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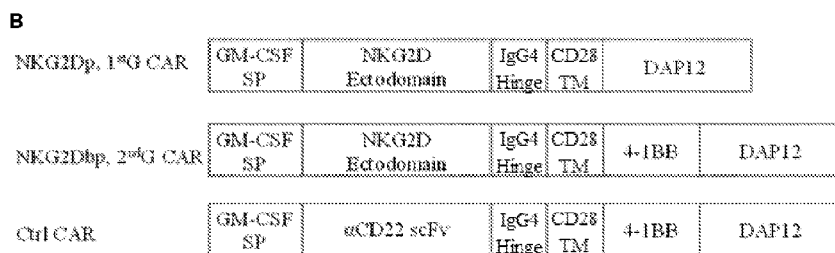


Fig. 2

(57) Abstract: The present invention refers to a $\alpha\beta$ T cell genetically modified to express a recombinant chimeric antigen receptor (CAR), wherein the recombinant CAR comprises (a) an extracellular domain comprising an antigen binding region, (b) a transmembrane domain, (c) a co-stimulatory signaling domain, and (d) an intracellular signaling domain, wherein the extracellular domain comprises the extracellular domain of an NKG2D receptor, and wherein the intracellular signaling domain comprises one immunoreceptor tyrosine-based activation motif (ITAM); the recombinant CAR used to modify the $\alpha\beta$ T cell; a pharmaceutical composition comprising the genetically modified $\alpha\beta$ T cell; method of treating cancer or tumor using the genetically modified $\alpha\beta$ T cell; and method of preparing the genetically modified $\alpha\beta$ T cell. In one embodiment, the genetically modified $\alpha\beta$ T cell comprises a CAR comprising the extracellular domain of NKG2D receptor, an IgG4 hinge region, a CD28 transmembrane domain, a 4-1 BB co-stimulatory signaling domain and a single ITAM-containing DAP 12 domain.



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T CELL MODIFIED WITH A SYNTHETIC RECEPTOR CONTAINING A SINGLE ITAM SIGNALING MOTIF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of the Singapore application No. 10201908256R, filed on 6 September 2019, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of biotechnology and cell therapy. In particular, the present invention relates to recombinant chimeric antigen receptors, genetically modified $\alpha\beta$ T cell for cancer immunotherapy, their preparation method and use in patients in need thereof.

BACKGROUND OF THE INVENTION

[0003] Recent years have seen tremendous progress in utilizing immune effector cells for cancer therapy. Cancer immunotherapy employing genetically engineered T lymphocytes that express chimeric antigen receptors (CARs) is an effective approach in treating several haematological cancers. CARs are composed of an extracellular antigen-binding domain, typically the single-chain variable fragment (scFv) derived from a monoclonal antibody, and an intracellular signaling domain. T cells are white blood cells that play a central role in immune responses that do not involve antibodies, otherwise known as the adaptive immune system. The adaptive immune system uses a combination of a diverse set of specialized receptors expressed on the surface of T-cells to provide protection against different pathogens. $\alpha\beta$ T cells are a type of T cell that express the alpha-beta T-cell receptor (TCR), and forms the majority of human T cells. $\alpha\beta$ T cells have the capacity for immunologic memory as they have the ability to adapt and recognize processed antigenic peptide presented on the multihistocompatibility complex (MHC). This immunologic memory arises from genetic recombination of the α and β chains of thymocytes in the thymus, thereby giving rise to a diverse repertoire of $\alpha\beta$ T cells and their ability to distinguish a vast array of peptides. Therefore, adoptive cell transfer therapy with $\alpha\beta$ T cells is one of the most promising immunotherapeutic modalities for cancer patients.

[0004] However, the approach has not been rather successful due to the risk of cytokine release syndrome (CRS). Despite recent success in clinical trials, several major limitations are associated with CAR-modified T cells. For example, CAR-T cell therapy holds an inherent risk of cytokine release syndrome (CRS), which is a systemic inflammatory response caused by the large, rapid release of cytokines when CAR-T cells are activated. Cytokine release syndrome (CRS) can result in various symptoms that can range from mild symptoms such as fever, nausea or rash, to being potentially life-threatening.

[0005] Furthermore, the translation of $\alpha\beta$ T cells therapy to non-haematological malignancies is challenging due to the special pathophysiological characteristics of solid tumors, including target antigen heterogeneity, obstacles to CAR immune cell trafficking, and intrinsic negative regulatory mechanisms of tumor microenvironment.

[0006] In addition, the need to generate an autologous CAR-T cell product for each individual patient is logistically demanding and restrictive for a wider adoption in the medical practice. Furthermore, it is not always possible to collect enough lymphocytes from heavily pretreated patients to generate sufficient quantities of CAR-T cells. An allogeneic “off-the-shelf” product could overcome these challenges, but allogeneic T cells pose significant risk of graft-versus-host disease (GvHD).

[0007] Therefore, it is an objective of the present invention to provide improved recombinant chimeric antigen receptors and improved $\alpha\beta$ T cells for cancer immunotherapy, which address some or all of the above mentioned problems.

SUMMARY

[0008] In one aspect, there is provided a $\alpha\beta$ T cell genetically modified to express a recombinant chimeric antigen receptor (CAR), wherein the recombinant CAR comprises (a) an extracellular domain comprising an antigen binding region, (b) a transmembrane domain, (c) at least one co-stimulatory signaling domain, and (d) an intracellular signaling domain, wherein the extracellular domain comprises the extracellular domain of an NKG2D receptor, and wherein the intracellular signaling domain comprises one immunoreceptor tyrosine-based activation motif (ITAM).

[0009] In another aspect, there is provided a pharmaceutical composition comprising a pharmaceutically effective amount of the $\alpha\beta$ T cell of the present invention and a pharmaceutically acceptable excipient.

[0010] In a further aspect, there is provided a method of treating cancer or tumor in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of the $\alpha\beta$ T cell of the present invention, or the pharmaceutical composition of the present invention.

[0011] In yet a further aspect, there is provided a method of treating cancer or tumor in a subject in need thereof, the method comprises: (i) obtaining $\alpha\beta$ T cells from the subject, or from a $\alpha\beta$ T cell donor which is different from the subject to be treated; (ii) providing a recombinant nucleic acid encoding a recombinant chimeric antigen receptor (CAR), wherein the recombinant CAR comprises (a) an extracellular domain comprising an antigen binding region, (b) a transmembrane domain, (c) at least one co-stimulatory signaling domain, and (d) an intracellular signaling domain, wherein the extracellular domain comprises the extracellular domain of an NKG2D receptor, and wherein the intracellular signaling domain comprises one immunoreceptor tyrosine-based activation motif (ITAM); (iii) transferring the recombinant nucleic acid encoding the recombinant CAR into the $\alpha\beta$ T cell to obtain genetically modified $\alpha\beta$ T cells; and (iv) administering to the subject a pharmaceutically effective amount of the $\alpha\beta$ T cells obtained from (iii).

[0012] In yet a further aspect, there is provided a method of preparing the $\alpha\beta$ T cell of the present invention, the method comprises: (i) obtaining or providing $\alpha\beta$ T cell; (ii) providing a recombinant nucleic acid encoding the recombinant chimeric antigen receptor (CAR); and (iii) transferring the recombinant nucleic acid encoding the recombinant CAR into the $\alpha\beta$ T cell.

[0013] In yet a further aspect, there is provided a recombinant chimeric antigen receptor (CAR) comprising (a) an extracellular domain comprising an antigen binding region, (b) a transmembrane domain, (c) at least one co-stimulatory signaling domain, and (d) an intracellular signaling domain, wherein the extracellular domain comprises the extracellular domain of an NKG2D receptor, and wherein the intracellular signaling domain comprises one immunoreceptor tyrosine-based activation motif (ITAM).

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0015] **Fig. 1** is an image showing the overall schematic of the different CAR-T construct and the effects on T cell activity and cytokine release. The different domains that make up the CAR-T constructs are provided in the legend.

[0016] **Fig. 2** is a collection of **Fig. 2A – Fig. 2F** showing the characterization of NKG2D CAR-T cells bearing a DAP12 activating domain. **Fig. 2A** is a set of schematic representation of the overview of the design concepts for the CAR constructs as described in the present disclosure. **Fig. 2B** is a set of schematic representation of three different CAR constructs: (*top*) NKG2Dp – first generation CAR construct with a granulocyte-macrophage colony-stimulating factor signal peptide (GM-CSF SP) domain, a NKG2D ectodomain, IgG4 hinge domain, a CD28 transmembrane (CD28 TM) domain and a DAP12 activating domain; (*middle*) NKG2Dbp – second generation CAR construct with a GM-CSF SP domain, a NKG2D ectodomain, IgG4 hinge domain, a CD28 TM domain, a 4-1BB co-stimulatory signaling domain and a DAP12 activating domain; (*bottom*) control (Ctrl) CAR – second generation anti-CD22 CAR construct with a GM-CSF SP domain, an α CD22 scFv ectodomain, IgG4 hinge domain, a CD28 TM domain, a single 4-1BB co-stimulatory signaling domain and a single DAP12 activating domain. **Fig. 2C** is a schematic representation of the pZTS4-Ef1a-NKG2D-IgG4H-CD28TM-DAP12 vector map. **Fig. 2D** is a line graph showing the antigen-dependent expansion of NKG2Dp CAR-T cells and NKG2Dbp CAR-T cells. The expansion of the two groups of CAR-T cells are performed with γ -irradiated parental K562 feeder cells that was driven by DAP12 activation and enhanced by 4-1BB co-stimulation. Data shown are cell numbers obtained from Trypan blue exclusion assay on day 17, 27 and 37 post DNA electroporation from one donor, representative of three independent experiments. The results show that both NKG2Dp and NKG2Dbp CAR-T cells underwent robust expansion in response to antigenic stimulation from K562 cells, with NKG2Dbp CAR-T cells showing higher fold of expansion after day 27. **Fig. 2E** is an image of two histogram diagrams that show the intensity of CAR expression and proportion of NKG2Dp CAR-T cells (left) and NKG2Dbp CAR-T cells (right). Within each CAR group, CAR expression intensity (mean fluorescence intensity (MFI) values) and proportion of CAR-expressing cells (%) were analyzed in the middle (area under the curve is colored black) and end (area under the curve is colored grey) of the CAR-T cell expansion phase. Unstained sample (black line with no color under the curve) is used as a background control. Within each CAR group, the histogram diagrams from middle and end of expansion

phase were superimposed to illustrate the enrichment of CAR-expressing T cells. The results show that the intensity of CAR expression and proportion of NKG2Dp CAR-T cells and NKG2Dbp CAR-T cells increased consecutively upon repeated cultures with K562 feeder cells. **Fig. 2F** is a column chart that shows the percentages of effector T cells, effector memory T cells, central memory T cells and stem cell memory T cells in the middle and at the end of the expansion phase. Memory T cell development was characterized for each NKG2D CAR group through detection of both CCR7 and CD45RA antigens. Effector T cell is CCR7⁻CD45RA⁺; effector memory T cell is CCR7⁺CD45RA⁻; central memory T cell is CCR7⁺CD45RA⁺; and stem cell memory T cell is CCR7⁻CD45RA⁻. Within each CAR group, composition of memory T subsets was analyzed both in the middle and at the end of each expansion phase and arranged side-by-side to illustrate the divergence in memory T subset development. The results show that the inclusion of 4-1BB co-stimulatory signaling domain in NKG2Dbp CAR-T cells enhanced the development of CCR7⁺/CD45RA⁻ effector memory T cells.

[0017] **Fig. 3** is a collection of **Fig. 3A** – **Fig. 3C** showing the effects of the NKG2Dp CAR-T cell and NKG2Dbp CAR-T cell in a mouse model of HCT116 human colorectal (CRC) cells. **Fig. 3A** shows photos of bioluminescent images of four groups of mice. The mice received intraperitoneal (i.p.) injection of 2×10^6 HCT116 cells (day 0) followed by i.p. injection of PBS, control CAR-T cells, NKG2Dp CAR-T cells, NKG2Dbp CAR-T cells on day 7 and day 30 (2×10^6 cells per mouse). Growth of HCT116 was monitored by bioluminescent imaging on the indicated days. Bioluminescent images of 5 mice per group are shown. A cross represents death or termination of the mouse by euthanasia. **Fig. 3B** is a line graph that shows the bioluminescence flux values from each mouse of respective groups from **Fig. 3A** to monitor growth of tumor. **Fig. 3C** is a line graph that shows the survival plot of the mice from **Fig. 3A**. The data was analyzed by the Kaplan-Meier method. The differences in survival were then compared using the log-rank test. ****P < 0.0001. The graph shows that all the mice treated with NKG2Dbp CAR-T cells continued to survive up to Day 90 of treatment. The results demonstrate that mice that received NKG2Dp CAR-T cells or NKG2Dbp CAR-T cells showed reduction in tumor burden on day 14 after receiving a single dose T cell injection. However, from day 21 onwards, tumor burden for mice that received NKG2Dp CAR-T cells increased progressively, and although a second CAR T cell injection was given at day 30, the tumor burden did not decrease. Mice that received

NKG2Dp CAR-T cells injection were all euthanized by day 62. In contrast, tumor burden for the group of mice that received NKG2Dbp CAR-T cells showed progressive reduction and became undetectable up to day 62.

