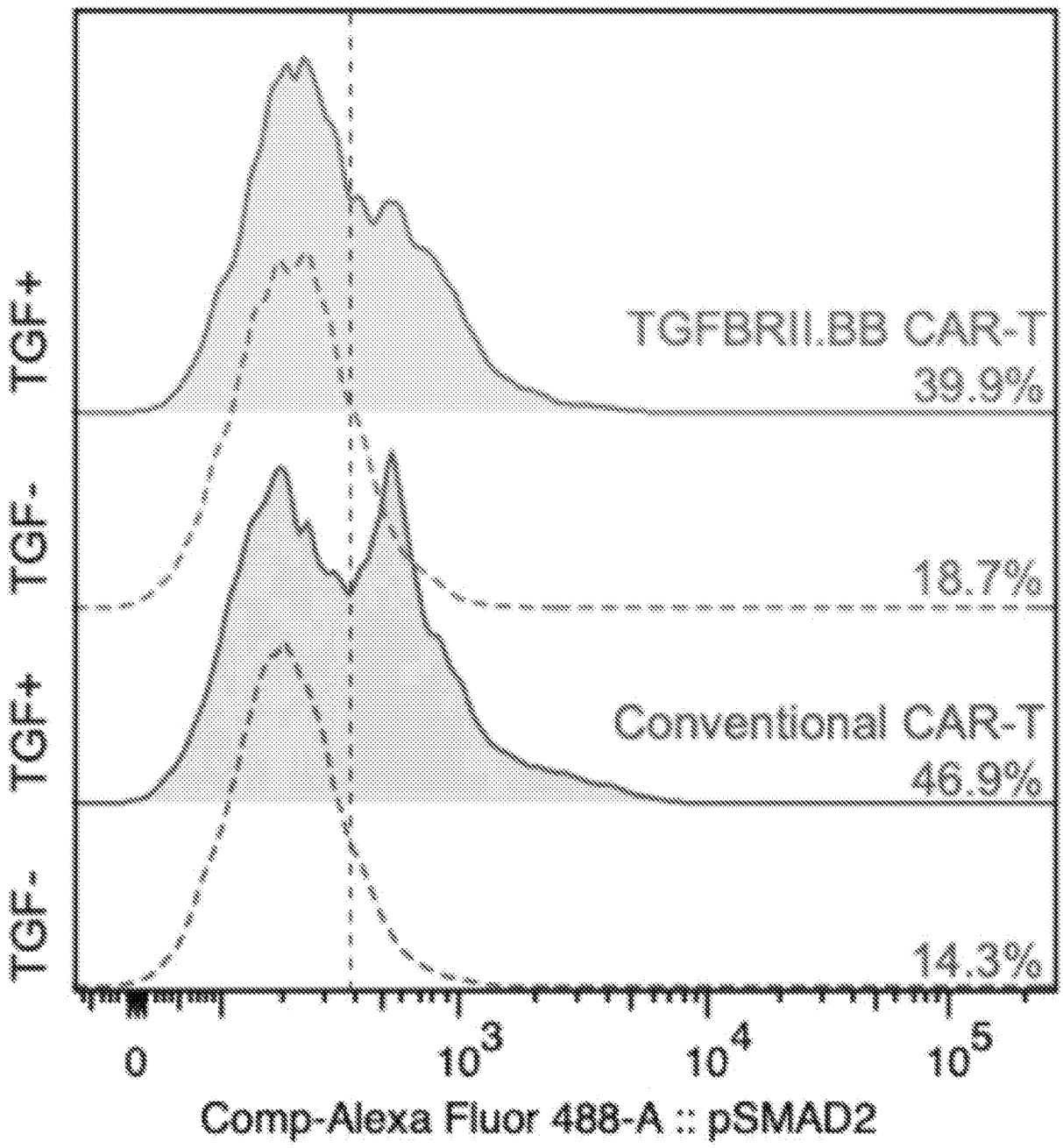


FIGURE 3

A.

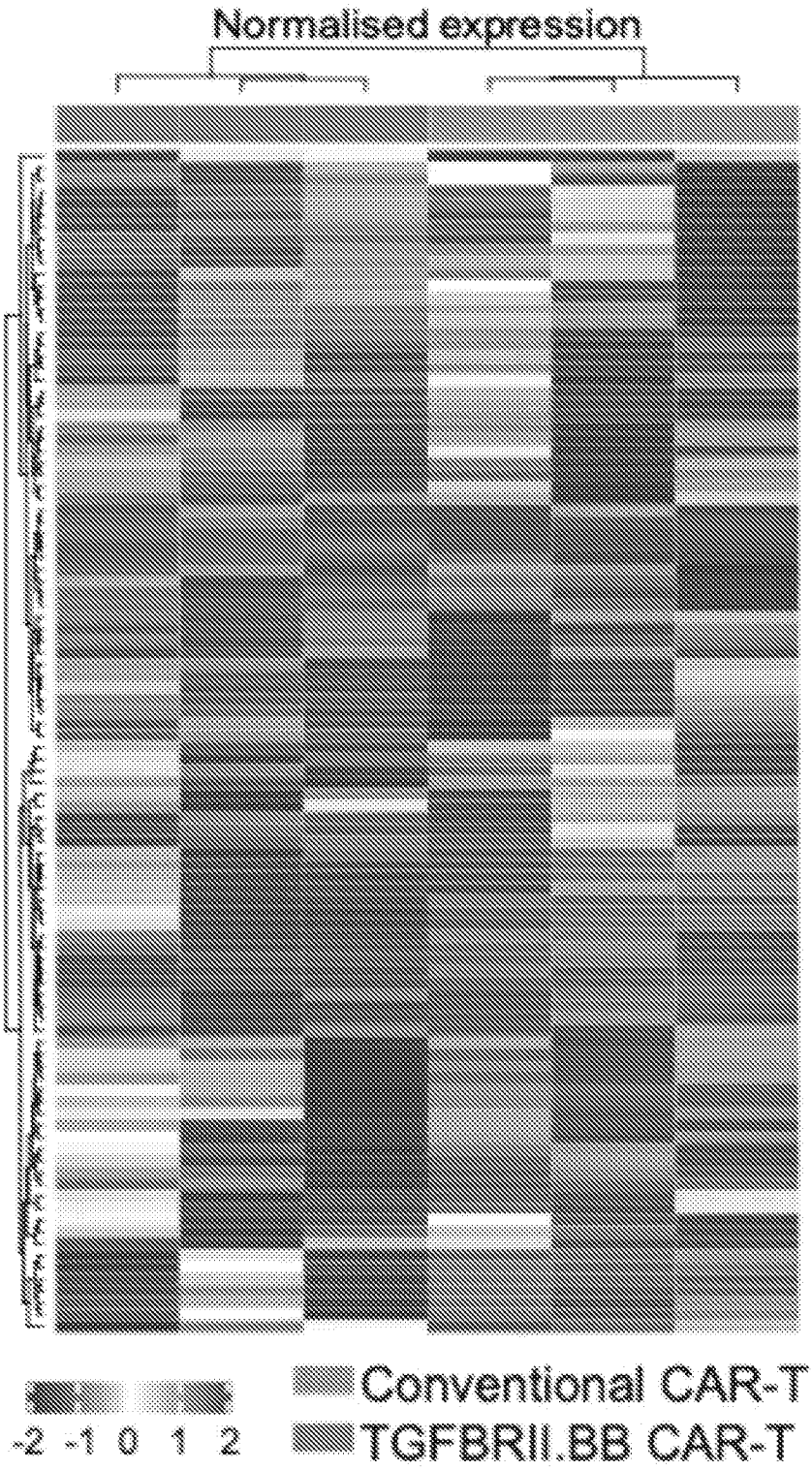

Normalised expression
-2 -1 0 1 2
Conventional CAR-T
TGFBRII.BB CAR-T

FIGURE 3 (continued)

B.

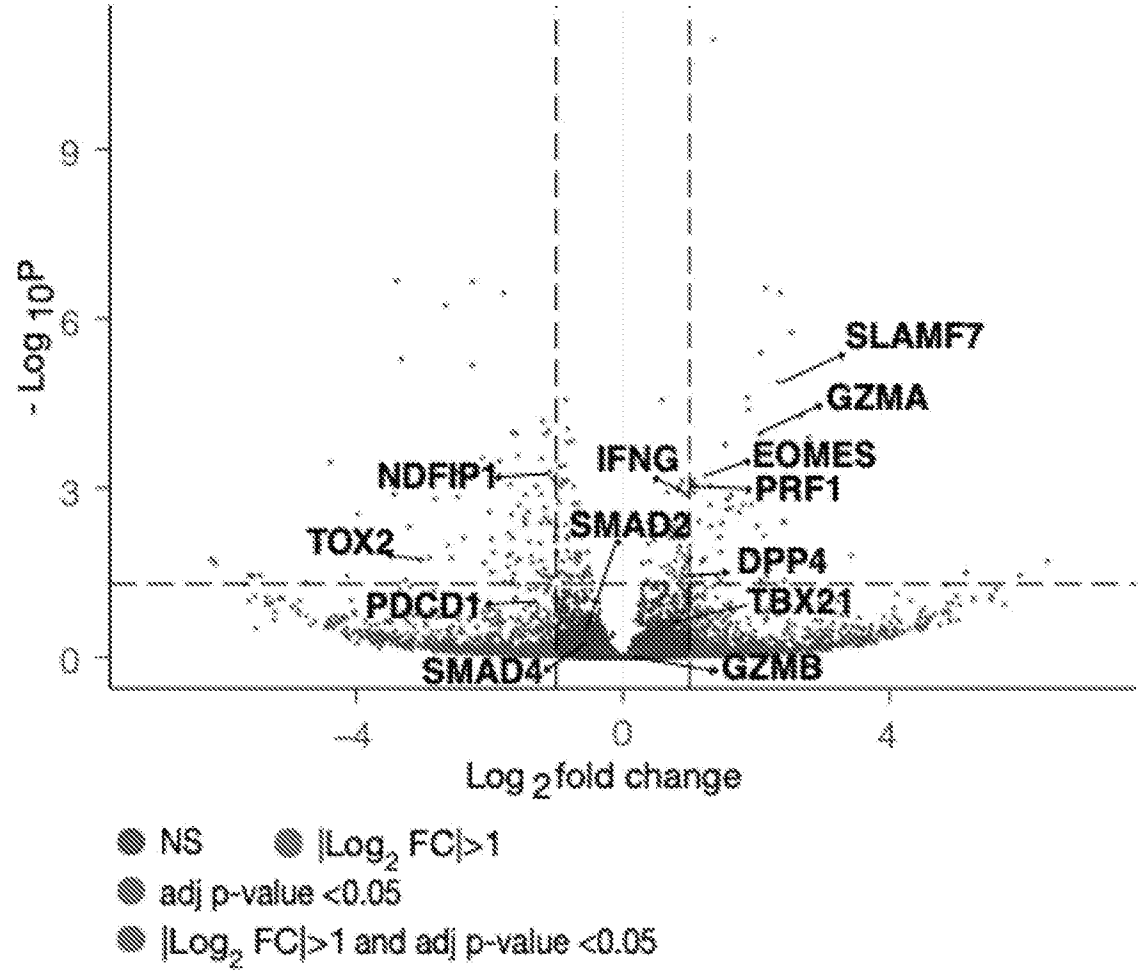


FIGURE 3 (continued)

C.

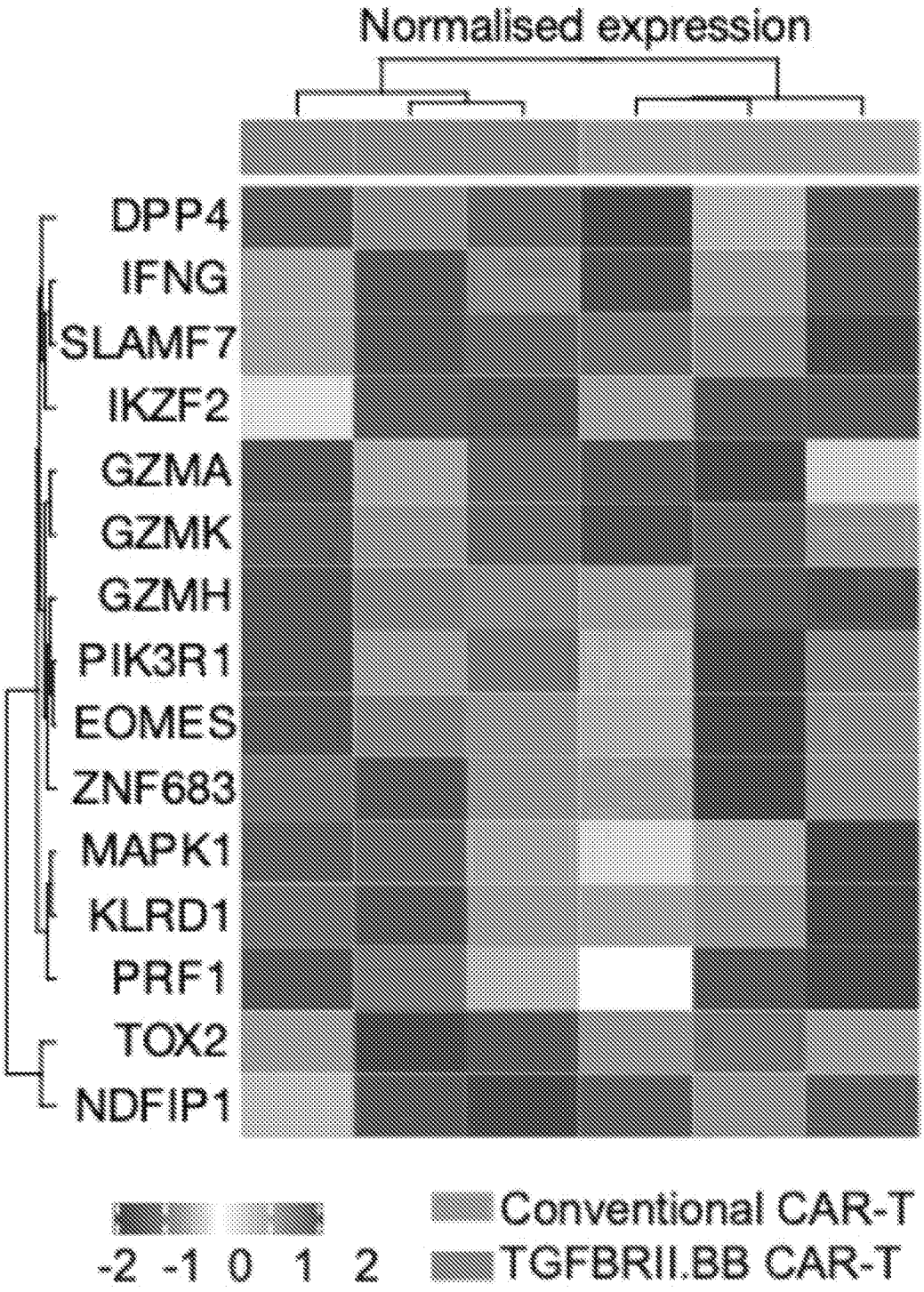


FIGURE 3 (continued)

D.

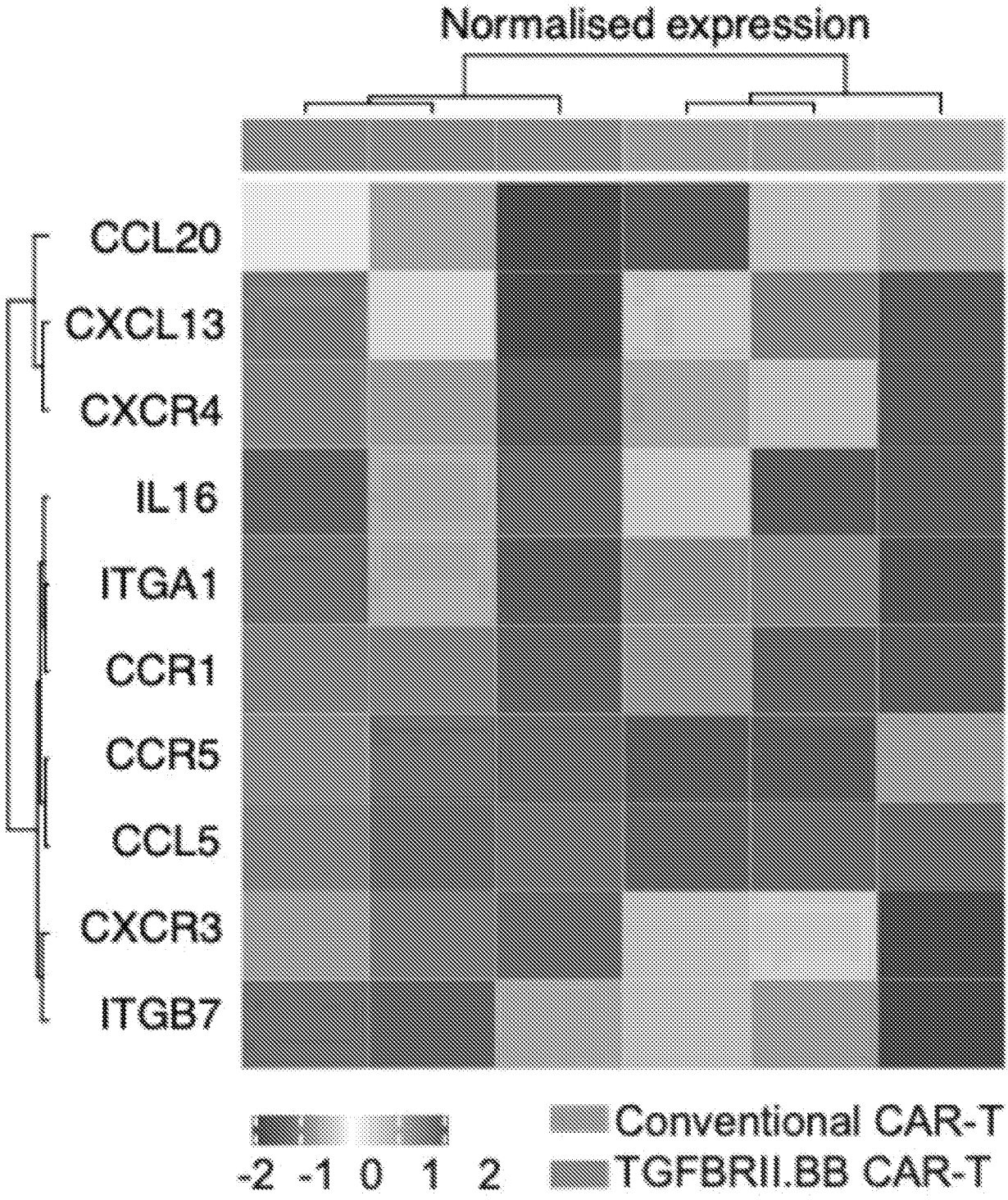


FIGURE 3 (continued)

E.