[0018] **Fig. 4** is a collection of **Fig. 4A – Fig. 4C** showing the characterisation of T-cell modified with different NKG2D CAR constructs. **Fig. 4A** is a set of schematic representation of three different CAR constructs: (*top*) NKG2Dbz – second generation CAR construct with a GM-CSF SP domain, a NKG2D ectodomain, IgG4 hinge domain, a CD28 TM domain, a 4-1BB co-stimulatory signaling domain and a full length CD3zeta activating domain; (*middle*) NKG2Dbz1 – second generation CAR construct with a GM-CSF SP domain, a NKG2D ectodomain, IgG4 hinge domain, a CD28 TM domain, a 4-1BB co-stimulatory signaling domain and a CD3zeta activating domain consisting the first ITAM only; (*bottom*) NKG2Dbp – second generation CAR construct with a GM-CSF SP domain, a NKG2D ectodomain, IgG4 hinge domain, a CD28 transmembrane (CD28 TM), a 4-1BB co-stimulatory signaling domain and a DAP12 activating domain. A ST2 (streptomycin tag II) tag was included to detect CAR expression for flow cytometry analysis. **Fig. 4B** is an image of four scatter plots that represent flow cytometry plots showing the expression of ST2 (NKG2Dbz, NKG2Dbz1 or NKG2Dbp CAR-T cells) 7 days after electroporation in T cells. The flow cytometry plots show the efficiency of T cell electroporated with NKG2D CARs using a Piggybac transposon system. Efficiency ranged about 50-80%. **Fig. 4C** is an image of four scatter plots that represent flow cytometry plots showing the expression of ST2 in electroporated T-cells after co-culture with K562#6 artificial antigen presenting cells (aAPC) at day 28. The results show that the expression of CARs in T cells can be enriched upon co-culture with K562#6.

[0019] **Fig. 5** is a collection of **Fig. 5A – Fig. 5B** showing T cell memory subsets in CAR-T cells electroporated with the three different NKG2D CAR constructs shown in **Fig. 4A**. **Fig. 5A** is an image of three scatter plots that represent flow cytometry plots showing the expression of CD45RO (x-axis) and CCR7 (y-axis). Staining of CD45RO and CCR7 showed that relative to NKG2Dbz with three ITAM motifs, the two CARs with a single ITAM (i.e. NKG2Dbz1 and NKG2Dbp CAR constructs), increased the fraction of effector memory CAR T cells and reduced the proportion of effector cells in response to *in vitro* stimulation with K562C6. T cells electroporated with NKG2Dbz, NKG2Dbz1 or NKG2Dbp CAR were subjected to three rounds of K562#6 artificial antigen presenting cells (aAPC) stimulation

before determining the percentage of T cell memory subsets based on CD45RO and CCR7 staining. Representative FACS staining shown for one PBMC donor. **Fig. 5B** depicts bar charts that show the percentage of each T cell memory subtypes from three different donors modified with either NKG2Dbz, NKG2Dbz1 or NKG2Dbp. Majority of the T cells showed an effector memory subtype.

[0020] **Fig. 6** is a collection of **Fig. 6A – Fig. 6C** showing the function of CAR-T cells electroporated with NKG2Dbz, NKG2Dbz1 or NKG2Dbp CAR constructs, measured in terms of cytolytic activity, cytokine release and cell expansion. **Fig. 6A** shows the cytolytic activity of the modified CAR-T cells against HCT116 colorectal cancer cell lines. HCT116 was labelled with DELFIA BATDA reagent (DELFLIA® EuTDA Cytotoxicity Reagents, Perkin Elmer) followed by co-culture with CAR-T cells at indicated effector to target ratios. Cytotoxicity assay was carried out over 4 hours (left) and 16 hours (right), % cytotoxicity is then calculated by measuring Europium release signal from the target tumor cells using a plate reader. The results shown are based on one representative experiment out of two independent experiments with 2 different donors. The results show that no difference in killing was observed in T-cell modified with three different NKG2D CAR constructs. **Fig. 6B** shows the cytokine release of CAR-T cells after co-culture with HCT116. Supernatants were collected to determine cytokine production by the CBA (Cytometric Bead Array) Human Th1/Th2 Cytokine Kit (BD Biosciences). Data shown in bar graph are mean $\pm$ SD for 3 cytokines in supernatants tested with CAR-T cells from 3 different PBMC donors. The results show that NKG2Dbz CAR-T cells in general promoted higher amounts of IL-2, IFN- γ , and TNF- α production, as compared to NKG2Dbz1 and NKG2Dbp CAR-T cells, although the statistical differences were significant ($p < 0.05$) only between NKG2Dbz-T and NKG2Dbz1-T for IL-2. The results also show that between NKG2Dbz1-T cells and NKG2Dbp-T cells, the latter stimulated the production of IL-2 and IFN- γ at lower levels ($p < 0.05$). **Fig. 6C** shows the expansion profile of the CAR-T cells enumerated at two different time points: (top) from Day 14 to Day 21; and (bottom) from Day 21 to Day 28. The CAR-T cells were expanded with K562C6 cells in the presence or absence of exogenous IL-2. It was observed that in the absence of exogenous IL-2, cell numbers were consistently lower in the CAR-T samples modified with NKG2Dbz1 and NKG2Dbp, as compared with those modified with NKG2Dbz, indicating that NKG2Dbz1 and NKG2Dbp CAR-T cells proliferated less than NKG2Dbz CAR-T without the support of exogenous IL-2. Nevertheless, in the presence of

exogenous IL-2 (300 IU/ml), there were no obvious differences in the antigen-stimulated CAR-T cell proliferation between the three types of CAR-T cells.

[0021] **Fig. 7** is a collection of **Fig. 7A** and **Fig. 7B** showing the function of CAR-T cells electroporated with NKG2Dbz or NKG2Dbp CAR constructs, measured in terms of cytolytic activity and IFN- γ production. **Fig. 7A** shows collection of four line graphs showing the percentage cytotoxicity of control, NKG2Dbz or NKG2Dbp CAR-T cells against solid tumor cell lines SKOV3, HCT116, U87 or FaDu. SKOV3, HCT116, U87 or FaDu were labelled with BATDA dye (DELFI[®] EuTDA Cytotoxicity Reagents, Perkin Elmer) followed by co-culture with CAR-T cells at indicated effector to target ratio. Cytotoxicity was calculated after 4 hours by measurement of Europium release signal using a plate reader. The results show that there were no observable differences in killing efficiency between NKG2Dbz and NKG2Dbp CAR-T cell groups. **Fig. 7B** shows one photo and one column chart that shows interferon-gamma (IFN- γ) release from NKG2Dbz or NKG2Dbp CAR-T cells against HCT116 human CRC cell line in each well. The photo shows IFN- γ production by control T cells (left column), NKG2Dbz CAR-T cells (middle column) and NKG2Dbp CAR-T cells (right column). IFN- γ production is shown by the number of spots per well. NKG2Dbz CAR-T cells or NKG2Dbp CAR-T cells were co-cultured with HCT116 at 1:1 ratio overnight to assess IFN- γ production using human IFN- γ Elispot kit (Mabtech). Number of spots per well were acquired by a plate reader and number of spots per well were plotted in a column chart. ****; $P < 0.0001$. The results indicate that NKG2Dbz CAR-T cells showed higher IFN- γ release compared to NK2Dbp CAR-T cells.

[0022] **Fig. 8** shows a collection of 10 column charts showing the levels of different cytokines released when control, NKG2Dbz or NKG2Dbp CAR-T cells were used against HCT116 human CRC cell lines. The cytokines measured are granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10) and interleukin 12 (IL-12). NKG2Dbz or NKG2Dbp CAR-T cells were co-cultured with HCT116 at 1:1 ratio overnight to access a panel of cytokine production. Concentration of cytokines were measured in 16 hour supernatants by LUNARIS[™] Human 11-Plex Cytokine Kit (Ayoxxa Biosystems). Shown in column charts are the mean $\pm$ standard deviation (SD) for 10 out of 11 cytokines tested from three different peripheral blood mononuclear cells (PBMC) donors. Statistical difference *

($P < 0.05$ denoted by asterisk) only for IFN- γ , TNF- α and IL-2 analysis between NKG2Dbz CAR-T cells vs NKG2Dbp CAR-T cells. This shows that HCT116 treated NKG2Dbp CAR-T cells has less IFN- γ , TNF- α and IL-2 release as compared to HCT116 treated with NKG2Dbz CAR-T cells.

[0023] **Fig. 9** is a collection of **Fig. 9A – Fig. 9C** showing the effects of NKG2Dbp or NKG2Dbz CAR-T cells in a mouse model of HCT116 human CRC Cells. **Fig. 9A** is an image showing the timeline and experimental outline for the investigation of NKG2Dbp CAR-T cells and NKG2Dbz CAR-T cells anti-tumor effect in an i.p HCT116 xenograft model. Four groups of mice received i.p. injection of 2×10^6 HCT116 cells (day 0) followed by i.p. injection of PBS, control CAR-T cells, NKG2Dbz CAR-T cells or NKG2Dbp CAR-T cells on day 7 (1×10^7 cells per mouse). Growth of HCT116 was monitored by bioluminescent imaging on the indicated days. **Fig. 9B** shows photos of bioluminescent images of four groups of mice from the experiment as described in Fig. 9A. The bioluminescent images of 5 mice per group are shown. Based on the images taken on Day 14, 21 and 28, the tumor sizes are smaller in NKG2Dbz CAR-T cells and NKG2Dbp CAR-T cells treated mice as compared to PBS and control T-cell treated mice. **Fig. 9C** is a line graph that shows the bioluminescence flux values on Day 7 and Day 28 from each mice of respective groups from Fig. 9B to monitor growth of tumor. Taken together, the results show that NKG2Dbp and NKG2Dbz CAR-T cells can reduce the size of the peritoneal tumor.

[0024] **Fig. 10** is a collection of **Fig. 10A – Fig. 10E** showing the effects of NKG2Dbp and NKG2Dbz human CAR-T cells on mice and death caused by graft versus host disease (GvHD). **Fig. 10A** shows photos of bioluminescent images of two groups of mice treated with NKG2Dbp and NKG2Dbz CAR-T cells in a mouse model of HCT116 human colorectal (CRC) cells. The growth of HCT116 was monitored by bioluminescent imaging on day 7 up to day 70. Mice from NKG2Dbz CAR-T cells group were euthanized starting from day 35 onwards due to evidence of xenogeneic GvHD despite negligible tumor burden based on bioluminescent imaging. A cross (“X”) represents death or termination of the mouse by euthanasia. (Criteria of xenogeneic GvHD included $>15\%$ weight loss, hunched posture, ruffled fur, reduced mobility). **Fig. 10B** is a line graph that shows the survival plot of the mice that are monitored up to 150 days post tumor inoculation. The data was analysed by the Kaplan-Meier method. **Fig. 10C** shows the serum cytokine concentration obtained from mice treated with either NKG2Dbp or NKG2Dbz CAR-T cells. It was observed that treatment with

NKG2Dbp CAR-T cells triggered significantly lower level secretions of the cytokines IL-2, TNF- α and IFN- γ . **Fig. 10D** shows the hematoxylin-eosin (H&E) staining of liver collected at euthanization. Multiple irregular patchy areas of hepatic coagulative necrosis were observed in liver sections from NKG2Dbz-T treated mice but not in those from NKG2Dbp-T treated mice. The pathological necrosis was characterized by hepatocellular loss and absence of nuclei, being accompanied with the infiltration of mononuclear inflammatory cells and bile duct hyperplasia. **Fig. 10E** shows that in mice treated with NKG2Dbz CAR-T cells, alopecia (which is one of the symptoms of GvHD) can be observed. In contrast, there were no signs of alopecia in mice treated with NKG2Dbp CAR-T cells.

[0025] **Fig. 11** is an image of three scatter plots that represent flow cytometry plots showing consecutive gating strategy (from left to right): based on cell morphology (left), CD3 and $\alpha\beta$ TCR expression levels (middle) and ST2 and NKG2D levels (right). The dots in the area marked up by dotted lines represent the cells that are selected for gating. 51.5% of the cells were gated based on cell morphology, wherein 94.9% of the gated cells T cells are CD3+ and express $\alpha\beta$ TCR. 93.2% of the gated cells expressing CD3 and $\alpha\beta$ TCR also express exogenous NKG2D CAR (ST2 and NKG2D double positive cells). Endogenous T cells (2.9%) express NKG2D and are not ST2 positive.

[0026] **Fig. 12** is a collection of **Fig. 12A** – **Fig. 12C** showing the effects of NKG2Dbp CAR-T cells in a mouse model of SKOV3 human ovarian cancer cells. **Fig. 12A** is an image showing the timeline and experimental outline for the investigation of the anti-tumor effect of NKG2Dbp CAR-T cells in an i.p SKOV3 xenograft model. Three groups of mice received i.p. injection of 1×10^7 SKOV3 cells (day 0) followed by i.p. injection of PBS, control CAR-T cells, or NKG2Dbp CAR-T cells on day 7 and day 14 (1×10^7 cells per injection per mouse). Growth of SKOV3 was monitored by bioluminescent imaging on the indicated days. **Fig. 12B** shows photos of bioluminescent images of three groups of mice from the experiment as described in **Fig. 12A**. The bioluminescent images of 5 mice per group are shown. Based on the images taken on Day 14, 28, 35 and 42, the SKOV3 tumor sizes are negligible in NKG2Dbp CAR-T cells treated mice as compared to PBS and control T-cell treated mice. A cross (“X”) represents death or termination of the mouse by euthanasia. **Fig. 12C** is a line graph that shows the survival plot of the mice from **Fig. 12B**. The data was analyzed by the Kaplan-Meier method. The differences in survival were then compared using the log-rank

test. $P < 0.01$. The graph shows that all the mice treated with NKG2Dbp CAR-T cells continued to survive up to Day 80 of treatment.

DEFINITIONS

[0027] A “genetically modified cell” means any cell of any organism that is modified, transformed, or manipulated by addition or modification of a gene, a DNA or RNA molecule, or protein or polypeptide.

[0028] A “T cell” is a type of lymphocyte which develops in the thymus gland and plays a central role in the immune response. T cells can be distinguished from other lymphocytes by the presence of a T-cell receptor on the cell surface. These immune cells originate as precursor cells, derived from bone marrow, and develop into several distinct types of T cells once they have migrated in to the thymus gland. T cells were initially grouped into a series of subsets based on their function, but also have been grouped into subsets based on associated gene or protein expression patterns. The T-cell receptor (TCR) is a molecule found on the surface of T cells, and is responsible for recognizing fragments of antigen as peptides bound to major histocompatibility complex (MHC) molecules. The TCR is composed of two different protein chains (i.e., it is a heterodimer). In humans, in the majority of the T cells the TCR consists of an alpha (α) chain and a beta (β) chain (encoded by TRA and TRB, respectively), thus being referred to as $\alpha\beta$ T cells. In a small portion of the T cells the TCR consists of a gamma (γ) and a delta (δ) chain (encoded by TRG and TRD, respectively), thus being referred to as $\gamma\delta$ T cells.

[0029] The term “chimeric antigen receptor” or the short form “CAR” as used herein refers to artificial receptor proteins, or chimeric immunoreceptors, and encompass engineered receptors that graft an artificial specificity onto a particular immune effector cell. CARs may be employed to impart the specificity of a monoclonal antibody onto a T cell, thereby allowing a large number of specific T cells to be generated, for example, for use in adoptive cell therapy. CARs typically comprise an intracellular activation domain, a transmembrane domain, and an extracellular domain comprising an antigen binding region. CARs can combine antibody-based specificity for a desired antigen with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific cellular immune activity such as anti-tumor cellular immune activity. In some cases, molecules can be co-

expressed with the CAR, including co-stimulatory molecules, reporter genes for imaging, gene products that conditionally ablate the T cells upon addition of a pro-drug, homing receptors, chemokines, chemokine receptors, cytokines, and cytokine receptors.

[0030] As used herein, the term "antigen" is a molecule capable of being bound by an antibody or cell surface receptor. An antigen may generally be used to induce a humoral immune response and/or a cellular immune response leading to the production of lymphocytes.

[0031] Natural-killer group 2, member D, also known as Klrk1 (NKG2D), is a C-type lectin-like receptor, which was firstly identified in NK cells as an activating immune receptor. NKG2D recognizes eight stress-induced ligands belonging to two families: two MHC class I chain-related proteins MICA and MICB and six HCMV UL16-binding proteins (ULBP1-6). These NKG2D ligands are not usually present on the cell surface of most healthy tissues, but can be up-regulated upon DNA damage, infection and transformation of cells, thus being commonly detected on hematopoietic tumors and carcinomas. NKG2D is a type II transmembrane glycoprotein, which does not contain any known signaling elements in the intracellular domain. Resembling many activating receptors, NKG2D depends on an adaptor molecule to initiate signaling transduction and cellular activation. In human, NKG2D is not only expressed by all NK cells, but is also expressed by all CD8⁺ T cells, and subsets of $\gamma\delta$ ⁺ T cells as a co-stimulatory receptor. NKG2D expression and signaling can be regulated by cytokines and tumor-derived factors. Cytokines, such as IL-2, IL-7, IL-12, IL-15, and type I interferons (IFNs) increase cell surface expression of NKG2D. Cytokines such as IL-21, IFN- γ , and TGF- β have been shown to decrease NKG2D expression. IL-21 has been reported to reduce expressions of NKG2D in human CD8⁺ T cells and NK cells.

[0032] The terms "polynucleotide", "nucleic acid" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogues thereof. Polynucleotides can have any three-dimensional structure and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified

nucleotides, such as methylated nucleotides and nucleotide analogues. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labelling component. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0033] The term “recombinant nucleic acid” as used herein refers to nucleic acid formed by laboratory methods of genetic recombination (such as molecular cloning) to bring together genetic material from multiple sources. The nucleic acid sequences used in the construction of recombinant nucleic acid molecules can originate from any species. For example, human nucleic acid may be joined with bacterial nucleic acid. In addition, nucleic acid sequences that do not occur anywhere in nature may be created by the chemical synthesis of nucleic acid, and incorporated into recombinant molecules. Proteins that can result from the expression of recombinant nucleic acid within living cells are termed recombinant proteins. When recombinant nucleic acid encoding a protein is introduced into a host organism, the recombinant protein is not necessarily produced. Expression of foreign proteins requires the use of specialized expression vectors and often necessitates significant restructuring by foreign coding sequences.

[0034] As used herein, the term “vector” refers to a non-chromosomal nucleic acid comprising an intact replicon such that the vector may be replicated when placed within a permissive cell, for example by a process of transformation. A vector may replicate in one cell type, such as bacteria, but have limited ability to replicate in another cell, such as mammalian cells. Vectors may be viral or non-viral. Exemplary non-viral vectors for delivering nucleic acid include naked DNA; DNA complexed with cationic lipids, alone or in combination with cationic polymers; anionic and cationic liposomes; DNA-protein complexes and particles comprising DNA condensed with cationic polymers such as heterogeneous polylysine, defined-length oligopeptides, and polyethylene imine, in some cases contained in liposomes; and the use of ternary complexes comprising a virus and polylysine-DNA.

[0035] The term “transfection” as used herein refers to the general process by which exogenous nucleic acid is transferred or introduced into the host cell, said process can be mechanical transfection (including electroporation), chemical transfection or viral transduction. A “transfected” cell is one which has been transfected with exogenous nucleic acid using any of the above mentioned methods. The cell includes the primary subject cell and its progeny.

[0036] As used herein, the term “autologous” is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

[0037] As used herein, the term “allogeneic” refers to any material derived from an individual other than the individual to which it is later to be introduced into.

[0038] As used herein, "percent identity" refers to sequence identity between two peptides or between two nucleic acid molecules. Percent identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are identical at that position.

[0039] As used herein, the terms “peptide”, “polypeptide”, and “protein” are used interchangeably, and refer to a compound having amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can include a protein's or peptide's sequence. Polypeptides include any peptide or protein having two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides, and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. “Polypeptides” include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

[0040] As used herein, the phrase "homologous" or "variant" nucleotide sequence, or "homologous" or "variant" amino acid sequence refers to sequences characterized by identity, at the nucleotide level or amino acid level, of at least a specified percentage. Homologous nucleotide sequences include those sequences coding for naturally occurring allelic variants

and mutations of the nucleotide sequences set forth herein. Homologous nucleotide sequences include nucleotide sequences encoding for a protein of a mammalian species other than humans. Homologous amino acid sequences include those amino acid sequences which contain conservative amino acid substitutions and which polypeptides have the same binding and/or activity. In some examples, a homologous nucleotide or amino acid sequence has at least 60% or greater, for example at least 70%, or at least 75%, or at least 80%, or at least 85%, or at least 90%, or at least 95%, or at least 98%, or at least 99%, with a comparator sequence. In some examples, a homologous nucleotide or amino acid sequence has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity with a comparator sequence. In some examples, a homologous amino acid sequence has no more than 15, or no more than 10, or no more than 5 or no more than 3 conservative amino acid substitutions. Percent identity can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for UNIX, Genetics Computer Group, University Research Park, Madison Wis.), using default settings, which uses the algorithm of Smith and Waterman. In some examples, the recombinant nucleic acid molecules used to modify the T cells according to the present disclosure are homologous to the exemplary nucleotide sequences disclosed herein, such as the sequences provided in any of SEQ ID NO: 1. In some other examples, the chimeric antigen receptor (CAR) expressed in the modified T cells are homologous to the exemplary amino acid sequences disclosed herein, such as the sequences provided in any of SEQ ID NO: 16.

[0041] The term “express” or “expression” refers to the production of a gene product in a cell.

[0042] The term “transient” when referred to expression means a polynucleotide is not incorporated into the genome of the cell. In contrast, the term “stable” when referred to expression means a polynucleotide is incorporated into the genome of the cell. Transient expression can occur from introduced constructs which contain expression signals functional in the host cell, but which constructs do not replicate and rarely integrate in the host cell, or where the host cell is not proliferating. Transient expression also can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, although such inducible systems frequently exhibit a low basal level of expression. Stable expression can be achieved by introduction of a nucleic acid construct that can integrate into the host genome or that autonomously replicates in the host cell. Stable expression of the

gene of interest can be selected for through the use of a selectable marker located on or transfected with the expression construct, followed by selection for cells expressing the marker. When stable expression results from integration, integration of constructs can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination with the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus. To achieve expression in a host cell, the transformed nucleic acid is operably associated with transcriptional and translational initiation and termination regulatory regions that are functional in the host cell.

[0043] The term “tumor” as used herein refers to a swelling of a part of the body, generally without inflammation, caused by an abnormal growth of tissue. Tumors can be benign or malignant (i.e. cancerous). A benign tumor does not invade nearby tissue or spread to other parts of the body. Common types of benign tumors include adenomas, fibromas (or fibroids), hemangiomas, lipomas, meningiomas, myomas, neuromas, and osteochondromas. Adenomas are benign tumors starting in the epithelial tissue of a gland or gland-like structure. A common type of adenoma is a polyp in the colon. Adenomas might also grow in the liver or the adrenal, pituitary, or thyroid gland. Fibromas (or fibroids) are tumors of fibrous or connective tissue that can grow in any organ. Hemangiomas are a buildup of blood vessel cells in the skin or internal organs. Lipomas grow from fat cells. They are the most common benign tumor in adults, often found in the neck, shoulders, back, or arms. Meningiomas are tumors that develop from the membrane surrounding the brain and spinal cord. Myomas are tumors that grow from muscle. Neuromas are tumors that develop from the nerves. Osteochondromas are tumors that develop from the bones.

[0044] The term “cancer” as used herein refers to an unregulated proliferation of cells due to loss or normal controls, resulting in unregulated growth, lack of differentiation, local tissue invasion, and, often, metastasis. There are several main types of cancer. Carcinoma is a cancer that begins in the skin or in tissues that line or cover internal organs. Sarcoma is a cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Leukemia is a cancer that starts in blood-forming tissue, such as the bone marrow, and causes large numbers of abnormal blood cells to be produced and enter the blood. Lymphoma and multiple myeloma are cancers that begin in the cells of the immune

system. Central nervous system cancers are cancers that begin in the tissues of the brain and spinal cord.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0045] The inventors of the present application have found that after genetically modifying T cells, in particular $\alpha\beta$ T cells, to express a chimeric antigen receptor (CAR) comprising at least one co-stimulatory signaling domain and an intracellular signaling domain comprising only one immunoreceptor tyrosine-based activation motif (ITAM), the genetically modified T cells display enhanced cell expansion, reduced cytokine release, as well as reduced xenogeneic graft versus host disease (GvHD).

[0046] Thus, in one example, the present invention refers to a recombinant chimeric antigen receptor (CAR), wherein the recombinant CAR comprises (a) an extracellular domain comprising an antigen binding region, (b) a transmembrane domain, (c) at least one co-stimulatory signaling domain, and (d) an intracellular signaling domain, wherein the extracellular domain comprises the extracellular domain of an NK cell activating receptor or a scFv fragment of a monoclonal antibody of an NK cell activating receptor, and wherein the intracellular signaling domain comprises one immunoreceptor tyrosine-based activation motif (ITAM). In some examples, the intracellular signaling domain of the recombinant CAR comprises only one ITAM, or not more than one ITAM. In some examples, the ITAM is a DAP12 ITAM.