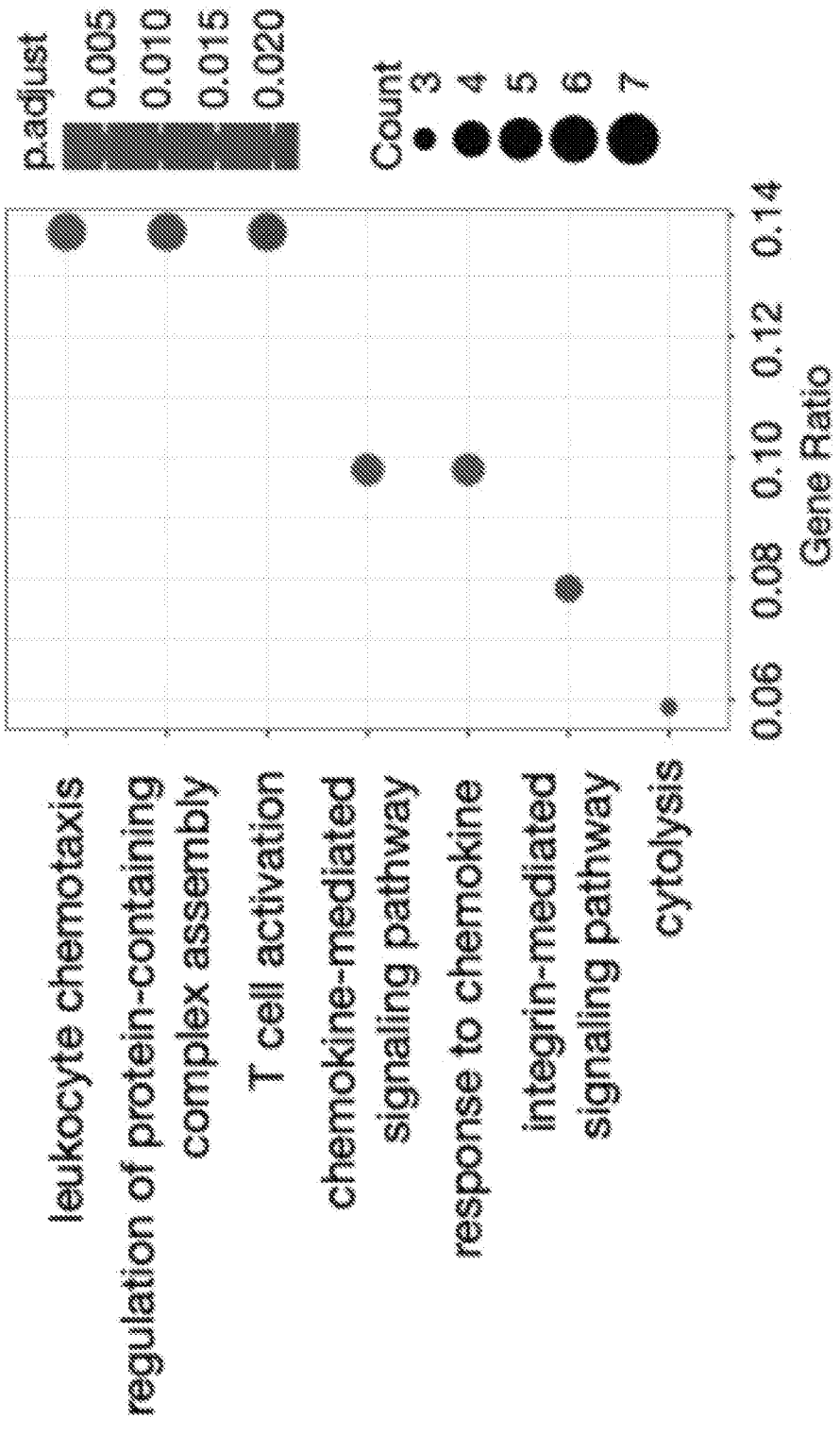
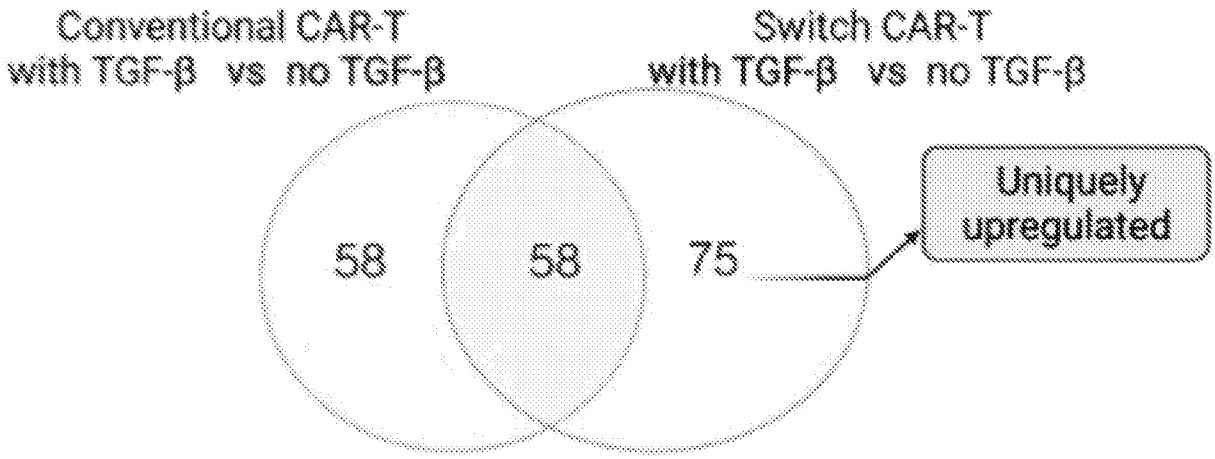


FIGURE 4

A.


Conventional CAR-T
with TGF-β vs no TGF-β
Switch CAR-T
with TGF-β vs no TGF-β
58
58
75
Uniquely
upregulated

B.

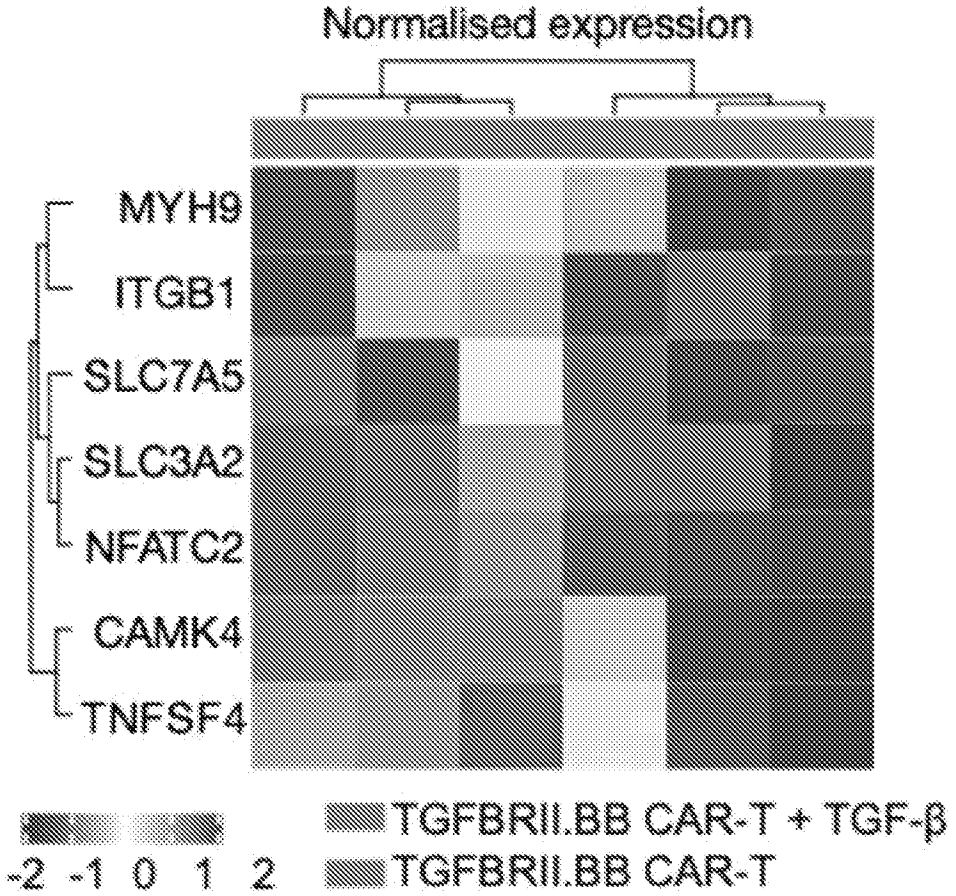

Normalised expression
MYH9
ITGB1
SLC7A5
SLC3A2
NFATC2
CAMK4
TNFSF4
-2 -1 0 1 2
TGFBRII.BB CAR-T + TGF-β
TGFBRII.BB CAR-T

FIGURE 4 (continued)

C.

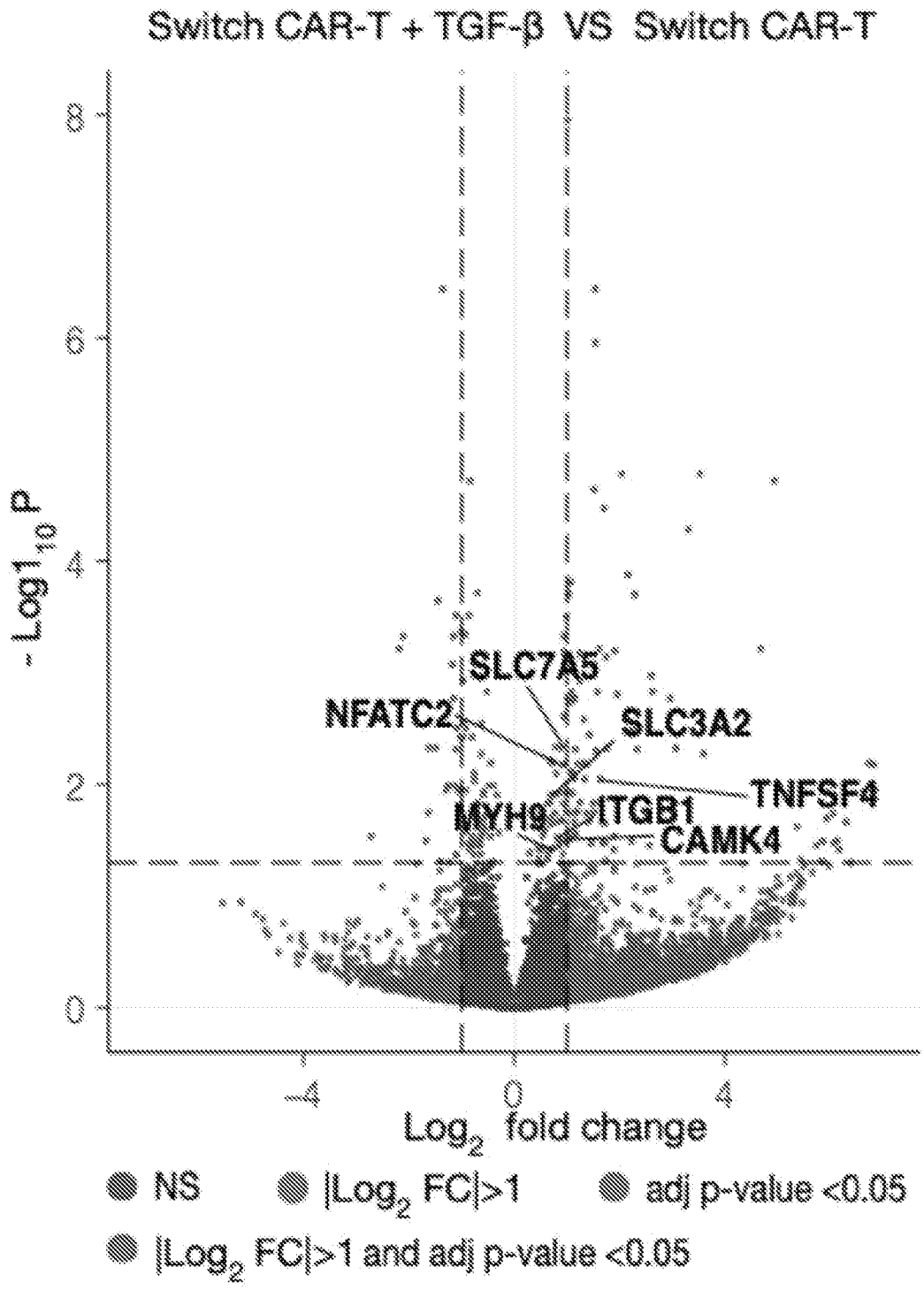
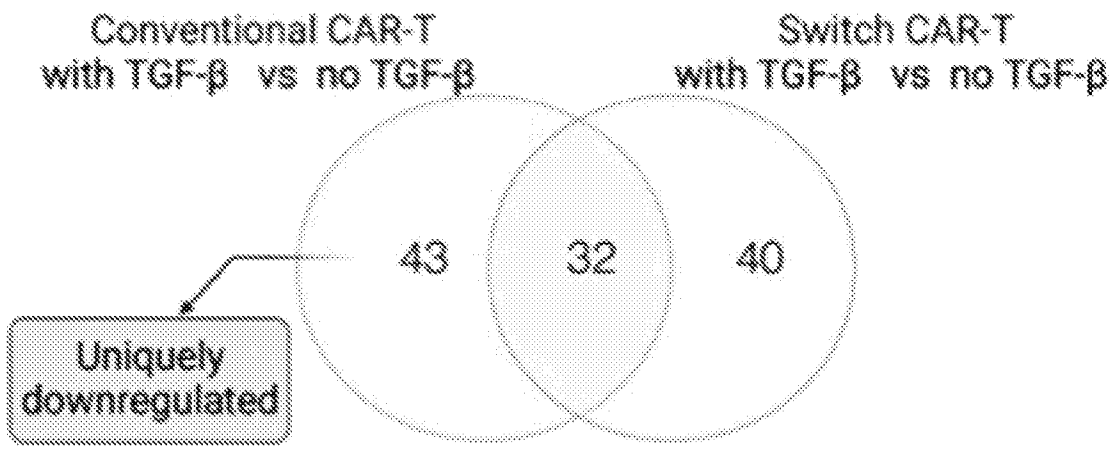


FIGURE 4 (continued)

D.



E.

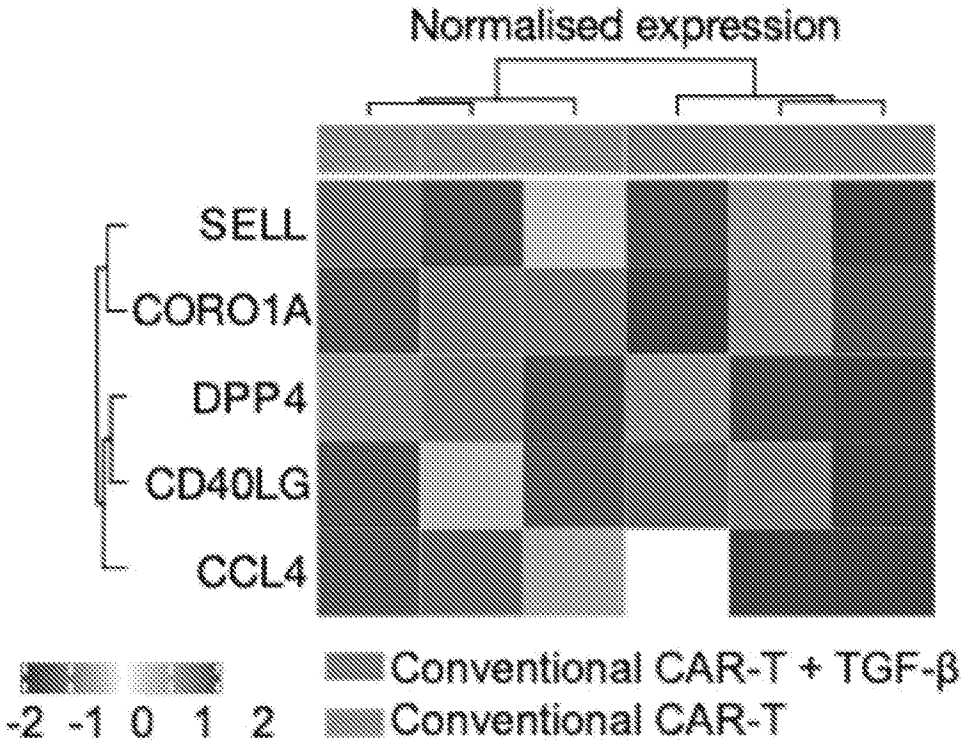


FIGURE 4 (continued)

F.