[0047] The antigen recognition domain includes a polypeptide that is selective for or targets an antigen, receptor, peptide ligand, or protein ligand of the target; or a polypeptide of the target. The antigen recognition domain may be obtained from any of the wide variety of extracellular domains or secreted proteins associated with ligand binding and/or signal transduction. The antigen recognition domain may include a portion of Ig heavy chain linked with a portion of Ig light chain, constituting a single chain fragment variable (scFv) that binds specifically to a target antigen. The antibody may be monoclonal or polyclonal antibody or may be of any type that binds specifically to the target antigen. In another embodiment, the antigen recognition domain can be a receptor or ligand.

[0048] The choice of the antigen binding region depends upon the type and number of ligands that define the surface of a target cell. For example, the antigen binding region may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated

with a particular disease state. Examples of cell surface markers that can act as ligands for the antigen binding region in the CARs include those associated with or specific to cancer and/or tumor cells, autoimmune diseases, and viral, bacterial and parasitic infections.

[0049] In some examples of CARs, the **antigen binding region** of the extracellular domain binds to a tumor associated antigen, a tumor specific antigen, or a pathogen-specific antigen. Cells comprising such CARs can then be redirected to the tumor associated antigen, tumor specific antigen or a pathogen-specific antigen based on the antigen binding specificity. A tumor specific antigen is unique to tumor cells and does not occur on other cells in the body. A tumor associated antigen is not unique to a tumor cell and instead is also expressed on a normal cell under conditions that fail to induce a state of immunologic tolerance to the antigen. The expression of the antigen on the tumor may occur under conditions that enable the immune system to respond to the antigen. Tumor associated antigens can be antigens that are expressed on normal cells during fetal development when the immune system is immature and unable to respond or they may be antigens that are normally present at extremely low levels on normal cells but which are expressed at much higher levels on tumor cells. Non-limiting examples of tumor associated or tumor specific antigens include the following: NKG2D, differentiation antigens such as MART-1/MelanA (MART-1), gp100 (Pmel 17), tyrosinase, TRP-1, TRP-2 and tumor-specific multilineage antigens such as MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, p15; overexpressed embryonic antigens such as CEA; overexpressed oncogenes and mutated tumor-suppressor genes such as p53, Ras, HER-2/neu; unique tumor antigens resulting from chromosomal translocations such as BCR-ABL, E2A-PRL, H4-RET, 1GH-IGK, MYL-RAR; and viral antigens such as the Epstein Barr virus antigens EBVA and the human papillomavirus (HPV) antigens E6 and E7. Other large, protein-based antigens include TSP-180, MAGE-4, MAGE-5, MAGE- 6, RAGE, NY-ESO, pl85erbB2, pl80erbB-3, c-met, nm-23H1, PSA, TAG-72, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, beta-Catenin, CDK4, Mum-1 , p15, p16, 43-9F, 5T4, 791Tgp72, alpha-fetoprotein, beta-HCG, BCA225, BTAA, CA 125, CA 15-3\CA 27.29\BCAA, CA 195, CA 242, CA-50, CAM43, CD68\I, CO-029, FGF-5, G250, Ga733VEpCAM, HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB/70K, NY-CO-1, RCAS1, SDCCAG16, TA-90\Mac-2 binding protein, Acyclophilin C-associated protein, TAAL6, TAG72, TLP, and TPS.

[0050] In some examples, the CAR recognizes cell-surface tumor associated antigen or tumor specific antigen independent of human leukocyte antigen (HLA) and employs one or

more signaling molecules to activate the genetically modified T cells for killing, proliferation, and/or cytokine production.

[0051] In some examples, the antigen binding region of the extracellular domain of the CARs target an antigen that includes but is not limited to CD19, CD20, CD22, ROR1, Mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, MY-ESO-1 TCR, MAGE A3 TCR, and the like.

[0052] In some specific examples, the tumor associated antigen is a ligand of NKG2D, and therefore the extracellular domain of the chimeric antigen receptor comprises NKG2D or an antigen binding fragment thereof.

[0053] In one example, the antigen binding fragment of NKG2D is or comprises the extracellular domain of NKG2D having the following amino acid sequence (from the N-terminal to the C-terminal):

FNQEVQIPLTESYCGPCPNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVY
SKEDQDLLKLKLSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIEMQKGDALYAS
SFKGYIENCSTPNTYICMQRTV (SEQ ID NO: 6) (UniProt P26718, amino acid residues 83-216), encoded by the following exemplary nucleotide sequence: 5'-
TTCAACCAAGAAGTTCAAATTCCCTTGACCGAAAGTTACTGTGGCCCATGTCCTA
AAAAGTGGATATGTTACAAAAATAACTGCTACCAATTTTTTGATGAGAGTAAAAA
CTGGTATGAGAGCCAGGCTTCTTGTATGTCTCAAATGCCAGCCTTCTGAAAGTA
TACAGCAAAGAGGACCAGGATTTACTTAAACTGGTGAAGTCATATCATTGGATG
GGACTAGTACACATTCCAACAAATGGATCTTGGCAGTGGGAAGATGGCTCCATT
CTCTCACCCAACCTACTAACAATAATTGAAATGCAGAAGGGAGACTGTGCACTCT
ATGCCTCGAGCTTTAAAGGCTATATAGAAAAGTGTCAACTCCAAATACGTACAT
CTGTATGCAAAGGACTGTG-3' (SEQ ID NO: 5). NKG2D ligands are structural homologs of MHC class I molecules. NKG2D ligands are absent or rarely expressed in normal tissues, but are extensively expressed in various malignancies and viral-infected tissues. Examples of human NKG2D ligands include class-I-related chains-related molecules A and B (MICA and MICB) proteins and retinoic acid early transcripts-1 (RAET1), also known as UL-16 binding proteins. Examples of mouse NKG2D ligands include five different RAET1 isoforms (RAET1 α , RAET1 β , RAET1 γ , RAET1 δ , and RAET1 ϵ), three different H60 isoforms (H60a, b, and c), and UL16 binding protein 1 (encoded by *MULT1* gene). Although NKG2D ligands are structural homologs of MHC class I molecules, they do not

present antigen to T cells or bind β 2-microglobulin. In some examples, the NKG2D ligands for which the extracellular domain of the chimeric antigen receptor binds to are membrane-bound ligands.

[0054] The extracellular domain of a chimeric antigen receptor generally also comprises a hinge region. The hinge region is a sequence positioned between for example, the **antigen binding region**, and the transmembrane domain. The sequence of the hinge region can be obtained from, for example, any suitable sequence from any genus, including human or a part thereof. In some examples, the hinge region includes the hinge region of a human protein including CD-8 alpha, CD28, 4-1BB, OX-40, T cell receptor α or β chain, a CD3 ζ chain, CD28, CD3e, CD45, CD4, CD5, CD8, CD8a, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, ICOS, CD154, functional derivatives thereof, and combinations thereof. In one specific example, the hinge region includes the CD-8 alpha hinge region. In one specific example, the CD-8 alpha hinge has the following amino acid sequence (from the N-terminal to the C-terminal): FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD (SEQ ID NO: 21), encoded by the following exemplary nucleotide sequence: 5'-TTCGTGCCCGGTCTTCCTGCCAGCGAAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCACCATCGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGGCGTGCCGGCCAGCGGCGGGGGGCGCAGTGCACACGAGGGGGCTGGAC-3' (SEQ ID NO: 22). In some examples, the hinge region can be one selected from, but is not limited to, immunoglobulin (e.g. IgG1, IgG2, IgG3, IgG4, and IgD). In one example, the hinge region is an IgG4 hinge region. In one specific example, the IgG4 hinge region has the following amino acid sequence (from the N-terminal to the C-terminal): ESKYGPPCPSCP (SEQ ID NO: 8) (UniProtKB - P01861 hinge domain, amino acid residues 99-110), encoded by the following exemplary nucleotide sequence: 5'-GAATCTAAATATGGCCCCCCTTGCCCACCATGTCCC-3' (SEQ ID NO: 7).

[0055] It is understood that the antigen binding region may include some variability within its sequence and still be selective for the targets disclosed herein. Therefore, it is contemplated that the polypeptide of the antigen binding region may be at least 95%, at least 90%, at least 80%, or at least 70% identical to the antigen binding region polypeptide sequences disclosed herein and still be selective for the targets described herein and be within the scope of the disclosure.

[0056] The transmembrane domain of a CAR includes a hydrophobic polypeptide that spans the cellular membrane. In particular, the transmembrane domain spans from one side of a cell membrane (extracellular) through to the other side of the cell membrane (intracellular or cytoplasmic).

[0057] In some examples, the transmembrane domain is artificially designed so that more than 25%, more than 50% or more than 75% of the amino acid residues of the domain are hydrophobic residues such as leucine and valine.

[0058] The transmembrane domain may be in the form of an alpha helix or a beta barrel, or combinations thereof. The transmembrane domain may include a polytopic protein, which has many transmembrane segments, each alpha-helical, beta sheets, or combinations thereof.

[0059] In one example, the transmembrane domain that is naturally associated with one of the domains in the CAR is used. In another example, the transmembrane domain is selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

[0060] For example, a transmembrane domain includes a transmembrane domain of a T-cell receptor α or β chain, a CD3 ζ chain, CD3 ϵ , CD8, CD45, CD4, CD5, CD7, CD9, CD16, CD22, CD28, CD33, CD37, CD64, CD80, CD86, CD68, CD134, CD137, ICOS, CD41, CD154, functional derivatives thereof, and combinations thereof. In one specific example, the transmembrane domain is a CD28 transmembrane domain. In one specific example, the transmembrane domain of the CAR has the following amino acid sequence (from the N-terminal to the C-terminal): FWVLVVVGVLACYSLLVTVAFIIFWV (SEQ ID NO: 10), encoded by the following exemplary nucleotide sequence: 5'-TTCTGGGTTTTGGTAGTTGTCGGCGGGGTGCTTGCTTGCTATTCATTGTTGGTTACGGTGGCTTTTATAATTTTCTGGGTA-3' (SEQ ID NO: 9).

[0061] The intracellular signaling domain of a chimeric antigen receptor is responsible for activation of at least one of the normal effector functions of the immune cell in which the chimeric antigen receptor has been placed. The term "effector function" refers to a specialized function of a differentiated cell, such as a T cell, or more specifically a $\alpha\beta$ T cell. The intracellular signaling domain known in the art generally includes multiple immunoreceptor tyrosine based activation motifs (ITAMs). However, the chimeric antigen receptors (CARs) of the present invention only contain one ITAM. The inventors found that

T cells, in particular $\alpha\beta$ T cells, that are genetically modified to express CARs containing only one ITAM in the intracellular signaling domain release lower levels of cytokines as compared to cells expressing CARs containing more than one ITAM in the intracellular signaling domain. Such genetically modified T cells or $\alpha\beta$ T cells in particular results in lower risk of xenogeneic graft versus host disease (GvHD) as compared to T cells or $\alpha\beta$ T cells expressing CARs containing more than one ITAM in the intracellular signaling domain. Each ITAM possesses two repeats of the consensus sequence Tyr-X-X-Leu/Ile (X being any amino acid) spaced by six to eight amino acids. The tyrosine residues within ITAM become phosphorylated following interaction of the receptor molecules with their ligands and form docking sites for other proteins involved in the signaling pathways of the cell. Examples of intracellular signaling domains containing only one ITAM include but are not limited to, DAP12, the cytoplasmic domain of CLEC2/CLEC1B and FcR γ . DAP12, also known as KARAP (killer cell activating receptor-associated protein), is a short 12 kDa transmembrane protein of 113 amino acids expressed on the cells surface. The protein consists of a 27 amino-acid leader, 14 amino-acid extracellular domain containing a cysteine residue, 24 amino-acid transmembrane segment with a negatively charged aspartic acid residue, and 48 amino-acid cytoplasmic domain containing a single ITAM. In one example, the peptide sequence of the ITAM of DAP12 is ESPYQELQGQRSDVYSDL (SEQ ID NO: 15), which has less than 25% homology with the ITAM motifs identified in human CD3zeta chain and Fc ϵ RI- γ chain. Ligation of a DAP12-associated receptor leads to activation of SRC-family kinases, phosphorylation of paired tyrosine residues in the ITAM of DAP12, and the subsequent recruitment of the cytoplasmic kinase ZAP-70, converting the ligation event into downstream signaling. In one specific example, the intracellular receptor signaling domain of the CAR has the following amino acid sequence (from the N-terminal to the C-terminal): YFLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSDVYSDLNTQRPYYK (SEQ ID NO: 14). CD3 (cluster of differentiation 3) is a protein complex and T cell co-receptor that is involved in activating both the cytotoxic T cell (CD8+ naive T cells) and T helper cells (CD4+ naive T cells). It is composed of four distinct chains. In mammals, the complex contains a CD3 γ chain, a CD3 δ chain, and two CD3 ϵ chains. These chains associate with the T-cell receptor (TCR) and the ζ -chain (zeta-chain) to generate an activation signal in T lymphocytes. Each CD3 zeta chain contains three distinct ITAMs. Exemplary sequences of the ITAMs in CD3 zeta chain include but are not limited to: CD3 zeta ITAM1 having the

following amino acid sequence (from the N-terminal to the C-terminal): APAYQQGQNQLYNELNLGRREEYDVLDKR (SEQ ID NO: 19), encoded by the following exemplary nucleotide sequence: 5'-GCCCCCGCGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGA-3' (SEQ ID NO: 18); CD3 zeta ITAM2 having the following amino acid sequence (from the N-terminal to the C-terminal): PQRRKNPQEGLYNELQKDKMAEAYSEIGM (SEQ ID NO: 23), encoded by the following exemplary nucleotide sequence: 5'-CCGCAAAGGAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATG-3' (SEQ ID NO: 24); CD3 zeta ITAM3 having the following amino acid sequence (from the N-terminal to the C-terminal): ERRRGKGHDGLYQGLSTATKDTYDALHMQ (SEQ ID NO: 25), encoded by the following exemplary nucleotide sequence: 5'-GAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGC-3' (SEQ ID NO: 26). In one specific example, the only ITAM comprised in the intracellular signaling domain of the recombinant CAR as disclosed herein is CD3zeta ITAM-1. C-type lectin-like receptor 2 (CLEC-2), also known as C-Type Lectin Domain Family 1 Member B (CLEC1B), is a 32 kDa, type II transmembrane glycoprotein and member of the C-type lectin-like family of receptors. CLEC-2 consists of a 33 amino acid cytoplasmic domain, a 21 aa transmembrane region, and a 175 aa extracellular domain (SwissProt #Q9P126). The cytoplasmic domain contains multiple threonine and serine residues which are sites of potential phosphorylation, and an ITAM through which CLEC-2 does its signaling. Ligand binding and cross-linking of CLEC-2 induces Src kinase-dependent tyrosine phosphorylation of the ITAM, inducing activation of the tyrosine kinase Syk and initiation of a signaling pathway that culminates in activation of phospholipase C gamma 2. The extracellular domain contains three potential sites of N-linked glycosylation, and a single carbohydrate recognition domain (CRD) which shows conservation of six cysteine residues. Human CLEC-2 shares 63% aa sequence identity with mouse CLEC-2. CLEC-2 is expressed preferentially in liver, and is also detected in myeloid cells (monocytes, dendritic cells, and granulocytes), platelets, and megakaryocytes. Fc receptor common γ chain (FcR γ) is an adaptor bearing an ITAM that transduces activation signals from various immunoreceptors.

[0062] The co-stimulatory signaling domain of a chimeric antigen receptor can enhance the proliferation, survival and/or development of the T cells. Examples of co-stimulatory signaling domains include but are not limited to: 4-1BB, CD27, CD28, OX-40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, functional derivatives thereof, and combinations thereof. In one example, the chimeric antigen receptor as described herein does not comprise more than one co-stimulatory signaling domain. In one specific example, the co-stimulatory signaling domain is 4-1BB. 4-1BB, also known as CD137, is a member of the tumor necrosis factor (TNF) receptor family.

[0063] As used herein, the at least one co-stimulatory signaling domain and intracellular signaling domain may be collectively referred to as the intracellular domain of the chimeric antigen receptor. As used herein, the hinge region and the antigen binding region may be collectively referred to as the extracellular domain of the chimeric antigen receptor.

[0064] In some examples, between the extracellular domain and the transmembrane domain of the CAR, or between the intracellular domain and the transmembrane domain of the CAR, there is incorporated a spacer domain. As used herein, the term "spacer domain" generally means any oligo- or polypeptide that functions to link the transmembrane domain to, either the extracellular domain or, the intracellular domain in the polypeptide chain. A spacer domain can comprise up to about 300 amino acids, or about 10 to about 100 amino acids, or about 25 to about 50 amino acids.

[0065] In one specific example, the desired CAR comprises the extracellular domain of an NKG2D receptor, IgG4 hinge, CD28 transmembrane domain, co-stimulatory signaling domain 4-1BB, and the intracellular signaling domain of DAP12. In another specific example, the desired CAR comprises the extracellular domain of an NKG2D receptor, IgG4 hinge, CD28 transmembrane domain, co-stimulatory signaling domain 4-1BB, and an intracellular signaling domain comprising CD3zeta ITAM-1. Unless otherwise specified, it is understood that each of the components of a chimeric antigen receptor, including but not limited to the extracellular domain, the hinge domain, the transmembrane domain, the co-stimulatory signaling domain, and the intracellular signaling domain, is covalently bonded to the chimeric antigen receptor component adjacent to it. For example, the co-stimulatory signaling domain of the recombinant CAR is covalently bonded to the intracellular signaling domain of the same recombinant CAR, and so forth.

[0066] In another example, there is provided a $\alpha\beta$ T cell genetically modified to express the recombinant chimeric antigen receptor (CAR) as disclosed herein. Expression of CARs in the $\alpha\beta$ T cells can be transient or stable.

[0067] In another example, there is provided a method of preparing the $\alpha\beta$ T cell genetically modified to express a recombinant chimeric antigen receptor (CAR) as disclosed herein, the method comprises: (i) obtaining or providing $\alpha\beta$ T cell; (ii) providing a recombinant nucleic acid encoding the recombinant CAR; and (iii) transfecting the recombinant nucleic acid encoding the recombinant CAR into the $\alpha\beta$ T cell. In some examples, the method further comprises, after step (i), culturing the $\alpha\beta$ T cells to expand the number of $\alpha\beta$ T cells. T cell expansion methods, particularly $\alpha\beta$ T cell expansion methods, known in the art can be used.

[0068] A recombinant nucleic acid encoding the recombinant CAR can be created using methods known in the art. A base sequence encoding an amino acid sequence can be obtained from the NCBI RefSeq IDs or accession numbers of GenBank for an amino acid sequence of each domain, and the nucleic acid as disclosed herein can be prepared using a standard molecular biological and/or chemical procedure. For example, based on the base sequence, a polynucleotide can be synthesized, and the polynucleotide of the present disclosure can be prepared by combining DNA fragments which are obtained from a cDNA library using a polymerase chain reaction (PCR). The sequence of the open reading frame encoding the CAR can be obtained from a genomic DNA source, a cDNA source, or can be synthesized (e.g. via PCR), or combinations thereof. Depending upon the size of the genomic DNA and the number of introns, it may be desirable to use cDNA or a combination thereof as it is found that introns stabilize the mRNA. Also, it may be further advantageous to use endogenous or exogenous non-coding regions to stabilize the mRNA.

[0069] The nucleotide sequence coding for the recombinant CAR can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.), insect cell systems infected with virus (e.g., baculovirus), microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA, transgenic plants or transgenic non-human animals. The expression elements

of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

[0070] Any of the known methods for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric nucleotide sequence consisting of appropriate transcriptional/translational control signals and the protein coding sequences. Exemplary methods include *in vitro* recombinant DNA and synthetic techniques. Expression of a recombinant nucleic acid sequence encoding the recombinant CAR may be regulated by a second nucleic acid sequence so that the recombinant CAR is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the recombinant CAR may be controlled by any promoter/enhancer element known in the art.

[0071] In one specific example, the recombinant CAR construct containing the 5'TR, the core insulator 1, Ef1a1178, the nucleotide sequence encoding the recombinant CAR, the Sv40L, the core insulator 2, the 3'TR, and the pUC, has the nucleotide sequence of SEQ ID NO: 1.

[0072] In some examples, the basic backbone of the recombinant expression vector is a commercially available vector into which each of the above elements is inserted.

[0073] The nucleic acid encoding the recombinant CAR can be cloned into the expression vector using molecular cloning techniques commonly known in the art.

[0074] The expression vector comprising the recombinant nucleic acid encoding the recombinant CAR can be transferred into the $\alpha\beta$ T cell using any techniques known in the art, such as electroporation, non-viral chemical transfection, and viral transduction. In one particular example, the $\alpha\beta$ T cells are genetically modified to express the CAR by electroporation.

[0075] In some examples, the $\alpha\beta$ T cells genetically modified to express the recombinant CAR are able to replicate *in vivo* resulting in long-term persistence that can lead to sustained tumor control.

[0076] The $\alpha\beta$ T cells as described herein can be provided as a composition or a pharmaceutical composition. Thus, in one example, there is provided a pharmaceutical composition comprising a pharmaceutically effective amount of the $\alpha\beta$ T cell as disclosed herein and a pharmaceutically acceptable excipient. The compositions as described herein may be administered in a number of ways depending upon whether local or systemic treatment is desired. Administration may be topical, pulmonary (e.g., by inhalation or

insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal) or systemic such as oral, and/or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. In some examples, the route of administration may be selected from the group consisting of systemic administration, oral administration, intravenous administration and parenteral administration.

[0077] A composition or pharmaceutical composition as described herein can be provided in unit dosage form wherein each dosage unit, e.g., an injection, contains a predetermined amount of the $\alpha\beta$ T cells as disclosed herein, alone or in appropriate combination with other active agents. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the $\alpha\beta$ T cells as disclosed herein, alone or in combination with other active agents, calculated in an amount sufficient to produce the desired effect, in association with a pharmaceutically acceptable diluent, carrier, or vehicle, where appropriate. The specifications for the unit dosage forms depend on the particular pharmacodynamics associated with the pharmaceutical composition in the particular subject. Unit dosage forms can be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid or semi-liquid carriers.

[0078] The compositions as described herein may additionally contain other adjunct components conventionally found in pharmaceutical compositions. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritic, astringents, local anaesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as buffer, dyes, preservatives, antioxidants, opacifiers, thickening agents and stabilizers or combination thereof appropriate for use with the pharmacologically active agent that may be added to solution in any concentration suitable for use in eye drops. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present

disclosure. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colourings, flavourings and/or aromatic substances and the like which do not deleteriously interact with the $\alpha\beta$ T cells of the formulation.

[0079] In some examples, a medical disease or disorder can be treated by administering a population of the $\alpha\beta$ T cell as disclosed herein. In some examples, the medical disease or disorder is cancer or tumor. In another example, there is provided a method of treating cancer or tumor in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of the $\alpha\beta$ T cell as disclosed herein, or the pharmaceutical composition as disclosed herein. In another example, there is provided use of a pharmaceutically effective amount of the $\alpha\beta$ T cell as disclosed herein in the manufacture of a medicament for treating cancer or tumor. In yet another example, there is provided the $\alpha\beta$ T cells or the pharmaceutical composition as disclosed herein for use in treating cancer or tumor. In some examples, the genetically modified $\alpha\beta$ T cells used to treat cancer or tumor are prepared during the course of treatment. Thus, in one example, there is provided a method of treating cancer or tumor in a subject in need thereof, the method comprises: (i) obtaining $\alpha\beta$ T cells from the subject, or from a $\alpha\beta$ T cell donor which is different from the subject to be treated; (ii) providing a recombinant nucleic acid encoding the recombinant chimeric antigen receptor (CAR) as disclosed herein; (iii) transfecting the recombinant nucleic acid encoding the recombinant CAR into the $\alpha\beta$ T cell to obtain genetically modified $\alpha\beta$ T cells; and (iv) administering to the subject a pharmaceutically effective amount of the $\alpha\beta$ T cells obtained from (iii). In some other examples, the method further comprises, after step (i), culturing the $\alpha\beta$ T cells to expand the number of $\alpha\beta$ T cells. $\alpha\beta$ T cell expansion methods are known in the art and exemplified in the Experimental Section of the present application.

[0080] The term “pharmaceutically effective amount” as used herein includes within its meaning a sufficient but non-toxic amount of the $\alpha\beta$ T cells as described herein to provide the desired treatment effect. Desirably an effective amount or sufficient number of the $\alpha\beta$ T cells as described herein is present in the composition and introduced into the subject such that long-term, specific, anti-tumor responses are established to reduce the size of a tumor or eliminate tumor growth or regrowth than would otherwise result in the absence of such treatment. Desirably, the amount of $\alpha\beta$ T cells introduced into the subject causes at least a

10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 100% decrease in tumor size when compared to otherwise same conditions wherein the $\alpha\beta$ T cells are not present. The exact amount of $\alpha\beta$ T cells required will vary from subject to subject depending on factors such as the species being treated, the age and general condition of the subject, the severity of the condition being treated (e.g. the stage and /or size of the tumor), the mode of administration, and so forth. In general, the concentration of $\alpha\beta$ T cells desirably should be sufficient to provide in the subject being treated at least from about 1×10^6 to about 1×10^9 $\alpha\beta$ T cells, even more desirably, from about 1×10^7 to about 5×10^8 $\alpha\beta$ T cells, although any suitable amount can be utilized either above, e.g., 5×10^8 cells, or below, e.g., 1×10^7 cells. The dosing schedule can be based on well-established cell-based therapies, or an alternate continuous infusion strategy can be employed. These values provide general guidance of the range of $\alpha\beta$ T cells to be utilized by the practitioner upon optimizing the method of treatment as disclosed herein. The recitation herein of such ranges by no means precludes the use of a higher or lower amount of a component, as might be warranted in a particular application. For example, the actual dose and schedule can vary depending on whether the compositions are administered in combination with other pharmaceutical compositions, or depending on inter-individual differences in pharmacokinetics, drug disposition, and metabolism. In any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation.