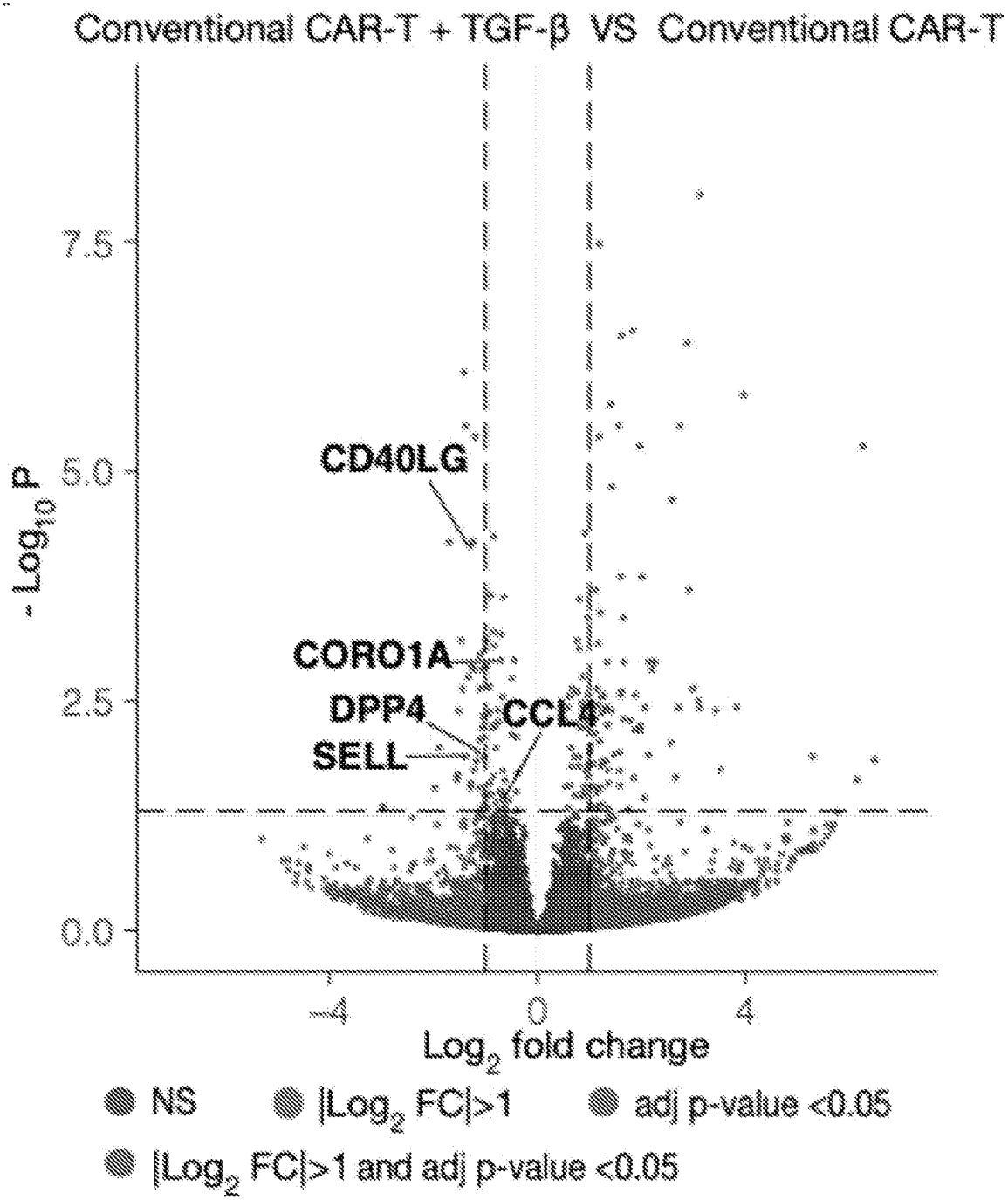


FIGURE 5

A.

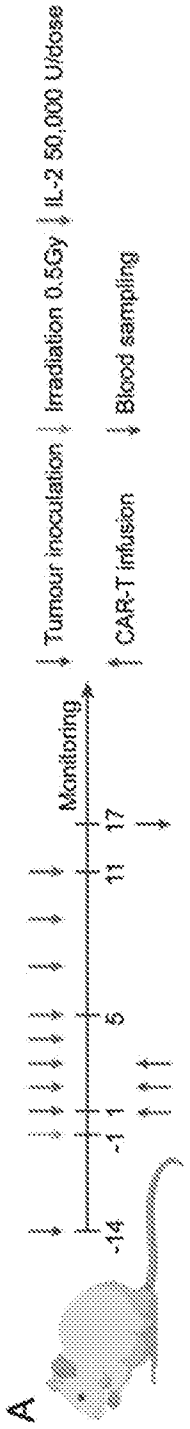

Tumour inoculation
Irradiation 0.5Gy
IL-2 50,000 U/dose
CAR-T infusion
Blood sampling
Monitoring
-14
-1
1
6
11
17
A

FIGURE 5 (continued)

B.

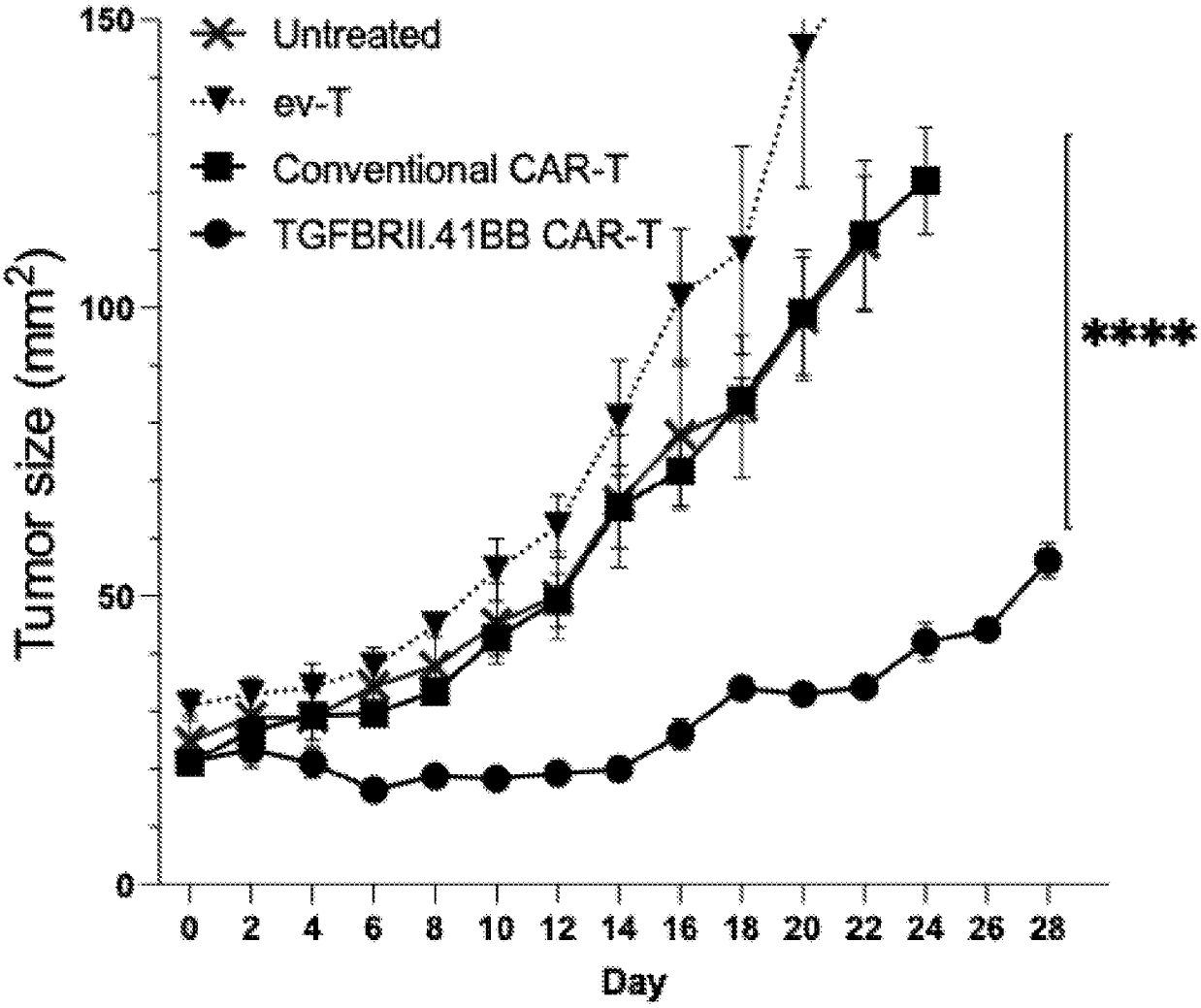


FIGURE 5 (continued)

C.

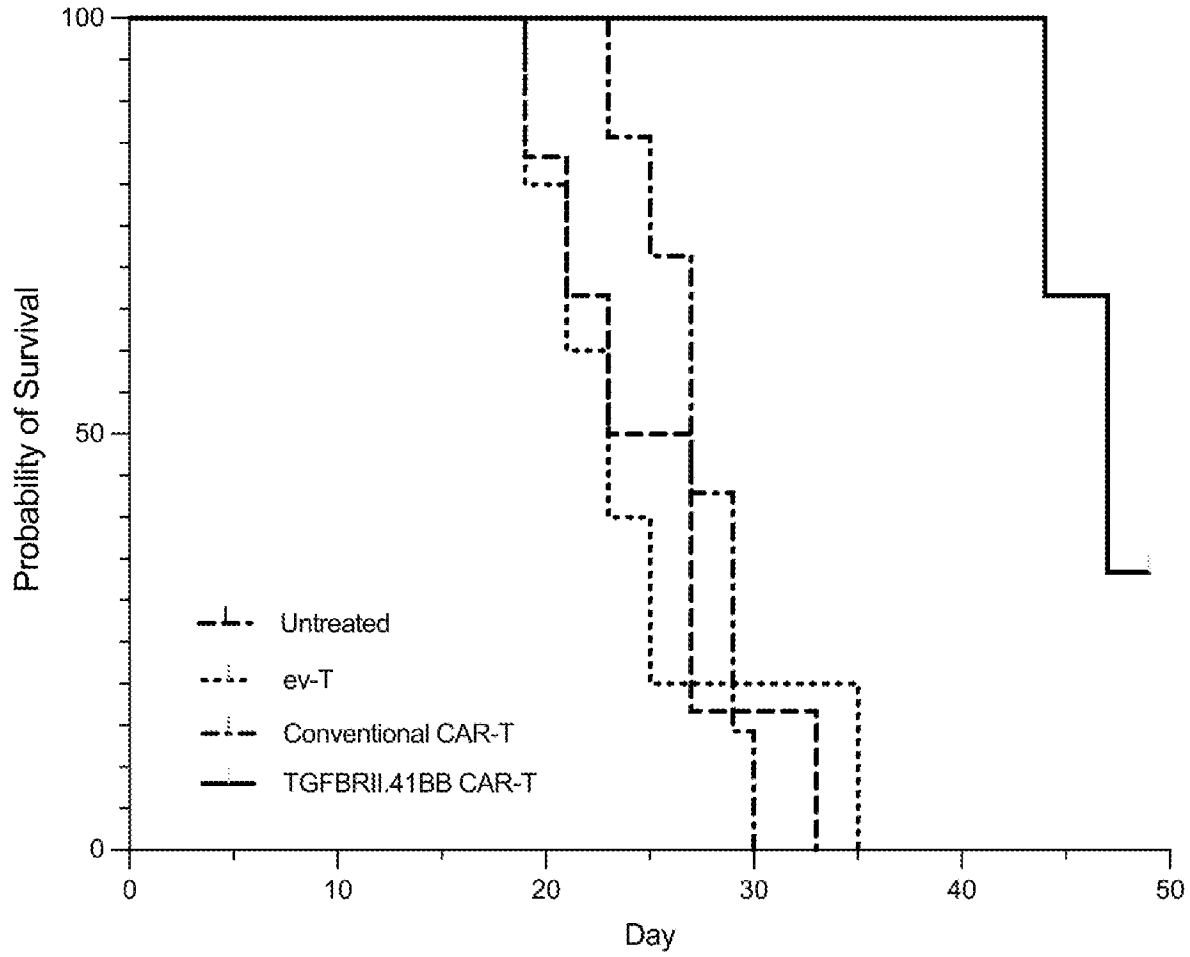
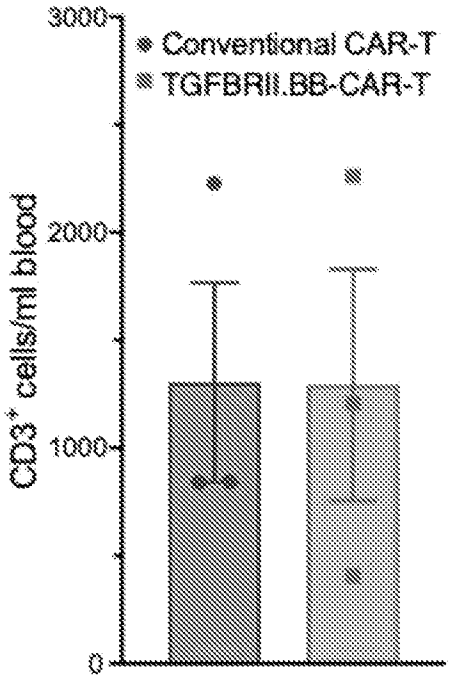


FIGURE 5 (continued)

D.



E.

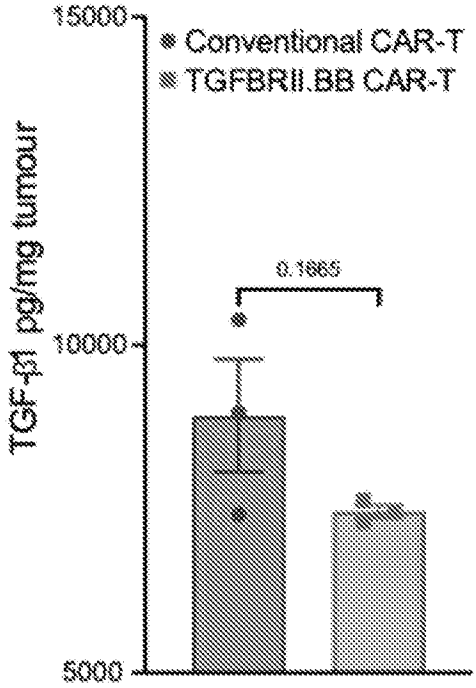


FIGURE 5 (continued)

F.

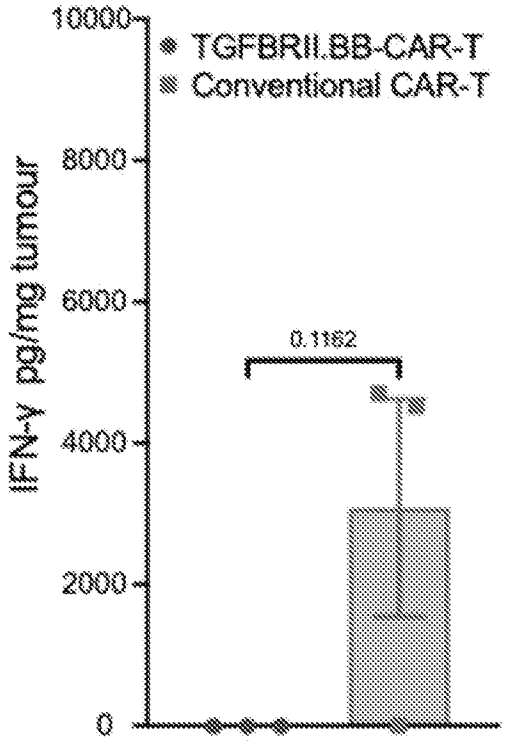


FIGURE 6

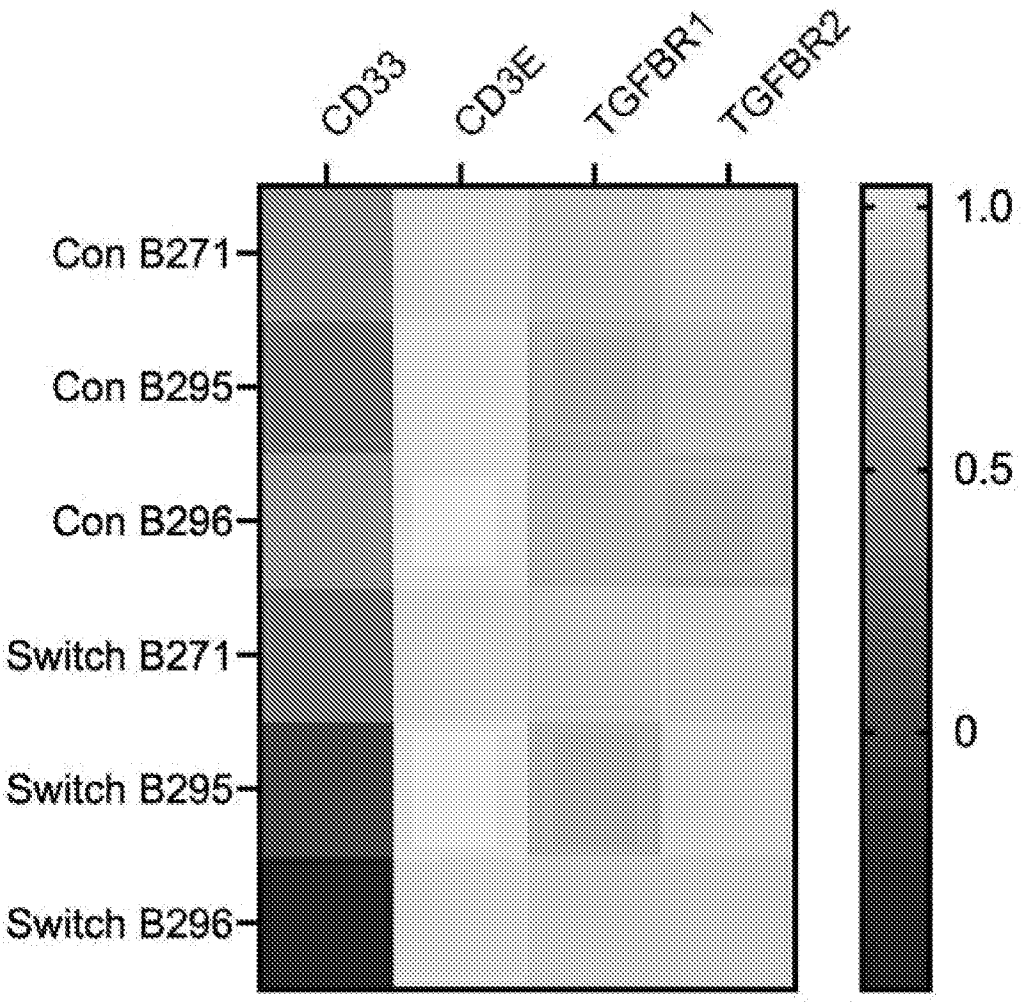

CD33
CD3E
TGFBR1
TGFBR2
Con B271
Con B295
Con B296
Switch B271
Switch B295
Switch B296
1.0
0.5
0

FIGURE 7

A.