[0081] Cancers or tumors for which the present treatment methods are useful include any malignant cell type, such as those found in a solid tumor or a hematological tumor. In some specific examples, the malignancy is a solid tumor. Exemplary solid tumors can include, but are not limited to, a tumor of an organ selected from the group consisting of pancreas, colon, cecum, stomach, brain, head, neck, ovary, kidney, larynx, sarcoma, lung, bladder, melanoma, prostate, and breast. In some specific examples, the malignancy is a hematological tumor. Exemplary hematological tumors include tumors of the bone marrow, T or B cell malignancies, leukemias, lymphomas, blastomas, myelomas, and the like. Further examples of cancers that may be treated using the methods provided herein include, but are not limited to, lung cancer (including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), cancer of the peritoneum, gastric or stomach cancer (including gastrointestinal cancer and gastrointestinal stromal cancer), pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer,

colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, various types of head and neck cancer, and melanoma. The cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; Sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; lentigo malignant melanoma; acral lentiginous melanomas; nodular melanomas; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial

sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; hodgkin's disease; hodgkin's; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-hodgkin's lymphomas; B-cell lymphoma; low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; Waldenstrom's macroglobulinemia; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; hairy cell leukemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); acute myeloid leukemia (AML); and chronic myeloblastic leukemia. In some examples, the cancer/tumor that can be treated using the genetically modified $\alpha\beta$ T cells provided herein include colorectal cancer, ovarian cancer, head and neck cancer, liver cancer, breast cancer, cervical cancer and glioma. In one specific example, the cancer/tumor that can be treated is colorectal cancer/tumor or ovarian cancer/tumor.

[0082] In some examples, the cancer/tumor expresses NKG2D ligand. In some other examples, the cancer/tumor expresses NKG2D ligand after treatment with another drug, radiation or biological agent.

[0083] The terms “treat,” “treatment,” and grammatical variants thereof, refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological condition, disorder or disease or obtain beneficial or desired clinical results. Such beneficial or desired clinical results include, but are not limited to, alleviation of symptoms; diminishment of extent of condition, disorder or disease; stabilized (i.e. not worsening) state of condition, disorder or disease; delay or slowing of condition, disorder or disease progression; amelioration of the condition, disorder or disease state, remission (whether partial or total), whether detectable or undetectable; or enhancement or improvement of condition, disorder or disease. Treatment includes eliciting a cellular response that is clinically significant, without excessive levels of side effects. Treatment also includes prolonging survival as compared to expected survival if not receiving treatment.

[0084] The terms "decrease" , "reduced", "reduction" , "decrease", “removal” or "inhibit" are all used herein generally to mean a decrease by a statistically significant amount. However, for avoidance of doubt, "reduced", "reduction" or "decrease", "removal", or "inhibit" means a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% decrease (e.g. absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level (e.g., in the absence of a treatment as described herein).

[0085] In some examples, the subject or patient to be treated is an animal, mammal, human, including, without limitation, animals classed as bovine, porcine, equine, canine, lupine, feline, murine, ovine, avian, piscine, caprine, corvine, acrine, or delphine. In one example, the patient is a human.

[0086] The source of $\alpha\beta$ T cells that can be used for treating a medical disease or disorder may be of any kind, but in specific examples the cells are obtained from a bank of umbilical cord blood, peripheral blood, human embryonic stem cells, or induced pluripotent stem cells.

[0087] In some examples, the $\alpha\beta$ T cells that can be used for treating a medical disease or disorder are autologous, i.e. obtained from the same individual to which the treatment is to be administered. For example, autologous source of $\alpha\beta$ T cells can be collected from a patient in need of treatment and $\alpha\beta$ T cells are activated and modified using the methods described

herein and known in the art and then infused back into the patient. Some autologous source of $\alpha\beta$ T cells include PBMCs, umbilical cord blood obtained when the patient was born and subsequently preserved, and induced pluripotent stem cells derived from cells obtained from the patient.

[0088] In some other examples, the $\alpha\beta$ T cells that can be used for treating a medical disease or disorder are allogeneic, i.e. derived from a different individual of the same species as the patient, such as an $\alpha\beta$ T cell donor. In some other examples, the $\alpha\beta$ T cells that can be used for treating a medical disease or disorder are xenogeneic, i.e. derived from an animal of a different species as the patient. Genetically modified $\alpha\beta$ T cells derived from allogeneic xenogeneic sources can provide an off-the-shelf product.

[0089] Allogeneic or autologous $\alpha\beta$ T cells induce a rapid immune response but disappear relatively rapidly from the circulation due to their limited lifespan. Thus, there is reduced concern of persisting side effects using the treatment methods as disclosed herein.

[0090] In certain examples, the $\alpha\beta$ T cells as described herein are administered in combination with a second therapeutic agent. For example, the second therapeutic agent may comprise T cells other than $\alpha\beta$ T cells, an immunomodulatory agent, a monoclonal antibody, or a chemotherapeutic agent. In non-limiting examples, the immunomodulatory agent is lenolidomide, the monoclonal antibody is alemtuzumab, rituxumab, trastuzumab, ibritumomab, gentuzumab, brentuximab, adotranstuzumab, blinatumomab, daratumumab or elotuzumab, and the chemotherapeutic agent is fludarabine or cyclophosphamide.

[0091] Following administration of the genetically modified $\alpha\beta$ T cells as disclosed herein for treating or preventing a cancer/tumor, the efficacy of the treatment can be assessed in various ways well known to the skilled practitioner. For instance, one of ordinary skill in the art will understand that a therapeutic genetically modified cell delivered in conjunction with the chemo-adjuvant is efficacious in treating or inhibiting a cancer in a patient by observing that the therapeutic genetically modified cell reduces the cancer cell load or prevents a further increase in cancer cell load. Cancer cell loads can be measured by methods that are known in the art, for example, using polymerase chain reaction assays to detect the presence of certain cancer cell nucleic acids or identification of certain cancer cell markers in the blood using, for example, an antibody assay to detect the presence of the markers in a sample (e.g., but not limited to, blood) from a subject or patient, or by measuring the level of circulating cancer cell antibody levels in the patient.

[0092] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0093] As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a genetic marker" includes a plurality of genetic markers, including mixtures and combinations thereof.

[0094] As used herein, the term "about", in the context of concentrations of components of the formulations, typically means $\pm 5\%$ of the stated value, more typically $\pm 4\%$ of the stated value, more typically $\pm 3\%$ of the stated value, more typically, $\pm 2\%$ of the stated value, even more typically $\pm 1\%$ of the stated value, and even more typically $\pm 0.5\%$ of the stated value.

[0095] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0096] Certain embodiments may also be described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the embodiments with a

proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0097] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0098] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

Materials and Methods

Cell lines and culture conditions

[0099] Human K562 myelogenous leukaemia feeder cells or K562#6 engineered to express CD64, CD86 and CD137L was maintained in Iscove's Modified Dulbecco's Media (IMDM). SKOV3-luc and HCT116-luc cells were maintained in McCoy's 5A medium, whereas FaDu and U87 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM). All types of medium were supplemented with 10% Fetal Bovine Serum (FBS) (Gibco).

NKG2D CAR-T cell preparation

[00100] Peripheral blood mononuclear cells (PBMCs) of healthy donors were isolated by gradient centrifugation from buffy coats obtained from Health Sciences Authority (HSA, Singapore) with ethics approved by the Institutional Review Board (IRB). PBMCs were seeded at 5×10^6 cells/ml in T cell media (AIM-V + 5% AB serum) and activated for 2-3 days with soluble OKT3 (Ebioscience) at 1 μ g/ml or TransAct (Miltenyi Biotec, Germany), 100 μ l of TransAct was added for every 1×10^7 PBMCs. Recombinant IL-2 (300 IU/ml; Peprotech) was at the start of the culture and replenished every other day.

[00101] On day 2 or 3, activated T cells were harvested and transduced with a non-viral electroporation method. Modified T cells were cultured in AIM-V medium supplemented with either 5% AB serum or 1% human plasma and 300IU/ml IL-2 for a further five days. On

day 8, genetically modified T cells were numerated and co-cultured with γ -irradiated K562 or K562#6 cells at 1:2 ratio in AIM-V medium supplemented with 300 IU/ml of IL-2. Re-stimulation of CAR T cells was done every 7 days with addition of new γ -irradiated K562 cells at 1:2 ratio. For propagation of non-modified T cells, T cells were co-cultured with γ -irradiated K562#6 at a 1:50 ratio supplemented with 60 ng/ml of OKT3 at the start of the co-culture. 300 IU/ml of IL-2 was added at the start of the culture and replenished every other day.

[00102] CD56+ cells were removed from day 17 CAR-T cell cultures based on manufacturer's protocol (Miltenyi Biotec, Germany). Briefly, up to 1×10^7 cells were suspended in 80 μ l of autoMACS running buffer and incubated with 20 μ l of CD56 microbeads at 4°C in the dark for 15 minutes. Cells were centrifuged at 300g for 10 minutes and resuspended in 500 μ l of autoMACS running buffer. LS columns were placed in a MACS MultiStand and equilibrated by rinsing twice with 1ml of autoMACS running buffer. Cell suspensions were introduced into the LS columns to capture tagged CD56+ cells. CD56-flow-through fraction was collected at the bottom of the column in a 15ml conical centrifuge tube (BD Biosciences, USA).

Flow cytometry

[00103] For phenotypic analysis of T cells, the following conjugated anti-human antibodies were used: CD3 (clone: OKT3; eBioscience), CD45RO (clone: UCHL1; BD Biosciences), CD45RA (clone HI100; eBioscience), CCR7 (clone: 3D12; eBioscience), $\alpha\beta$ TCR (clone IP26, BD Biosciences), NKG2D (clone 1D11, BD Biosciences) Appropriate isotype controls were used to validate gating. The expression of NKG2D CAR (which included a Strep Tag) on T cell was determined using THETM NWSHPQFEK Tag (ST2) Antibody (Genescript) at day 5 post electroporation and day 12 after one week of co-culture with γ -irradiated K562#6. Flow cytometry analysis was performed and analysed using BD Accuri™ C6 (BD Biosciences).

In vitro cytotoxicity assay

[00104] The cytolytic activity of CAR-modified T cells was examined with the DELFIA EuTDA Cytotoxicity Reagents kit (PerkinElmer). The effector to target (E:T) ratios used ranged from 40:1 to 1:1. Control groups were set up to measure spontaneous release (only target cells added), maximum release (target cells added with 10 μ l lysis buffer), and medium background (no cell added). Killing efficacy was calculated by using the following formula:

% Specific release

$$= \frac{\text{Experimental release (counts)} - \text{Spontaneous release (counts)}}{\text{Maximum release (counts)} - \text{Spontaneous release (counts)}} \times 100$$

Multiplex cytokine quantification assay

[00105] Non-modified or CAR T cells were stimulated for 18 hours with HCT116 at a 1:1 effector to target (E:T) ratio. The cells were lysed, and the supernatants were collected and analysed with LUNARIS™ Human 11-Plex Cytokine kit (Ayoxxa Biosystems) or BD™ Cytometric Bead Array Human Th1/Th2 Cytokine Kit (BD Biosciences)

In vivo experiments

[00106] Animal experiments were performed according to protocols reviewed and approved by Institutional Animal Care and Use Committee (IACUC), the Biological Resource Centre (BRC), the Agency for Science, Technology and Research (A*STAR), Singapore (Permit number BRC IACUC#181324).

[00107] Non-obese diabetic/severe combined immuno-deficiency/IL-2R γ null (NSG) mice (The Jackson Laboratory) were maintained and used in the current study. All luminescent signals and images were acquired and analysed with the Xenogen living imaging software v3.2.

[00108] For the study to determine the effects of NKG2D 1st Generation (NKG2Dp) CAR-T cell and NKG2D 2nd Generation (NKG2Dbp) CAR-T cells, 8-10 weeks old male NSG mice were used. Male mice were subjected to intraperitoneal (i.p.) injection with 2×10^6 HCT116-luc cells on day 0 to establish a human colorectal cancer (CRC) xenograft model. On day 7 post-tumor inoculation, tumor engraftment was confirmed by live bioluminescence imaging (BLI) monitored using an IVIS Spectrum Imaging platform with Living Image software (PerkinElmer). Mice with similar BLI signal intensity randomly divided into 4 different treatment groups containing 5 mice per group. On day 7, 100 μ l of PBS or 100 μ l cell suspension containing 2×10^6 T cells of three different groups: (1) Control T cell, (2) NKG2D 1st Generation (NKG2Dp) CAR-T cells or (3) NKG2D 2nd Generation (NKG2Dbp) CAR-T cells were injected intraperitoneally (i.p.). A second intraperitoneal (i.p.) injection at the same dose (2×10^6 T cells/mice) was given at day 30 post tumor inoculation. Tumor progression was monitored by bioluminescence imaging (BLI) every week.