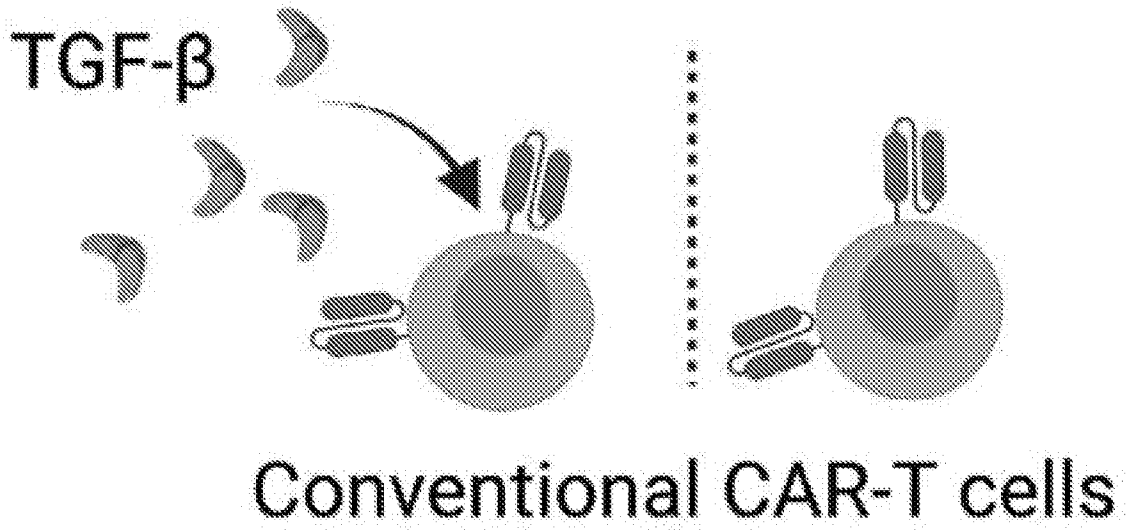


FIGURE 7 (continued)

B.

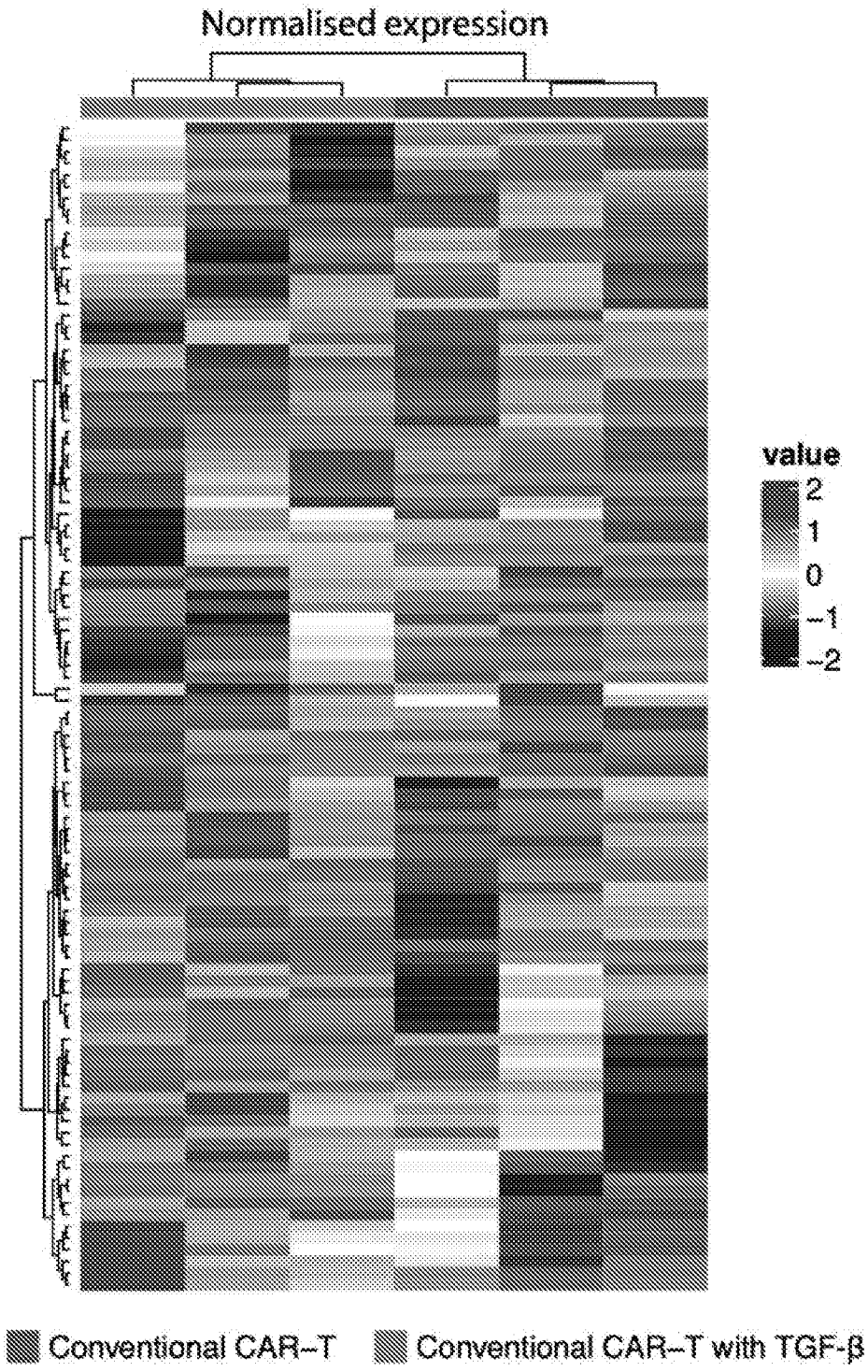


FIGURE 7 (continued)

C.

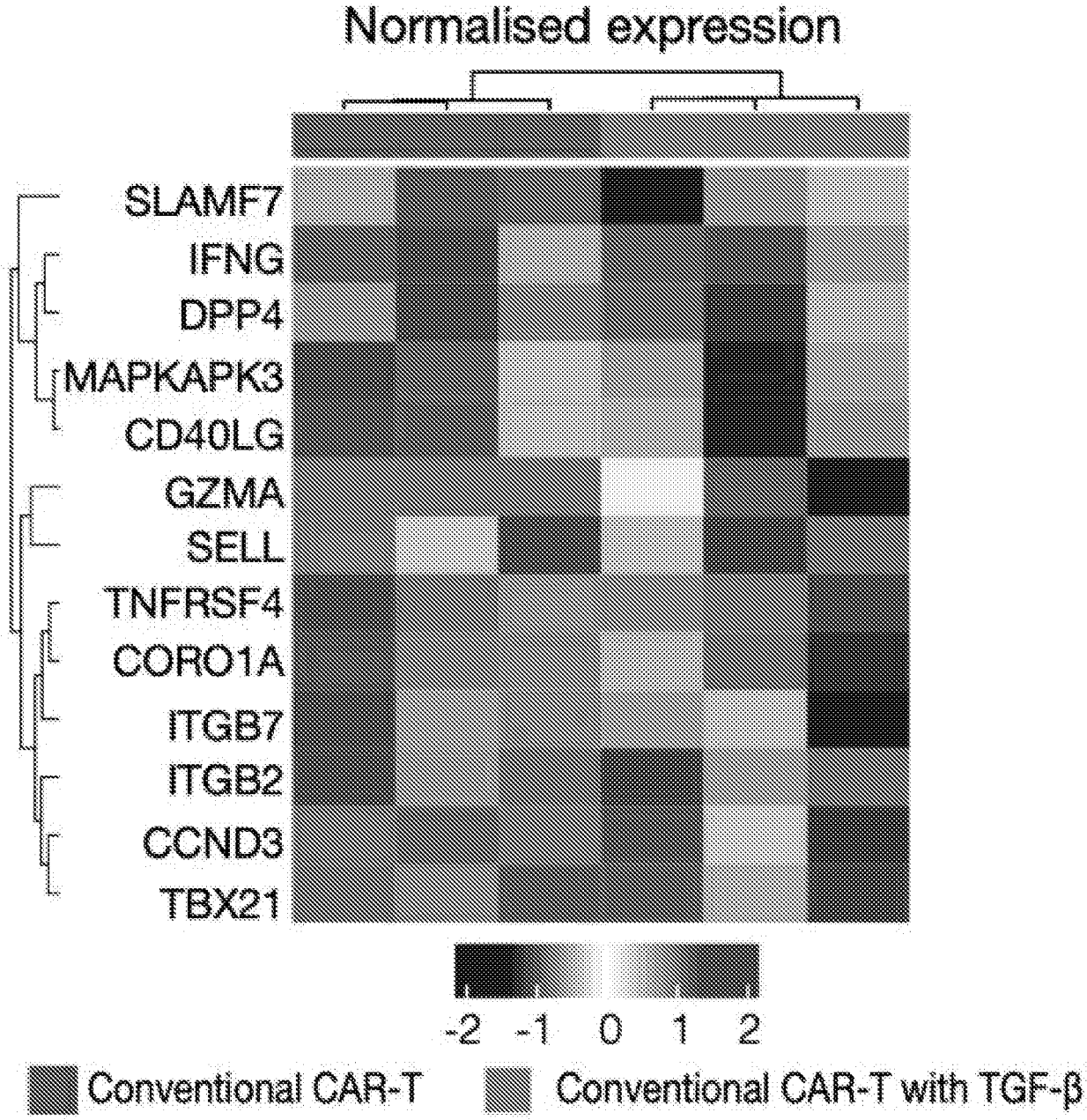


FIGURE 7 (continued)

D.

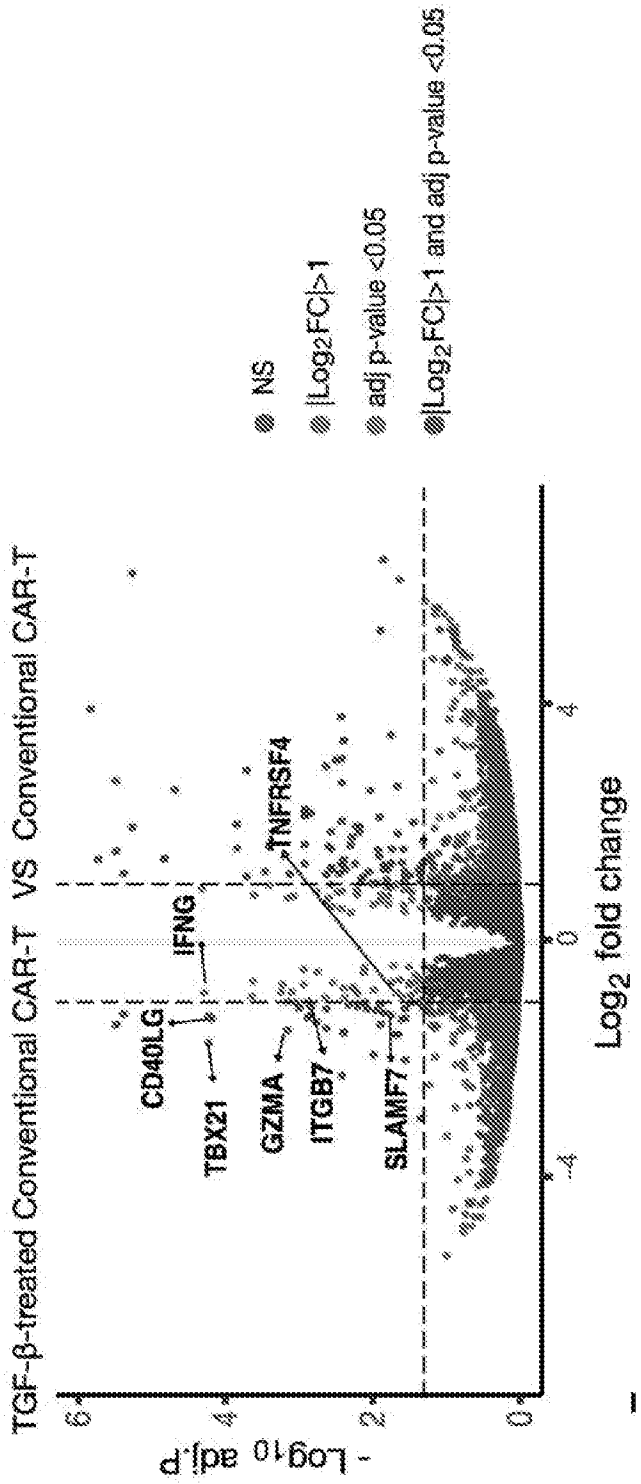


FIGURE 7 (continued)

E.

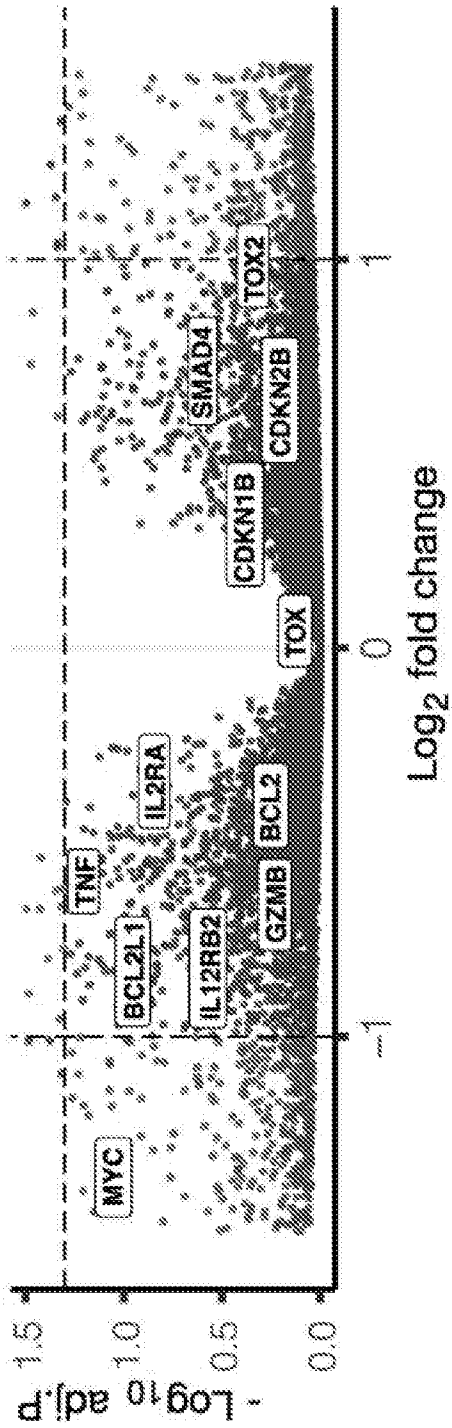


FIGURE 8

A.

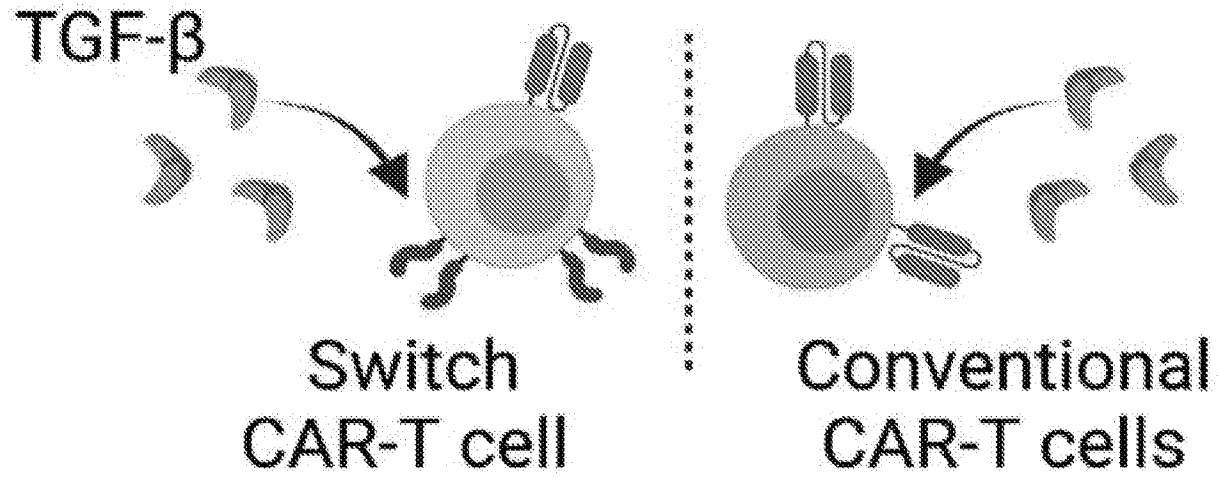


FIGURE 8 (continued)

B.

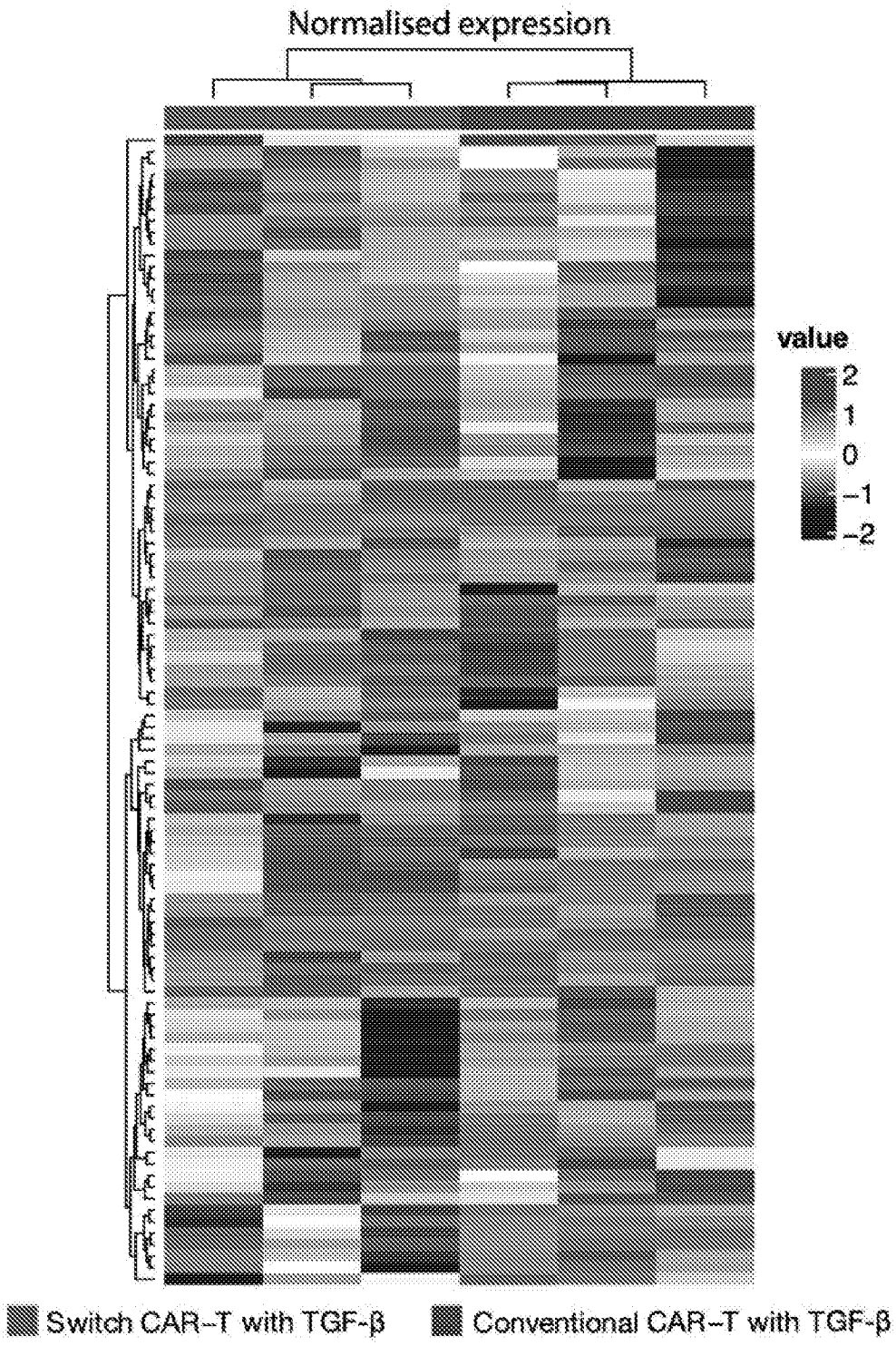


FIGURE 9

A.

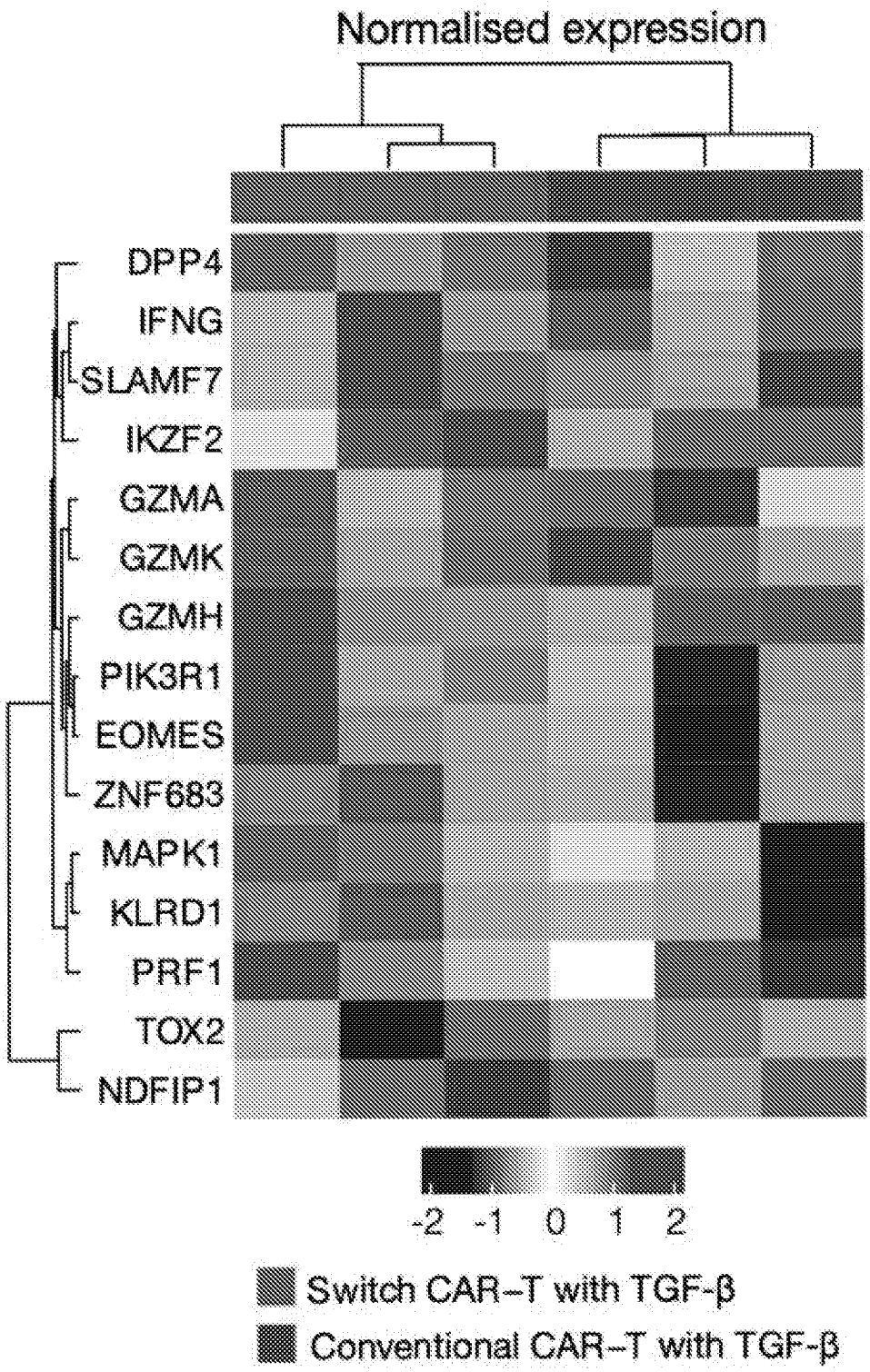

Normalised expression
DPP4
IFNG
SLAMF7
IKZF2
GZMA
GZMK
GZMH
PIK3R1
EOMES
ZNF683
MAPK1
KLRD1
PRF1
TOX2
NDFIP1
-2 -1 0 1 2
Switch CAR–T with TGF-β
Conventional CAR–T with TGF-β

FIGURE 9 (continued)

B.

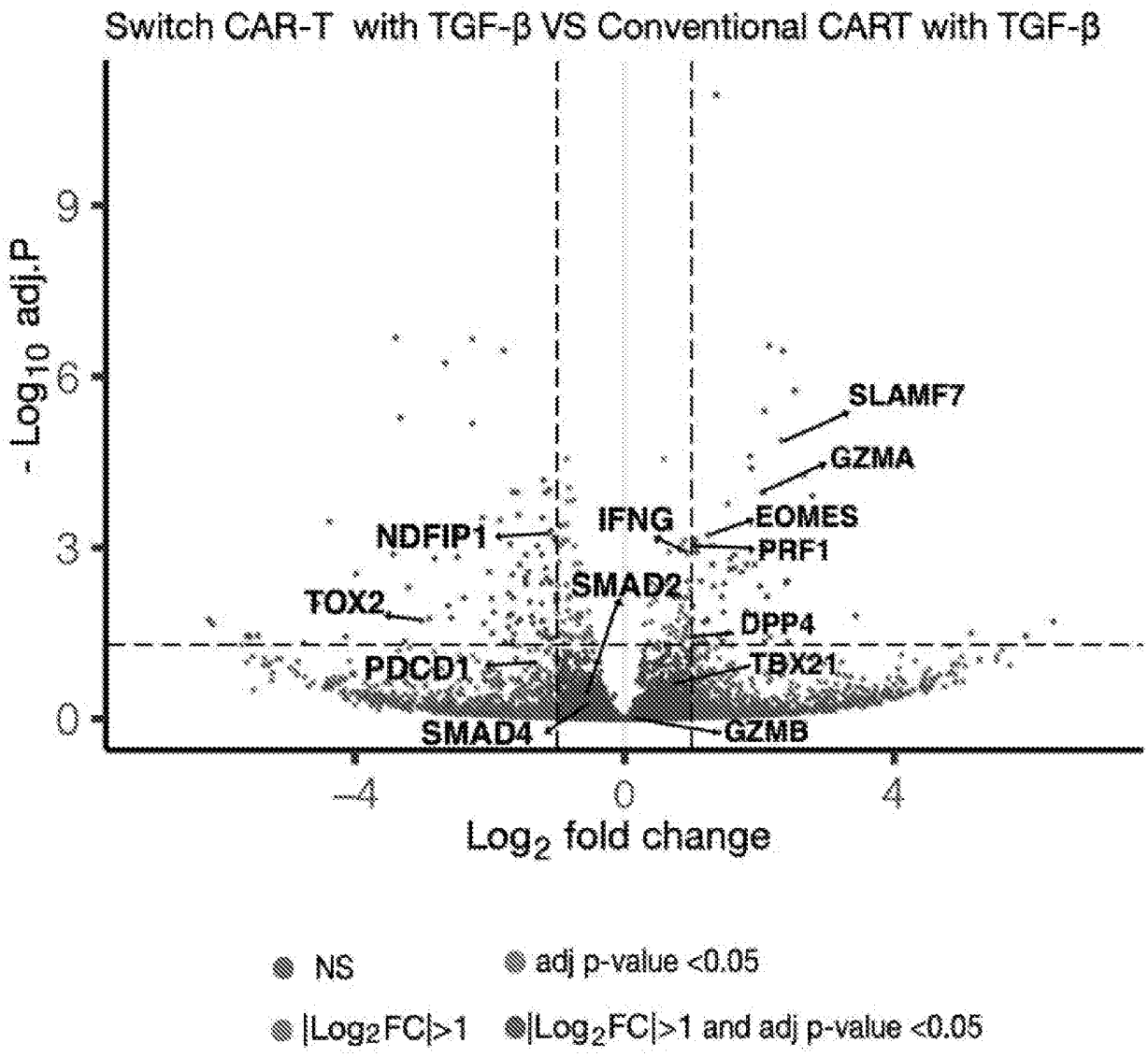


FIGURE 9 (continued)

C.

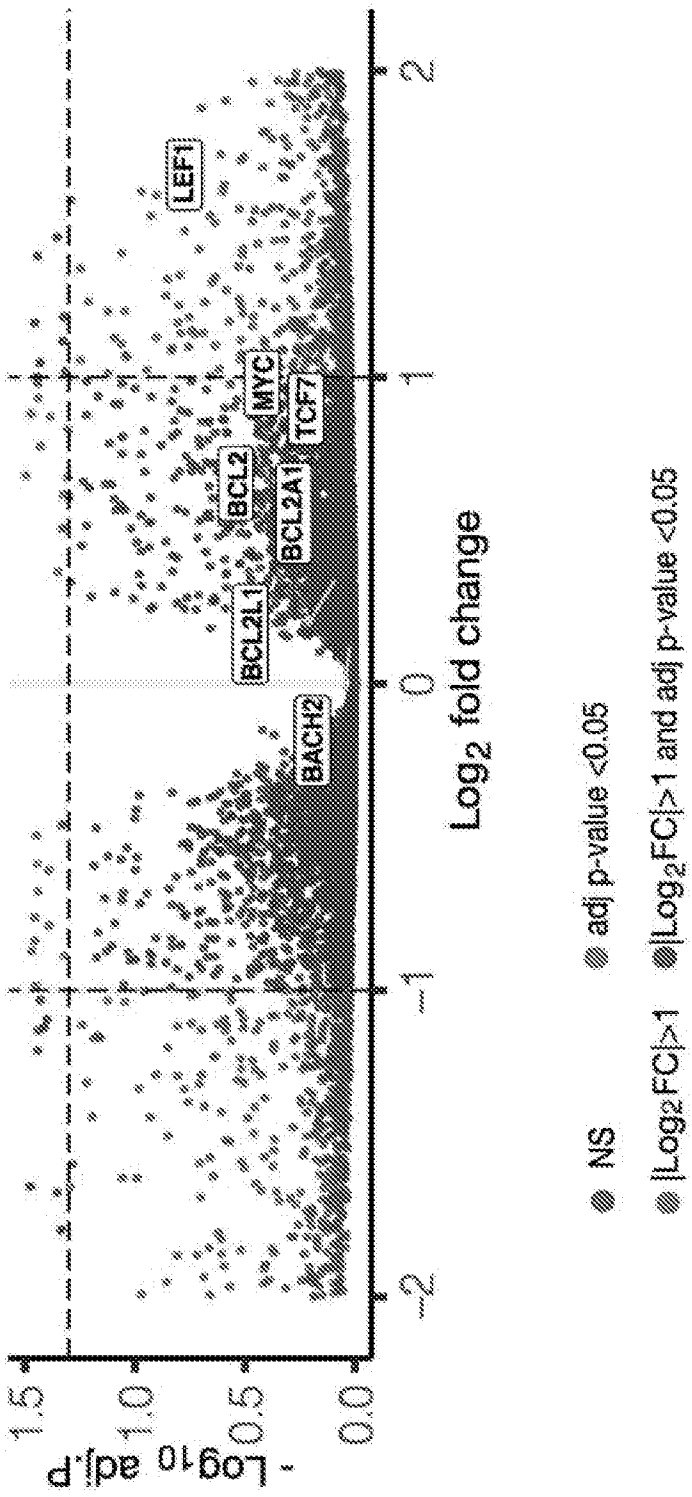


FIGURE 10

A.

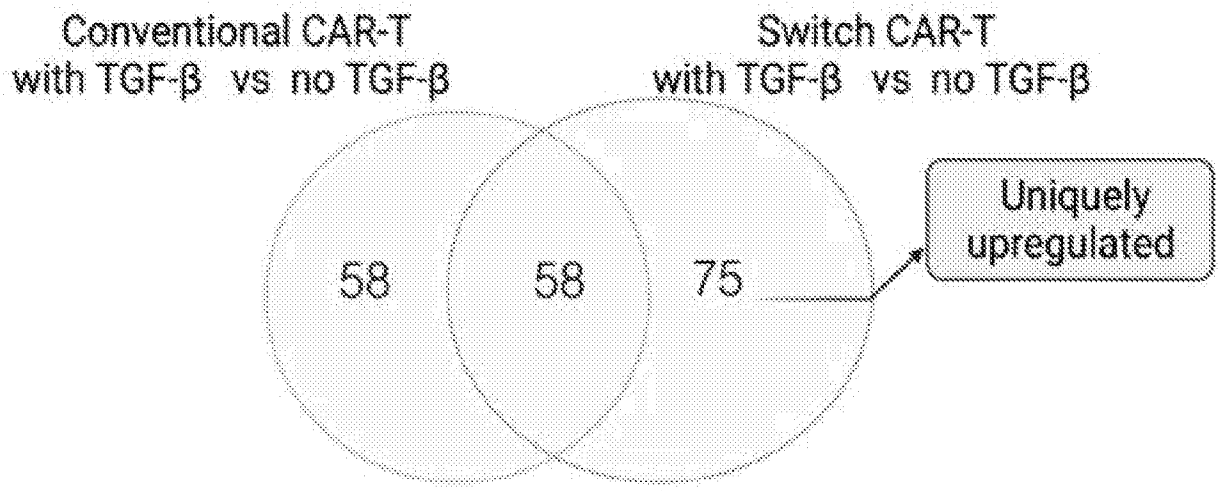

Conventional CAR-T
with TGF-β vs no TGF-β
Switch CAR-T
with TGF-β vs no TGF-β
58
58
75
Uniquely
upregulated

FIGURE 10 (continued)

B.