[00109] For the study to determine the effects of NKG2D-41BB-3 ζ CAR (NKG2Dbz) and NKG2D-41BB-Dap12 CAR (NKG2Dpb), 8-10 weeks old female NSG mice were used.

Female mice were injected intraperitoneally (i.p.) with 2×10^6 HCT116-luc cells on day 0 to establish a human CRC xenograft model. On day 7 post-tumor inoculation, tumor engraftment was confirmed by live imaging and mice with similar BLI signal intensity randomly divided into 4 different treatment groups containing 5 mice per group. On day 7, 100 μ l of PBS or 100 μ l cell suspension containing 1×10^7 T cells of three different group: (1) Control T cell (2) NKG2D-41BB-3 ζ CAR T Cell or (3) NKG2D-41BB-Dap12 CAR T cell was injected i.p. vein. A second injection was given at day 32 at a reduced dose of 2×10^6 T cells/mice post tumor inoculation. Tumor progression was monitored by BLI every week.

[00110] Mice were monitored closely and humanely euthanized after observing the development of moribund condition characterized by obvious abdominal bloating due to ascites, palpable hypothermia, inability to walk, and/or lack of overt response to manipulation. Similarly, mice were monitored closely and humanely euthanized after showing signs of xenogeneic graft versus host disease (GvHD) characterized by >15% loss in weight, ruffled fur, hunched posture and/or hind leg paralysis.

[00111] For the study to determine the effects of NKG2D-41BB-Dap12 CAR (NKG2Dpb) in a human SKOV3 ovarian cancer xenograft model, 8-10 weeks old female NSG mice were used. Female mice were injected intraperitoneally (i.p.) with 1×10^7 SKOV3-luc cells on day 0 to establish a human ovarian cancer xenograft model. On day 7 post-tumor inoculation, tumor engraftment was confirmed by live imaging and mice with similar BLI signal intensity randomly divided into 3 different treatment groups containing 5 mice per group. On day 7, 100 μ l of PBS or 100 μ l cell suspension containing 1×10^7 T cells of two different group: (1) Control T cell or (2) NKG2D-41BB-Dap12 CAR T cell was injected i.p.. A second injection was given at day 14 post tumor inoculation with the same dose of 1×10^7 T cells/mice. Tumor progression was monitored by BLI every week. Mice were monitored closely till day 80 and humanely euthanized after observing the development of moribund condition characterized by obvious abdominal bloating due to ascites, palpable hypothermia, inability to walk, and/or lack of overt response to manipulation.

Statistics

[00112] For *in vitro* and *in vivo* experiments, unpaired Student's t test was used to evaluate continuous variable of 2 groups, and 1-way ANOVA with post-test Bonferroni to evaluate continuous variables of more than 2 groups. Survival was analysed by the Kaplan-Meier method and the log-rank (Mantel-Cox) test to compare pairs of groups. Statistics were

computed using GraphPad Prism 7.0 (GraphPad Software). Differences were considered significant when the P value was less than 0.05.

Interferon-gamma (IFN- γ) release assay

[00113] To detect IFN γ secretion and up-regulation by CAR T cell after co-culture with HCT116, 10^5 T cells were incubated with 10^5 HCT116 cells for 16 hours using the IFN γ ELISPOT assays according to the provided protocols of ELISPOT kits (Mabtech, Nacka Strand, Sweden). The plates were analyzed by an ELISPOT scanner (CTL, Ltd., of Cleveland, OH).

Results

[00114] The 1st-generation DAP12 CAR construct was generated by cloning the type II NKG2D ectodomain sequence (amino acids 82 – 216) downstream of the GM-CSF signal peptide, followed by the hinge region and transmembrane domain of the CD8 protein. The single ITAM-containing DAP12 coding sequence was cloned downstream of the transmembrane. Accordingly, the 2nd-generation CAR construct incorporated an additional 4-1BB co-stimulatory signaling domain upstream of the DAP12 activating domain (**Fig. 2B**).

[00115] NKG2D CAR-T cells were generated by electroporating day 2 beads-activated T cells with NKG2D CAR-encoding and piggyBac transposase-encoding plasmids, and cultured for a further five days in IL-2 alone. For large-scale expansion and longitudinal study of memory T development, day 7 CAR-T cells (5 days post-electroporation) were then co-cultured with the human K562 myelogenous leukaemia cell line at an effector to target (E:T) ratio of 1:2. Both NKG2Dp and NKG2Dbp CAR-T cells underwent robust proliferation in response to antigenic stimulation from K562 cells (**Fig. 2D**). As the use of K562 feeder cells would inevitably cause NK cells to increase in numbers because NK cells naturally lyse K562 feeder cells, this leads to the competition of NK cells with CAR-T cells for the recognition of K562 feeder cells. Therefore, a CD56 depletion step was incorporated on day 17 prior to further co-culture procedures, wherein the depletion of CD56-expressing NK cells would allow for selective enrichment of NKG2D CAR-T cells.

[00116] Concomitantly, CAR expression intensity and proportion of CAR-expressing T cells increased as antigen-dependent proliferation took place (**Fig. 2E**). By the end of CAR-T cell expansion, nearly 100% of T cells in both NKG2Dp and NKG2Dbp groups were CAR-positive. While proportions were comparable, the expression intensity of NKG2Dbp (mean

fluorescence intensity (MFI) value: 19037) was lower compared to NKG2Dp (mean fluorescence intensity (MFI) value: 28263).

[00117] The incorporation of 4-1BB co-stimulatory signaling domain also increased the formation of memory T cell subsets. The memory T cell subsets can be determined by the combination of expression of the cell surface markers. For example, effector T cell is CCR7⁻CD45RA⁺; effector memory T cell is CCR7⁻CD45RA⁻; central memory T cell is CCR7⁺CD45RA⁻; and stem cell memory T cell is CCR7⁺CD45RA⁺. As seen in **Fig. 2F**, NKG2Dbp CAR-T cells showed increased development of CCR7⁻CD45RA⁻ effector memory T cells than NKG2Dp CAR-T cells. The capacity for differentiation and proliferation increases in the following order: effector T cells, effector memory T cells, central memory T cells, stem cell memory T cells. Conversely, their effector functions, for example, cell-mediated cytotoxicity and cytokine release, decrease in the same order. The clinical efficacy of CAR-T cells is directly correlated with the capacity to proliferate and persist in the body. Thus, a higher memory order allows CAR-T cells to persist in the patient's body longer and thus exert longer-lasting anti-tumor activity. While composition of memory T subsets was comparable in the middle of expansion phase (Effector memory T cell: 67.1% for NKG2Dp vs 76.4% for NKG2Dbp; effector T cell: 32.7% for NKG2Dp vs 23.4% for NKG2Dbp), there was a clear divergence in memory T cell development by the end of the expansion phase (Effector memory T cell: 39.6% for NKG2Dp vs 87.5% for NKG2Dbp; effector T cell: 60.3% for NKG2Dp vs 12.4% for NKG2Dbp). While the expansion folds from both groups were comparable in the initial phases of co-culture with K562, a higher proportion of effector memory T cells in NKG2Dbp group resulted in a more persistent expansion of T cells driven by 4-1BB signaling.

[00118] The *in vivo* tumor killing effects between T cells stably expressing NKG2D 1st generation CAR (NKG2Dp) and T cells stably expressing NKG2D 2nd generation CAR (NKG2Dbp) were analysed. A mouse colorectal model was established in NOD scid gamma (NSG) mice by intraperitoneal (i.p.) injection of HCT116-luc cells. Tumor progression was monitored by whole body bioluminescence imaging (**Fig. 3A**). As shown in **Fig. 3A** and **3B**, the bioluminescence intensities, which is indicative of tumor burdens, in the PBS group and the control CAR T cell groups increased rapidly. Due to the aggressiveness of the model, mice in both PBS and control CAR T cell groups were all euthanized prior to day 28 due to developing moribund condition.

[00119] Mice that received NKG2D 1st generation CAR-T cells or 2nd generation CAR-T cells showed reduction in tumor burden on day 14 after receiving a single dose T cell injection (**Fig. 3A** and **3B**). However, from day 21 onwards (**Fig. 3B**), tumor burden for mice that received NKG2D 1st generation CAR-T cells increased progressively and although a second CAR T cell injection was given at day 30, the tumor burden did not decrease (**Fig. 3B**). Mice that received NKG2D 1st generation CAR-T cells injection were all euthanized by day 62. In contrast, tumor burden for the group of mice that received NKG2D 2nd generation CAR-T cells showed progressive reduction and became undetectable up to day 62. Mice continued to survive up to day 90 at the termination of the animal study (**Fig. 3C**).

[00120] After showing that NKG2D 2nd generation CAR (NKG2Dbp) had impressive *in vitro* and *in vivo* tumor killing effects, the NKG2Dbp CAR construct was compared against two NKG2D CAR constructs that use CD3 ζ (instead of Dap12) as the activation and signaling domain. As a comparison, the full length sequence of CD3 ζ , and CD3 ζ consisting only one single ITAM, were used. As shown in **Fig. 4**, the CAR construct that consists of NKG2D-41BB-CD3 ζ was termed NKG2Dbz, the CAR construct that consists of NKG2D-41BB-CD3 ζ (ITAM1) was termed NKG2Dbz1, while NKG2D-41BB-Dap12 construct was termed NKG2Dbp. A 3x streptomycin – tag II (ST2) was included in the CAR construct to detect CAR expression in T cells. Using the Piggybac transposon system, about 50-70% efficiency five was achieved days after electroporation (**Fig. 4B**). The expression of CAR on modified T cells can be further enriched upon co-culture with K562#6 that is engineered to express CD64, CD86 and CD137L, as shown in **Fig. 4C**. This is because the presence of the CD86 and CD137L expressed on K562#6 allows the CAR T cell to proliferate better and therefore improve the CAR expression on total T cells.

[00121] The calibration of CAR activation potential determines alternative T cell fate and therapeutic potency. Therefore, the generation of T cell memory subsets amongst the three different groups of CAR-T cells was determined. Staining of CD45RO and CCR7 showed that relative to NKG2Dbz with three ITAM motifs, the two CARs with a single ITAM, NKG2Dbz1 and NKG2Dbp, increased the fraction of effector memory CAR T cells and reduced the proportion of effector cells in response to *in vitro* stimulation with K562C6 cells (**Fig. 5A** and **5B**).

[00122] NKG2Dbp CAR-T cells, NKG2Dbz1 CAR-T cells, and NKG2Dbz CAR-T cells were then tested for their anti-tumor cytotoxicity using HCT116-luc (colorectal cancer) in a 4

hour and 16 hour cytotoxicity assays. It was found that the three CAR-T types showed indistinguishable tumor cell lysis activities, while all of them were highly effective in killing the investigated tumor cell lines (**Fig. 6A**). Both NKG2Dbp CAR-T cells and NKG2Dbz CAR-T cells displayed high cell lysis activity towards a panel of solid tumour cancer cells, including SKOV3 luc (ovarian cancer), HCT116-luc (colorectal cancer), FaDu (head and neck cancer) and U87 (glioblastoma). However, there were no significant differences in cytotoxicity between the two NKG2D CAR-T cell groups (**Fig. 7A**).

[00123] To further distinguish function of CAR-T cells electroporated with NKG2Dbz, NKG2Dbz1 or NKG2Dbp CAR constructs, cytokine release and proliferation were investigated. NKG2Dbz CAR-T cells in general promoted higher amounts of IL-2, IFN- γ , and TNF- α production as compared to NKG2Dbz1 and NKG2Dbp CAR-T cells, although the statistical differences were significant ($p < 0.05$) only between NKG2Dbz CAR-T cells and NKG2Dbz1 CAR-T cells for IL-2, and between NKG2Dbz CAR-T cells and NKG2Dbp CAR-T cells for all three examined pro-inflammatory cytokines (**Fig. 6B**). ELISPOT assay as shown in **Fig. 7B** also confirmed that NKG2Dbz CAR-T cells showed higher level of IFN- γ production compared to NKG2Dbp CAR-T cells after 18 hours co-culture with HCT116.

[00124] The production of other pro-inflammatory cytokines was also assayed in the supernatants collected from NKG2Dbp CAR-T cells and NKG2Dbz CAR-T cells. As shown in **Fig. 8**, NKG2Dbz CAR-T cells produced significantly higher amounts of IFN- γ , TNF- α and IL-2 as compared to NKG2Dbp CAR-T cells ($p < 0.05$). Notably, it was observed that NKG2Dbz CAR-T cells produced higher levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) as compared to NKG2Dbp CAR-T cells although it did not reach statistically significant level ($p = 0.0519$).