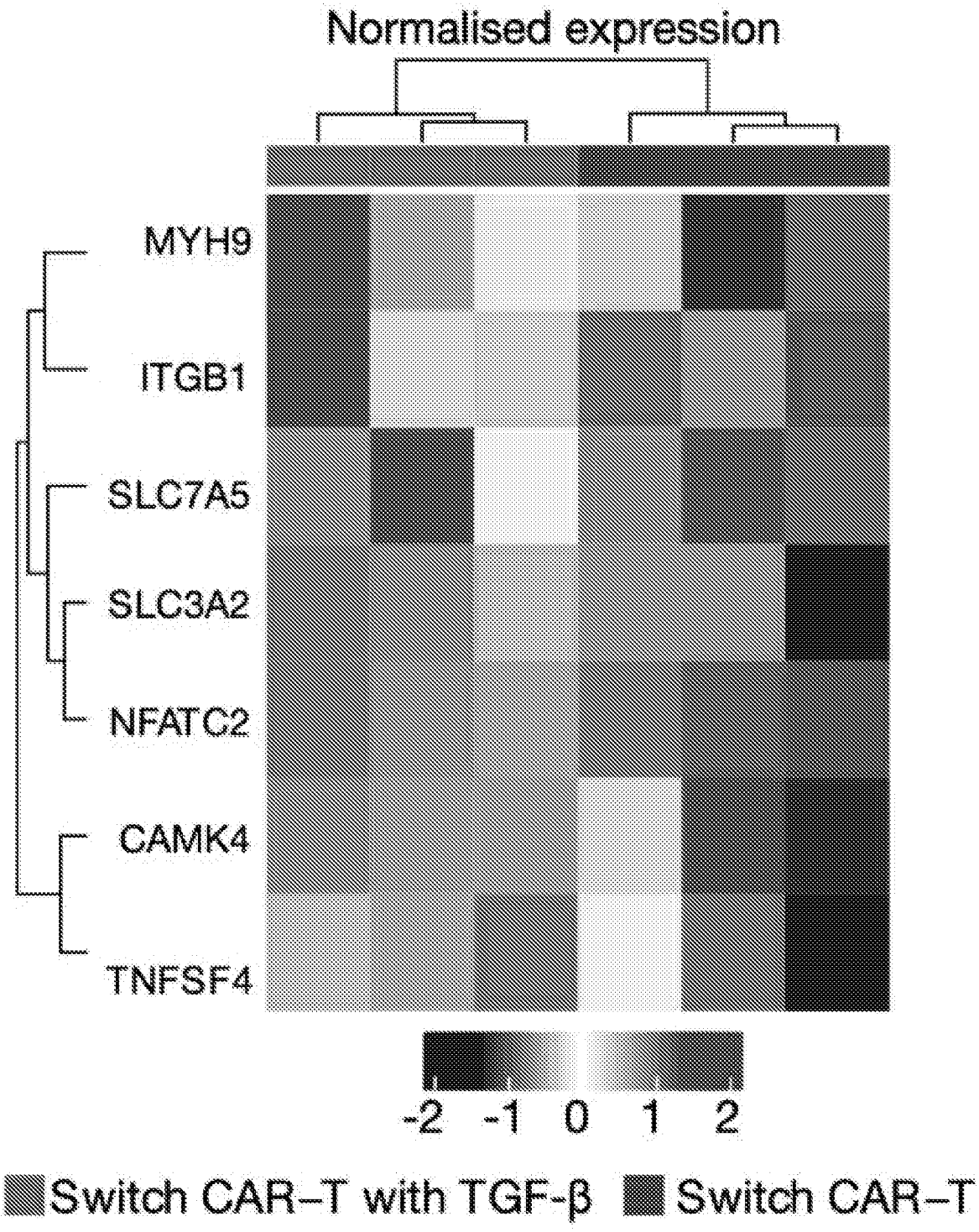


FIGURE 10 (continued)

C.

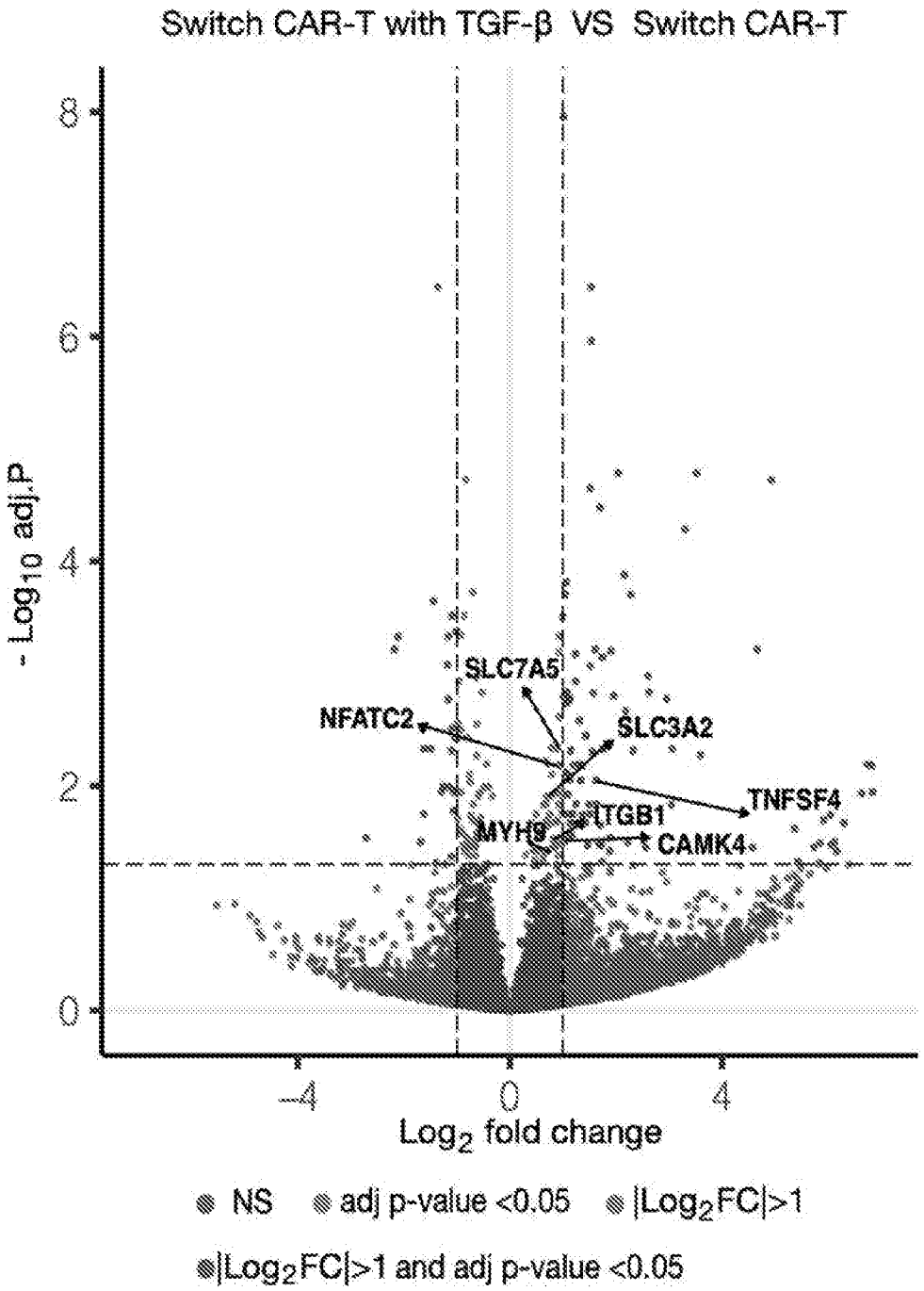


FIGURE 11

A.

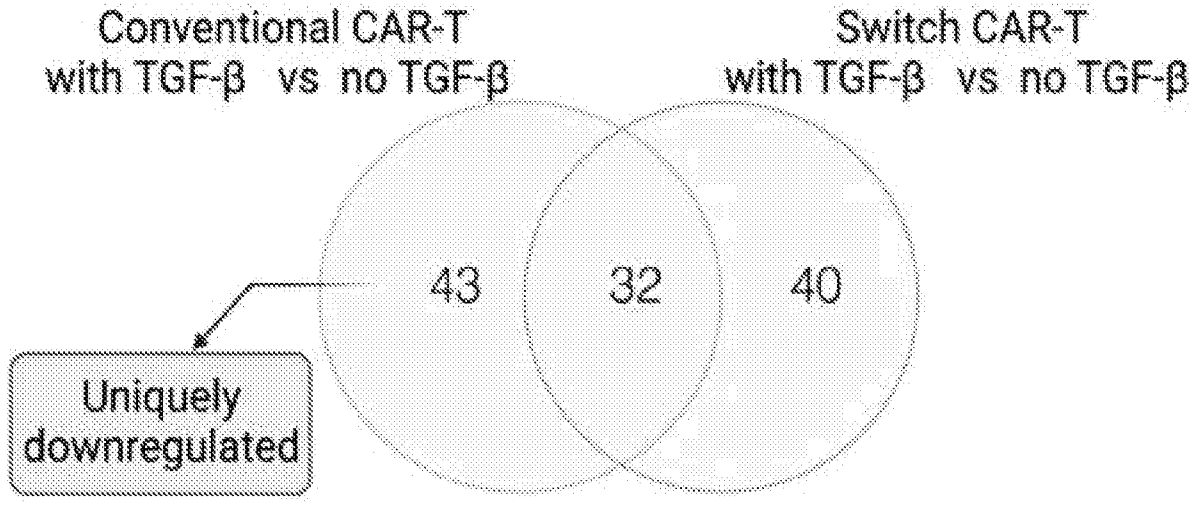

Conventional CAR-T
with TGF-β vs no TGF-β
Switch CAR-T
with TGF-β vs no TGF-β
43
32
40
Uniquely
downregulated

FIGURE 11 (continued)

B.

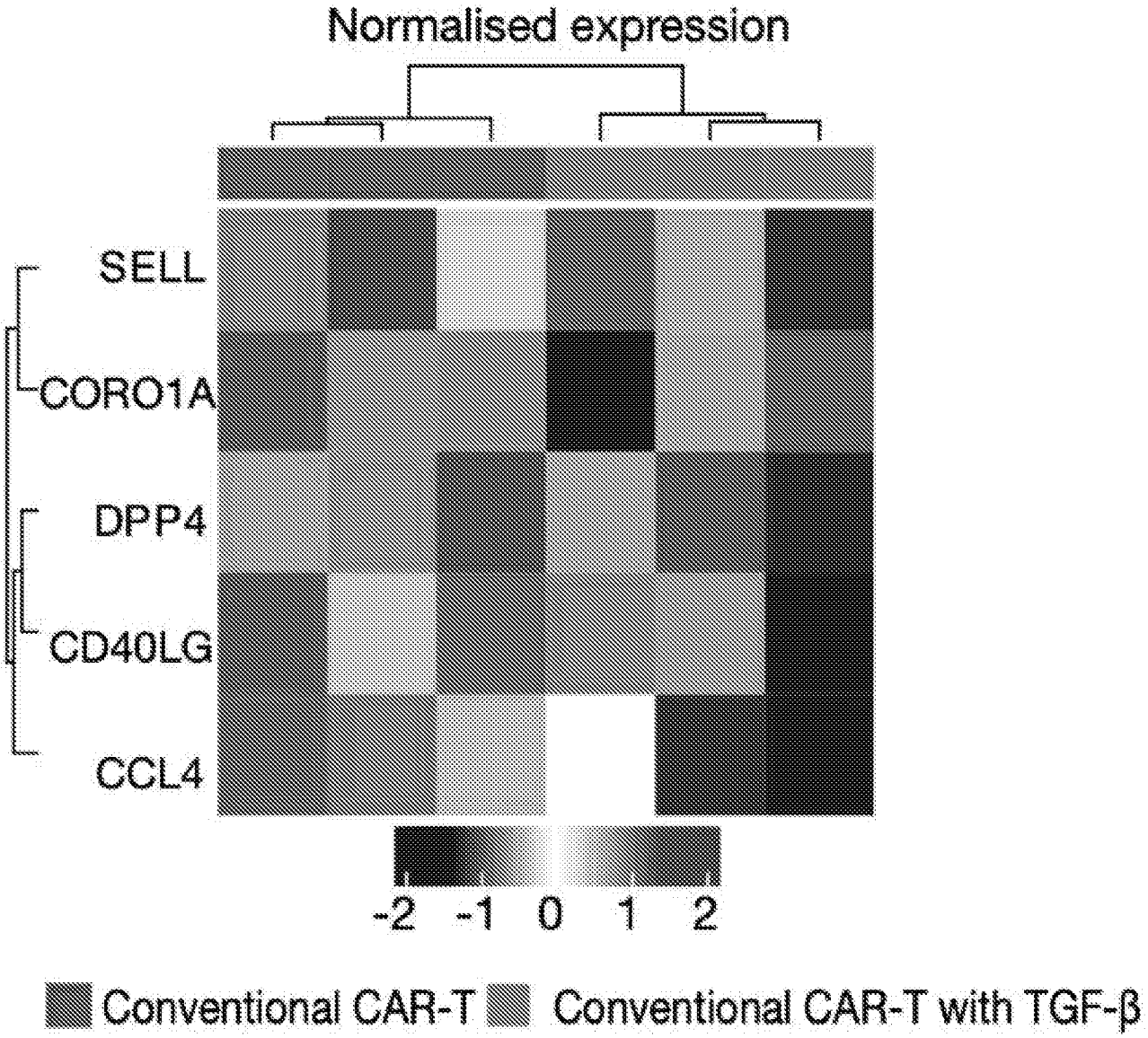


FIGURE 11 (continued)

C.

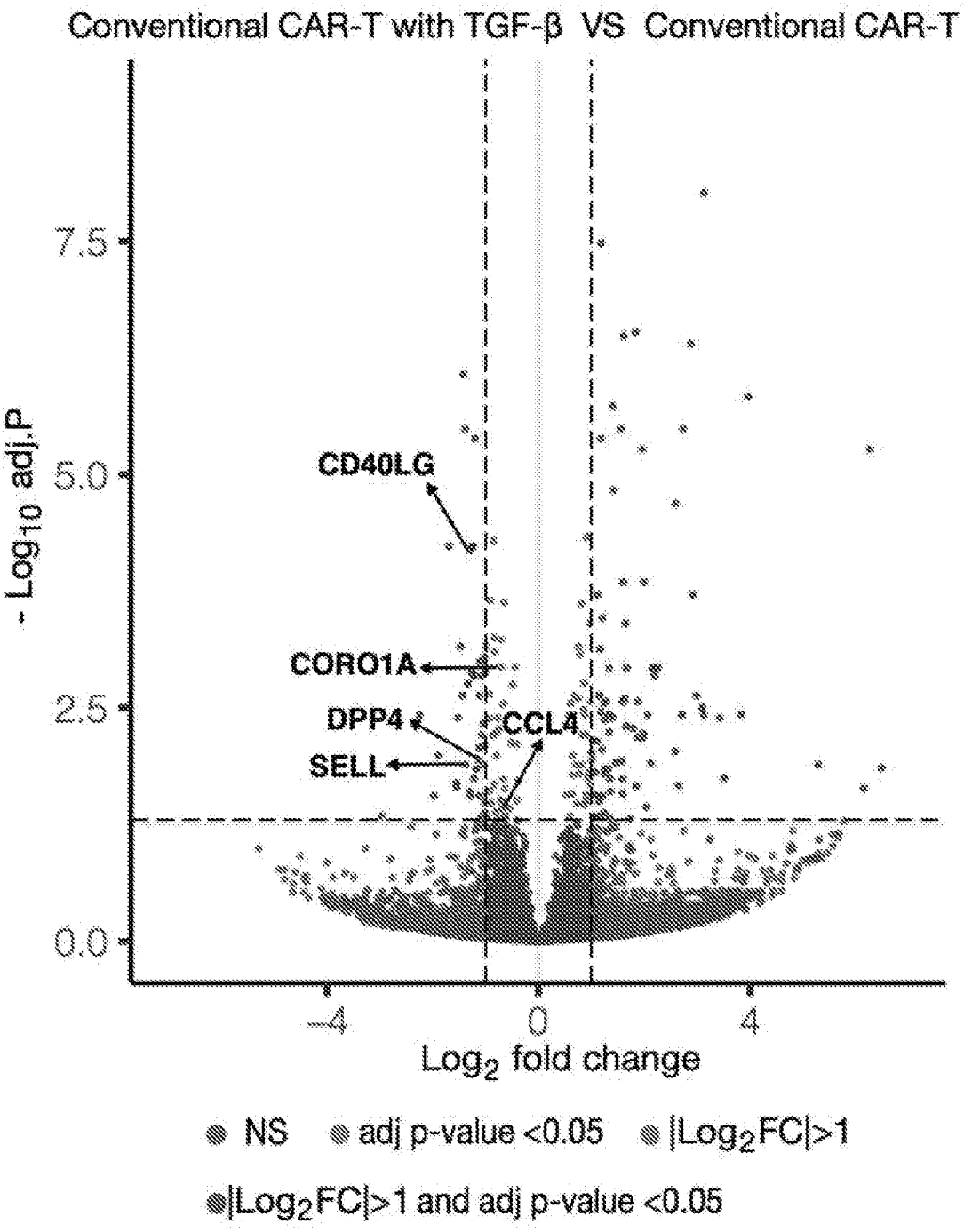
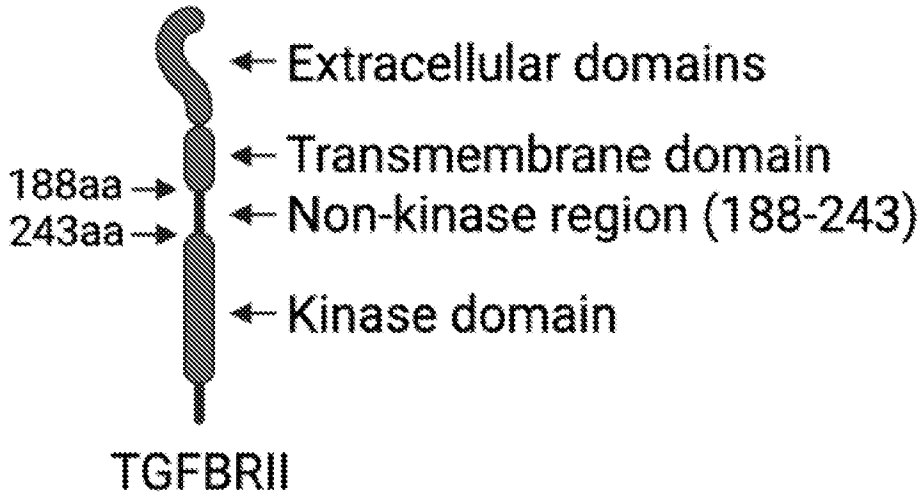


FIGURE 12

A.


Extracellular domains
Transmembrane domain
188aa
243aa
Non-kinase region (188-243)
Kinase domain
TGFBRII

B.

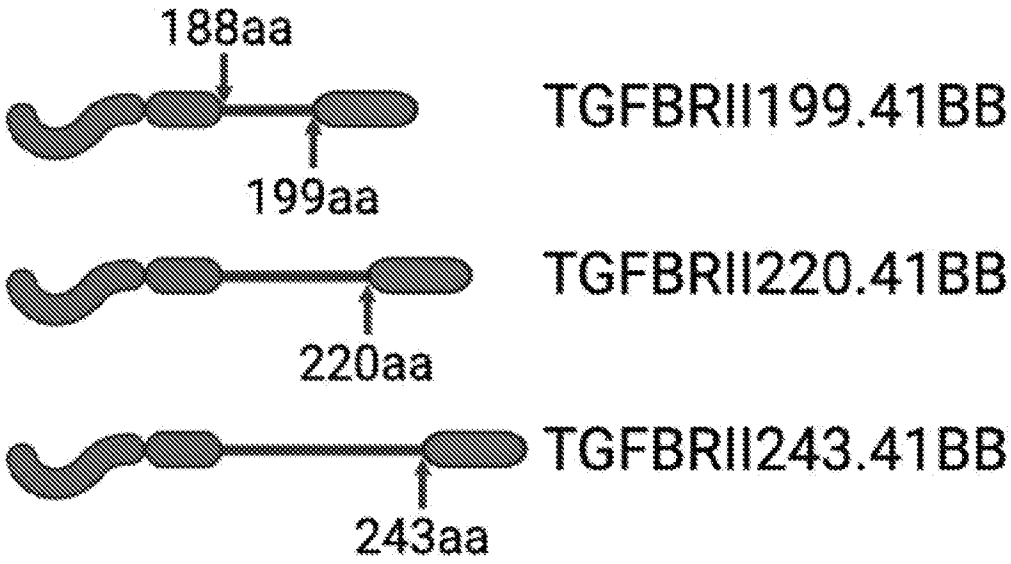

188aa
TGFBRII199.41BB
199aa
TGFBRII220.41BB
220aa
TGFBRII243.41BB
243aa

FIGURE 12 (continued)

C.

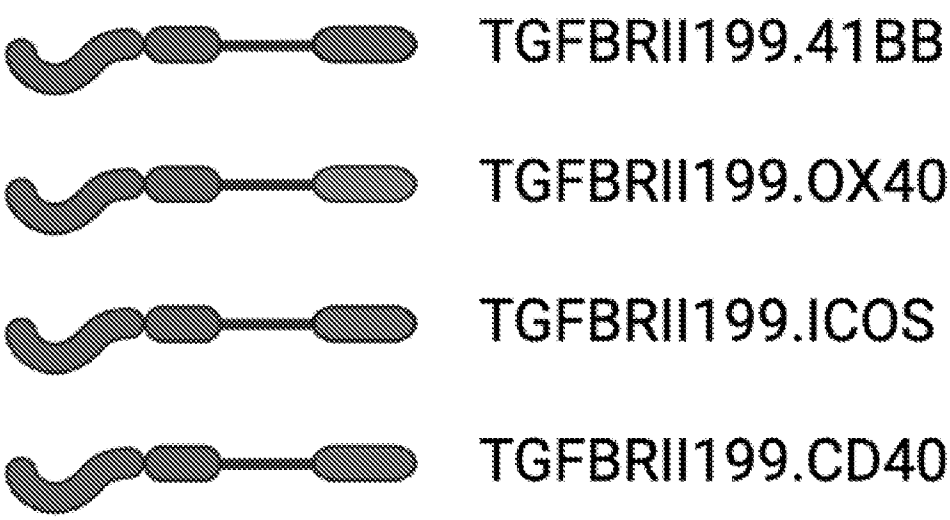


FIGURE 13

A.

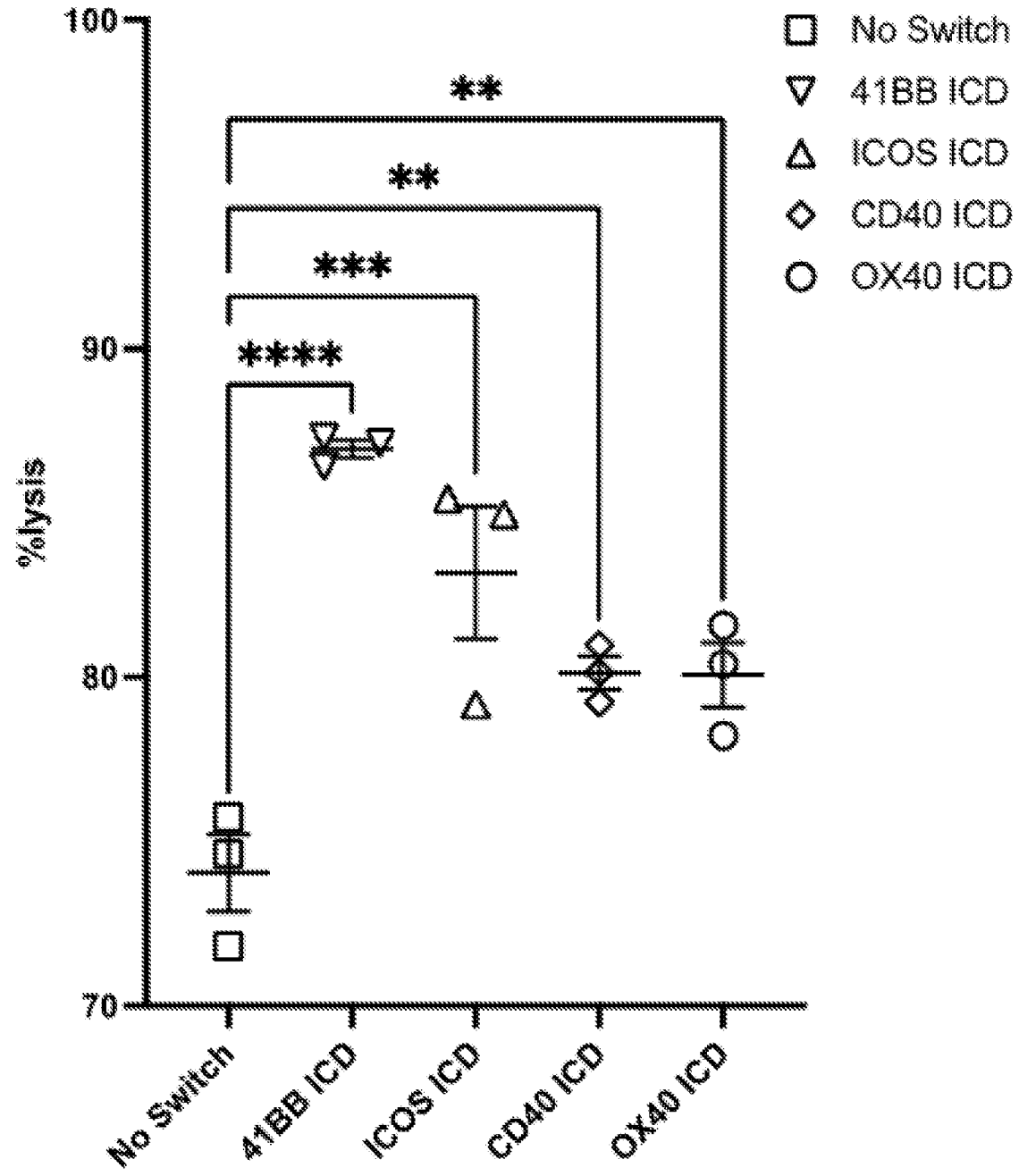

100
90
80
70
%lysis
**
**
***
****
No Switch
41BB ICD
ICOS ICD
CD40 ICD
OX40 ICD
No Switch
41BB ICD
ICOS ICD
CD40 ICD
OX40 ICD

FIGURE 13 (continued)

B.

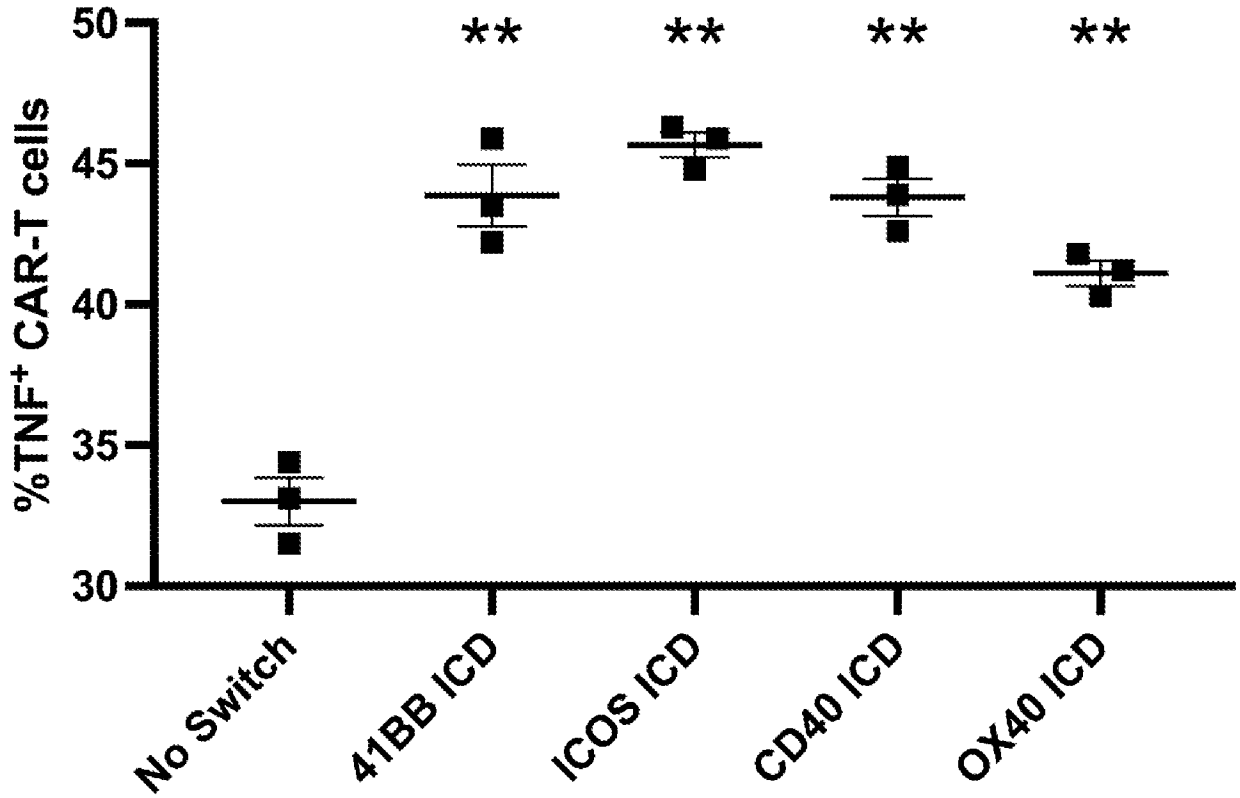


FIGURE 14

A.

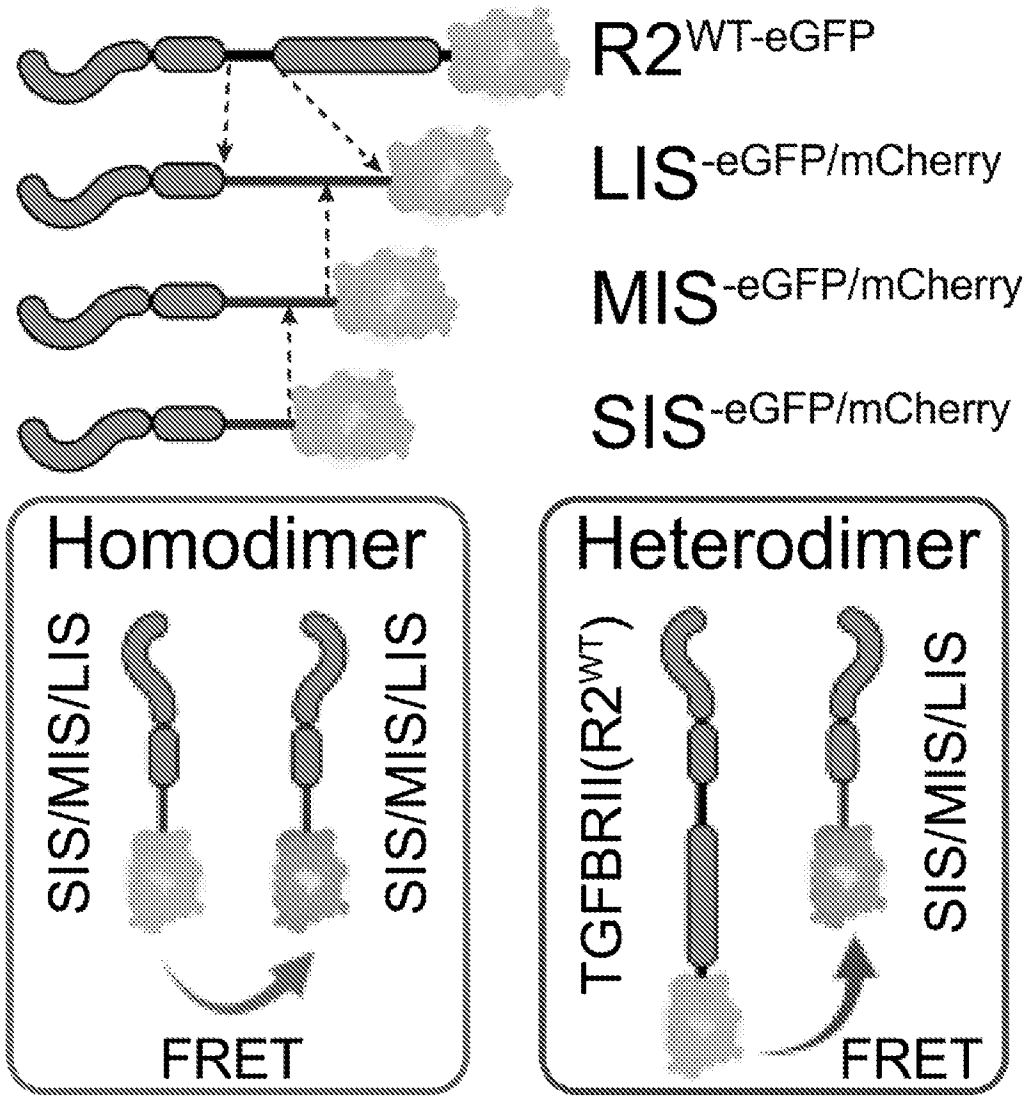

R2WT-eGFP
LIS-eGFP/mCherry
MIS-eGFP/mCherry
SIS-eGFP/mCherry
Homodimer
SIS/MIS/LIS
SIS/MIS/LIS
FRET
Heterodimer
TGFBRII(R2WT)
SIS/MIS/LIS
FRET

FIGURE 14 (continued)

B.

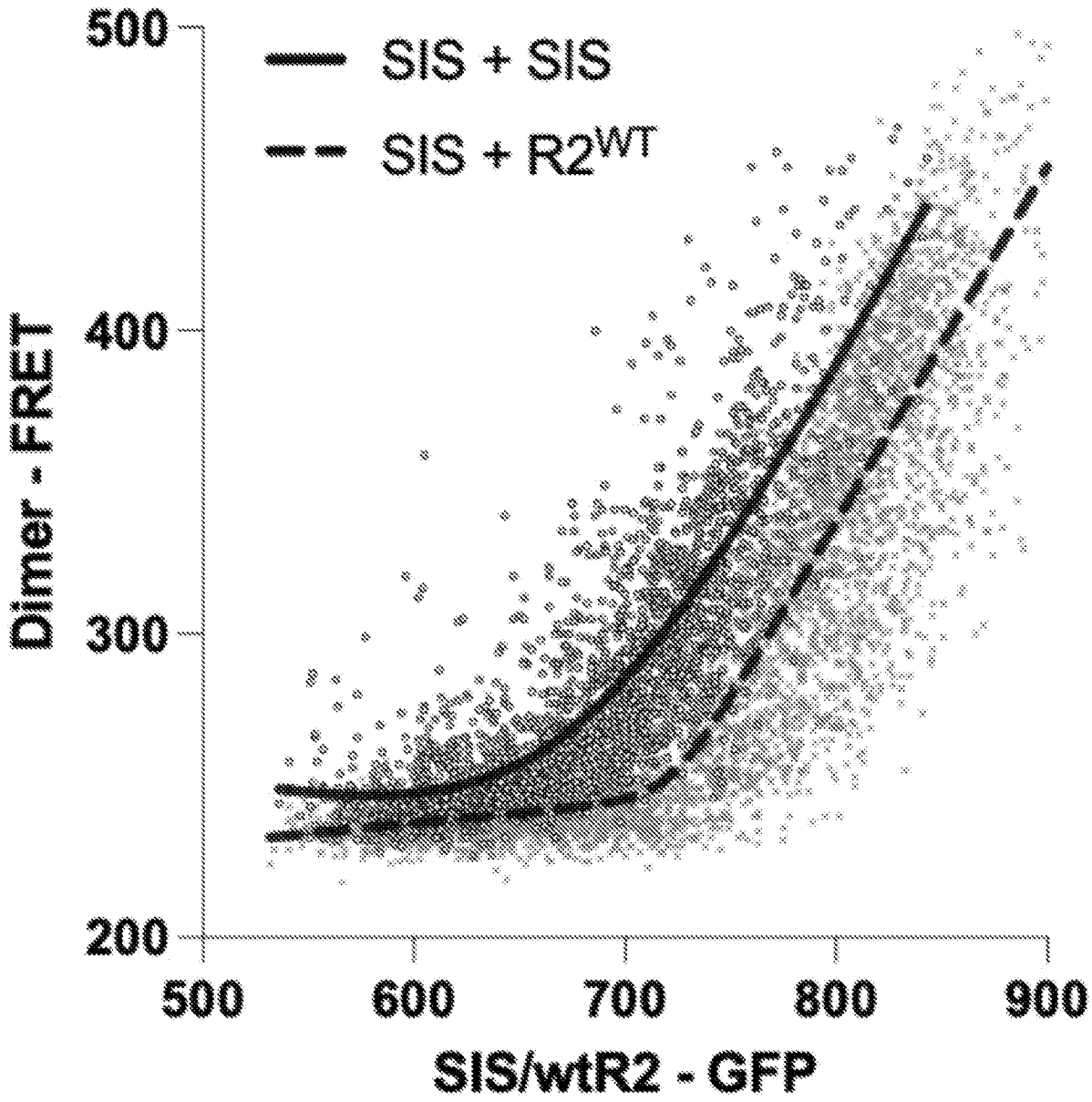


FIGURE 14 (continued)

C.

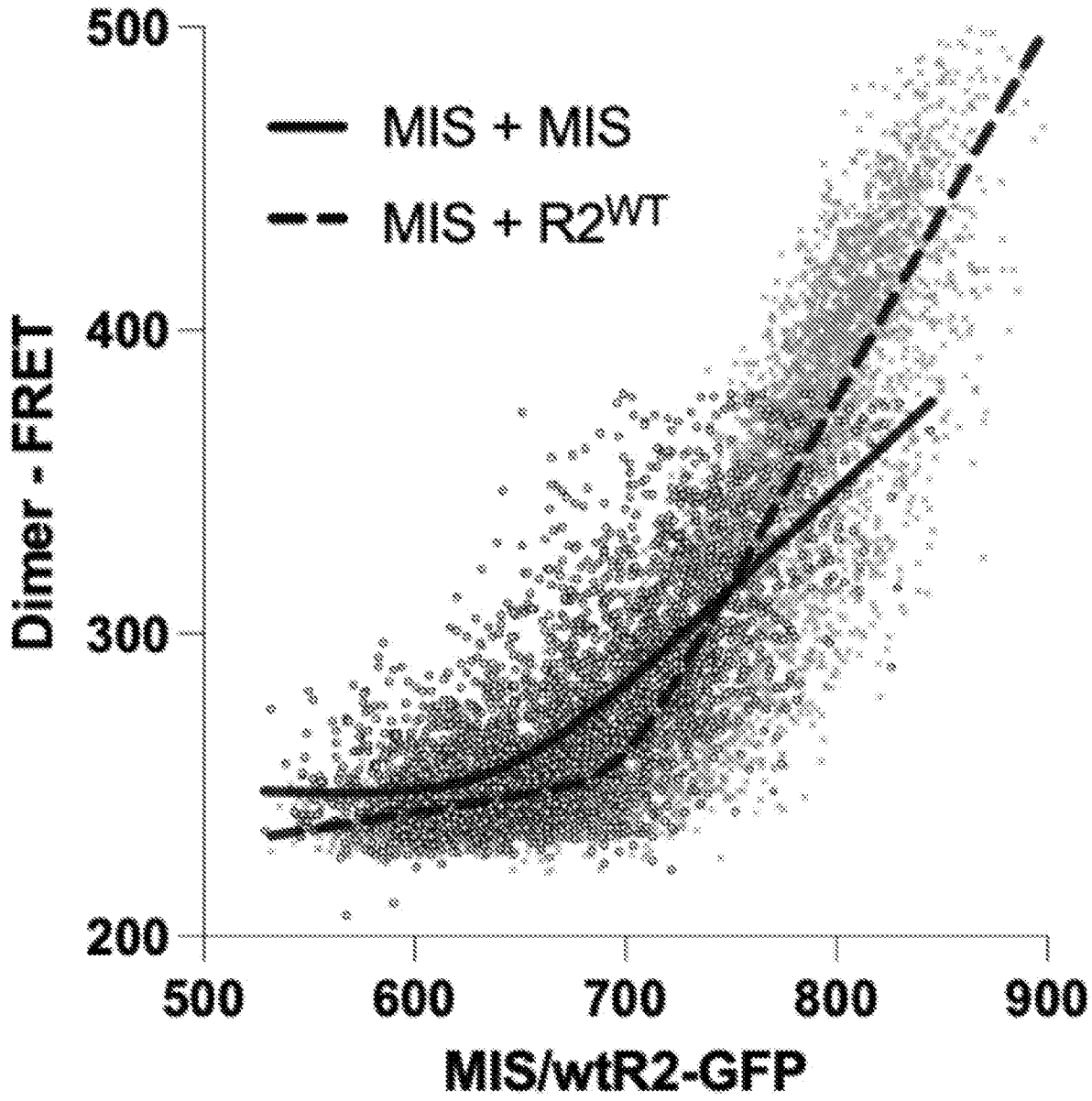


FIGURE 14 (continued)

D.

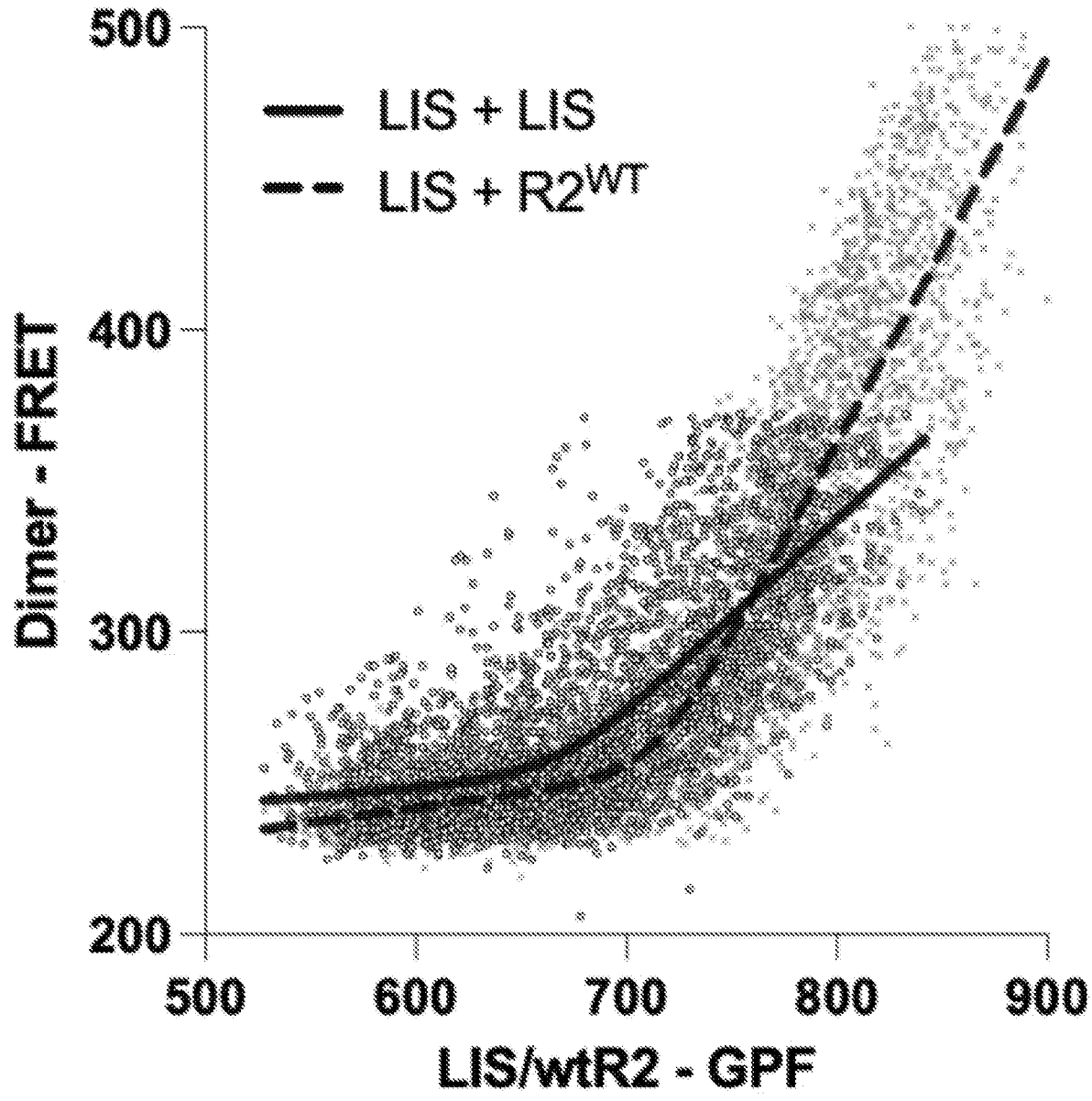


FIGURE 15

A.

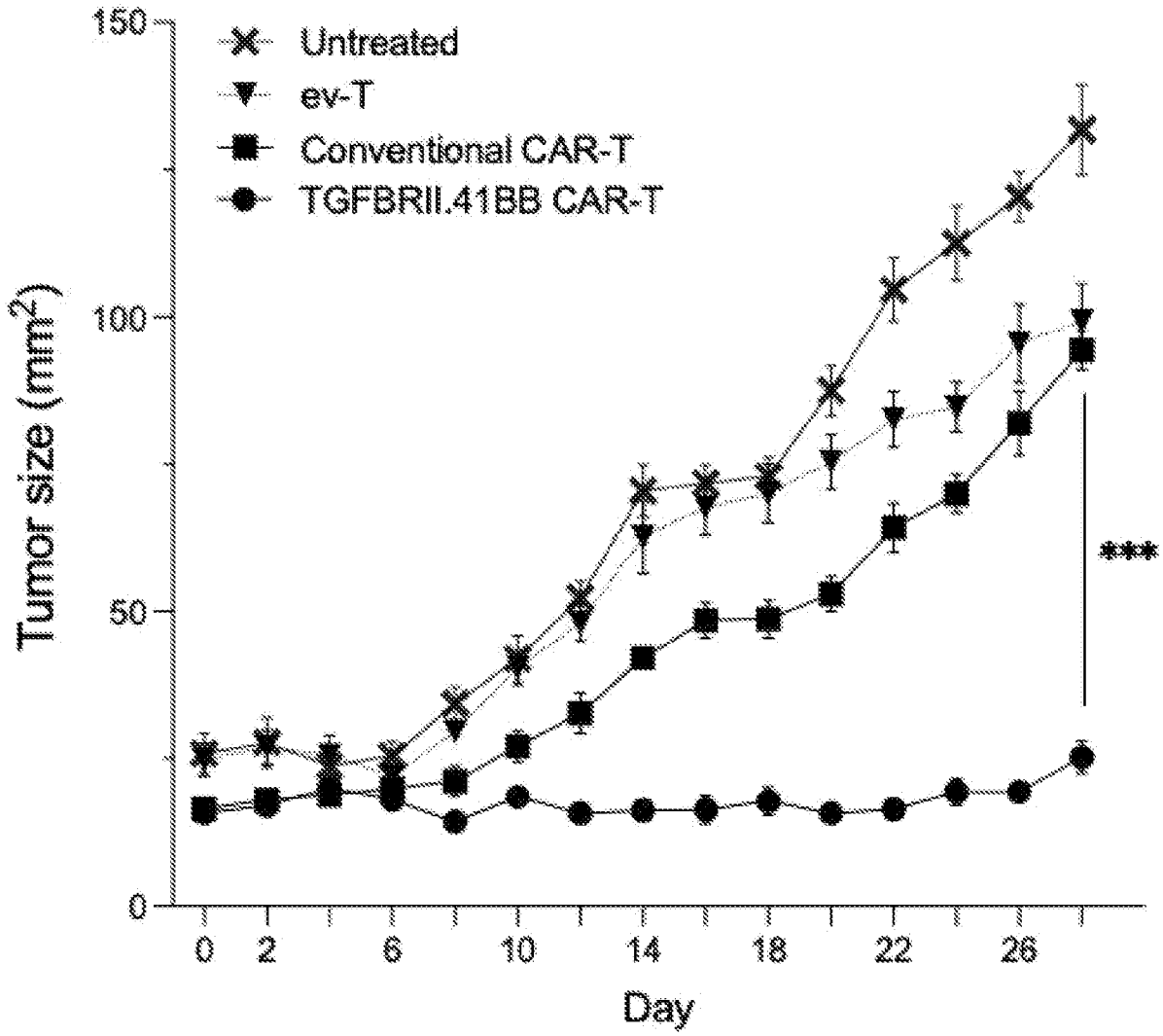
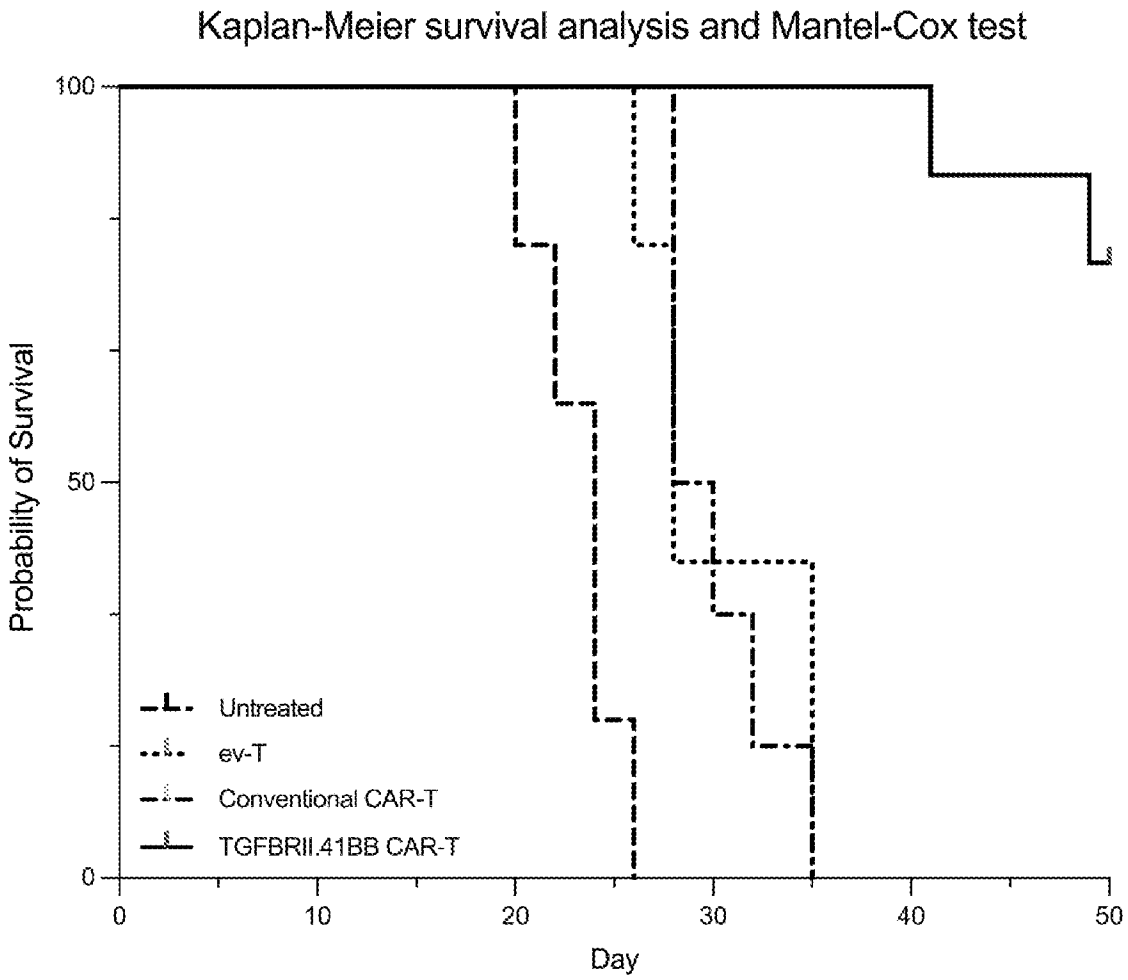

Untreated
ev-T
Conventional CAR-T
TGFBRII.41BB CAR-T
Tumor size (mm2)
150
100
50
0
0
2
6
10
14
18
22
26
Day
***

FIGURE 15 (continued)

B.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2023/051150

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/71 (2006.01) ***A61K 38/00 (2006.01)*** ***A61K 38/18 (2006.01)*** ***A61K 39/00 (2006.01)*** ***A61P 35/00 (2006.01)***
C07K 14/715 (2006.01) ***C07K 14/725 (2006.01)*** ***C12N 5/0783 (2010.01)***

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: Registry, Caplus: Sequence search based on residues 1-187 of SEQ ID NO:1 (the extracellular domain of TGFβRII)

GenomeQuest: Claim 1, Residues 1-187 of SEQ ID NO:1 at 100% Sequence identity, results limited to those with a sequence length not equal to 567 residues. Claim 8, Sequence ID NOs: 11-16 at 90% sequence identity.

Preliminary search tool provided by IP Australia, Esp@cenet and Google Scholar: Peter Maccallum Cancer Institute (Applicant); Neeson, P (Inventor).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2024	22 January 2024
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Colton Payne AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 3 9935 9609

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2023/051150**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2020/186219 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 17 September 2020 SEQ ID NO:51, sequence listing; claims 58-59, 63, 73, 93; pages 163-167, 169, and 174	1-22
X	WO 2022/218402 A1 (NANJING LEGEND BIOTECH CO., LTD.) 20 October 2022 SEQ ID NOs: 20-22, and 50; Examples 1-2; claims 33-37 and 42-45; pages 127-131, 141-142,153, and 169-170	1-22
X	WO 2022/232277 A1 (TCR2 THERAPEUTICS INC.) 03 November 2022 SEQ ID NO: 285; claims 42, 87, 89-91, 104, 106, and 110-113; pages 193-194, 205, and 208-211	1-22
X	WO 2022/006451 A2 (TCR2 THERAPEUTICS INC.) 06 January 2022 SEQ ID NOs: 283 and 285, sequence listing; claims 160-161, 398-399, 507, and 519-523; pages 269-270, 291, and 300-301	1-22
X	WO 2019/173324 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 12 September 2019 SEQ ID NO:125, sequence listing; claims 40, 47, and 90-92; pages 267, 269, and 274	1-22
X	WO 2019/118508 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 20 June 2019 SEQ ID NO: 18; claims 36, 38, and 113; pages 47, 135, and 144	1-22
X	WO 2022/056321 A1 (TCR2 THERAPEUTICS INC.) 17 March 2022 SEQ ID NOs: 216 and 218, sequence listing	1 and 5-8
P,X	WO 2023/034220 A2 (TCR2 THERAPEUTICS INC.) 09 March 2023 Sequence listing; Table 6; claims 113, 180-182, and 210-212; pages 271, 289, 294, and 297	1, 5-11, and 18-22
P,X	WO 2023/288281 A2 (FRED HUTCHINSON CANCER CENTER) 19 January 2023 Sequence listing; claims 34-35, 56, 74-75, 81, and 101-102; pages 171, 182-183, and 187	1, 5-6, 9-16, and 18-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2023/051150

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2023/051150**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2023/051150**

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End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.