[00125] The *in vivo* tumor killing effects between NKG2Dbp CAR-T cells and NKG2Dbz CAR-T cells are determined using a mouse colorectal model. The mouse colorectal model is established in (NOD scid gamma) NSG mice by intraperitoneal (i.p.) injection of HCT116-luc cells. Tumor progression was monitored by whole body bioluminescence imaging (**Fig. 9**). On day 7, when all mice had established tumors in the peritoneal cavity, animals were randomly divided into 4 groups for treatment: group 1 was subjected to one intraperitoneal (i.p.) injection of PBS, group 2 was subjected to one intraperitoneal (i.p.) injection of 1×10^7 control T cells; and groups 3 and 4 received one intraperitoneal (i.p.) injection of 1×10^7 NKG2Dbp CAR-T cells and NKG2Dbz CAR-T cells respectively. Bioluminescence

intensities, which are indicative of tumor burdens, are used for analysis. As shown in **Fig. 9B**, the bioluminescence intensities taken in the PBS group remain high during the treatment period from day 7 to 28. In addition, tumor progression for mice that received control CAR T cell groups was slower compared to PBS group. This finding is represented in a bar graph as shown in **Fig. 9C**.

[00126] In comparison, the groups of mice that received NKG2Dbp CAR-T cells or NKG2Dbz CAR-T cells showed reduction in tumor burden after receiving a single dose T cell injection (**Fig. 9B**). At Day 28, tumor burden remained undetectable (**Fig. 9B** and **9C**) in most of the mice in the two groups. Bioluminescence signals were detected only at the site of injection for two mice, suggesting tumor relapse (**Fig. 9B**). A second T cell injection was given at a lower dose (2×10^6 cells/mice) in an attempt to eradicate the relapsed tumor and was successful (**Fig. 10**).

[00127] The group of mice that had received NKG2Dbz CAR-T cells injection began to show signs of xenogeneic graft versus host disease (x-GvHD) as evidenced by $> 15\%$ loss of weight, ruffled fur and hunched posture from day 35 onwards. Mice that showed signs of xenogeneic graft versus host disease (x-GvHD) were euthanized according to IACUC protocol. Xenogeneic graft versus host disease (x-GvHD) was not observed in mice that had received NKG2Dbp CAR-T cells. The animal study was terminated at day 150 post tumor inoculation, where all mice that received NKG2Dbp CAR-T cells remained healthy. Only one mouse from the NKG2Dbz CAR-T cell treatment group survived. Therefore, treatment with NKG2Dbp CAR-T cells is less susceptible to xenogeneic graft versus host disease (x-GvHD). This could be explained by the lower production of pro-inflammatory cytokines present in the mice serum compared to treatment with NKG2Dbz CAR-T cells (**Fig. 10C**). One of the causes of x-GvHD is excess cytokine-release after T-cell activation, which triggers a high level of proliferation of T cells and observed only in $\alpha\beta$ T cells, not other immune cells such as gamma delta T cells and natural killer (NK) cells. When NKG2Dbp CAR is used, reduced cytokine release was observed, as compared to NKG2Dbz CAR. Therefore, NKG2Dbp CAR can control the proliferation of CAR $\alpha\beta$ T cells, thereby reducing the occurrence of x-GvHD.

[00128] To demonstrate that NKG2Dbp CAR-T cells exhibit broad anti-tumor effects beyond colorectal cancer, a mouse model of SKOV3 human ovarian cancer cells in NSG mice was established (**Fig. 12**). The mice, 5 per group, received i.p. injection of 1×10^7 SKOV3 cells (day 0), followed by i.p. injection of PBS, control CAR-T cells, or NKG2Dbp

CAR-T cells on day 7 and day 14 (1×10^7 cells per injection per mouse). Growth of SKOV3 was monitored by bioluminescent imaging on the indicated days (**Fig. 12A**). **Fig. 12B** shows photos of bioluminescent images of three groups of mice from the experiment as described in **Fig. 12A**. The bioluminescent images of 5 mice per group demonstrated that the SKOV3 tumors in mice receiving NKG2Dbp CAR-T cells were eliminated from Day 14 onwards. Consequently, the CAR-T treatment significantly prolonged the survival of the mice in this group over the two controls (**Fig. 12C**) ($P < 0.01$). While all mice died in the two control groups by day 42, the mice treated with NKG2Dbp CAR-T cells survived for at least 80 days. Together with the results from the above CRC model, the data demonstrates that NKG2Dbp CAR-T cells are able to reduce and/or eradicate established solid tumors, thus prolonging the survival of tumor-bearing mice.

CAR triggering lower cytokine release

[00129] The presently disclosed NKG2Dbp CAR-T cell has only one ITAM motif per CAR molecule, which can stimulate relatively lower levels of cytokine release upon interaction with target cells, in comparison to T cells expressing NKG2Dbz that contains three ITAM motifs per CAR molecule. This suggests that cytokine release upon CAR activation could be controlled by simply decreasing the ITAM density of the CAR construct. We hypothesize that the reduction in cytokine release by NKG2Dbp is primarily attributable to the use of the single ITAM configuration, which can affect downstream functional output through the mitogen-activated protein kinase pathway. This effect provided by NKG2Dbp CAR-T would possibly provide a potential clinical advantage in reducing the risk of CRS and is the most important finding of this study. In addition, both NKG2Dbp and NKG2Dbz CAR-T products result in similar *in vivo* anti-tumor activities. This demonstrates that a single functional ITAM is sufficient for potent *in vivo* antitumor efficacy and superior to that afforded by the triple-ITAM-containing wild-type CD3 ζ chain. Moreover, the single ITAM configuration favours the persistence of highly functional CARs, balancing the replicative capacity of long-lived memory cells and the acquisition of effective antitumor function, thereby yielding CAR designs with enhanced therapeutic profiles.

[00130] The most common toxicities observed after CAR T-cell therapy are cytokine release syndrome (CRS) and cerebral edema/neurotoxicity. These involve the release of excess amounts of cytokines, and could be potentially life-threatening. Several factors including, for example, high tumor burden, high CAR-T cell dose, and lymphodepletion

using cyclophosphamide and fludarabine, have been identified as being associated with a higher risk of cytokine release syndrome (CRS) and/or neurotoxicity among patients receiving CD19-targeted CAR T cell therapies.

[00131] The NKG2Dbp construct comprises a CD8a hinge and a transmembrane domain. Furthermore, the CAR construct was designed to comprise only one ITAM motif so as to reduce the ITAM density of NKG2Dbp. This is to reduce the cytokine release that is commonly seen in present CAR-T therapies, and yet retain the tumor killing capacity of NKG2Dbp CAR T cells.

Conclusion

[00132] A single-chain CAR design targeting NKG2D ligand has been developed by fusing the NKG2D extracellular domain to the DAP12 internal signaling domain containing a single ITAM of the amino acid sequence ESPYQELQGQRSDVYSDL (NKG2D-DAP12). A functional synthetic NKG2D-DAP12 protein has also been demonstrated in human T cells. The T cells genetically modified with NKG2D-DAP12 stimulate a lower level of cytokine release during tumor cell lysis, and can eliminate established tumor xenografts in mice.

Tables

[00133] Table 1: Table of sequences used in the construction of recombinant CARs

SEQ ID NO	Sequence	Description
1	ggtctcctcccaataacgataatagatacggacggacacgcagctagattaa ccctagaaagataatcatattgtgacgtacgttaaagataatcatgtgtaaaattg acgcatgaaaacttttatgagggacagccccccccaaagccccagggatg taattacgtccctccccgctagggggcagcagcagccgccccggggtcc gctccgggtccggcgtccccccgcacccccgagccggcagcgtgcgggga cagccccgggcacggggaagggtggcaggggacgctttcctctgaacgcttct cgctgctctttgagcctgcagacacctggggggatacggggaaaaggcggg atccactgaggggtaccgctccgggtgcccgtcagtgggcagagcgcacatc gccacagtccccgagaagttggggggaggggtcggcaattgaaccggtgc ctagagaagggtggcgcggggtaaactgggaaagtgtgctgtactggctc cgccttttcccgagggtgggggagaaccgtatataagtgagtagtcgccgt gaacgttcttttgcgaacgggtttgccgccagaacacaggttaagtccgtgtg tggttcccgcgggcctggcctctttacgggttatggcccttgcgtgcctgaatt actccacctgggtgcagtacgtgattcttgatcccgagcttcgggttggagtg ggtgggagagttcgaggccttgcgttaaggagcccccttcgctcgtgcttga gttgaggcctggcctgggcgctggggccgccgcgtgcgaatctggtggcac	DNA sequence of the pZTS4-Ef1a-NKG2D-IgG4H-CD28TM-DAP12 construct

cttcgcgcctgtctcgtgctttcgataagtctctagccatttaaaattttgatgac ctgtctgcgacgctttttctggcaagatagtcttgtaaagcggggccaagatctg cacactgggtatttcgggttttggggccgcccggcgacggggcccgtgcgt cccagcgcacatgttcggcgaggcggggcctgcgagcgcggccaccgaga atcggacgggggtagtctcaagctggccggcctgctctgggtgcctggcctcg cgccgccgtgtatgccccgccctggcgggcaaggctggcccggtcggcac cagttgcgtgagcggaaagatggccgcttcccggccctgctgcaggagctc aaaatggaggacggggcgctcgggagagcgggggggtgagtcaccacac aaagggaaaaggcctttccgtcctcagccgtcgttcatgtgactccacggag taccgggcgccgtccaggcacctcgattagttctcgagctttggagtagctcg tctttaggttggggggagggttttatgcgatggagtttccccacactgagtgg gtggagactgaagttaggccagcttggcacttgatgtaattctccttgaatttg cccttttgagtttgatcttgggtcattctcaagcctcagacagtgggtcaaagttt ttttctccatttcaggtgtcgtgaaagcttagctgaattctaatacgactcactata gggaaataagagagaaaaagaagtaagaagaaataaagagccaccatgc ttctcctggtgacaagccttctgctctgtgagttaccacaccagcattcctcctg atcccgagcgcgcatgccttcaaccaagaagttcaaattcccttgaccgaaagt tactgtggcccatgtcctaaaaactggatatgttataaaaaataactgctaccaatt ttttgatgagagtaaaaaactggatgagagccaggcttcttgatgtctcaaaatg ccagccttctgaaagtatacagcaaaaggaccaggatttacttaaaactggtga agtcatatcattggatgggactagtagacattccaacaaatggatcttggcagtg ggaagatggctccattctctcaccacactactaacaataattgaaatgcagaa gggagactgtgcactctatgcctcgagctttaaaggctatatagaaaactgttca actccaaatacgtacatctgtatgcaaaggactgtggctagcaactggtcacac ccccagtttgagaaagggtgggggaggggtcaggtgggtgggggggtcaaattggt ctcatccccagttcgagaaggcgaggaggttcaggtgggggtggggagca actggagccaccctcaattgaaaaaggaggtggaggaggtggaggtggtg gatctgaatctaaatatggcccccttggccaccatgtcccttctgggttttgta gttgctggcggggtgcttgcttctcattgttggttacggtggcttttataattt ctgggtaagattctctgtgtggaagaggggtagaagaagctcctctatatcttt aagcaaccttttatgcggccagtacagactactcaagaggaggacggatgttc atgccgattccccgaagaggaagaagggggggtgcgagttgtacttctggga cgacttgttctcggggggcgggggtgctgcagaagctgcaacgcgaaaacaa agaattaccgagactgagagtccgtatcaagaactgcaggggccaaagaagc gacgtttactccgatctcaataccaacggccttactacaaatgagtcgactga gtttggacaaaccacaactagaatgcagtgaaaaaaatgctttatttgtaaattt gtgatgctattgctttattgtaaccattataagctgcaataaacaagttaacaaca acaattgcattcattttatgttcagggtcagggggaggtgtgggaggtttttaaa gcaagtaaaacctctacaaatgtggtacttaagaatgcatgcttttccccgtatc ccccaggtgtctgcaggctcaaagagcagcgagaagcgttcagaggaaag cgatcccgtgccaccttccccgtgccggggtgtccccgcacgtgccggct cggggatgcggggggagcggcgaccggagcggagccccggcgggctc	
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	gctgctgccccctagcgggggagggacgtaattacatccctgggggctttgg gggggggctgtccctgatatctataacatgcgtcaattttacgcagactatcttc tagggttaatctagctgcagtgtatgaaatgctttaaggaggagagggggaga ccaaagggcgagacgttaaggcctcacgtgacatgtgagcaaaaggccagc aaaaggccaggaaccgtaaaaaggccgcgttgctggcgttttccataggctc cgccccctgacgagcatcacaaaaatcgacgctcaagtcagagggtggcga aaccgcagaggactataaagataaccaggcggttccccctggaagctccctcgt gcgctctcgtttccgacctgccgttaccggatacctgtccgcctttctccctt cgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcgggtga ggcgttcgctccaagctgggctgtgtgcacgaacccccgttcagcccagcc gctgcgccttatccggtaactatcgtcttgagccaacccgtaagacacgact tatgccactggcagcagccactggtaacaggattagcagagcgaggatgt aggcgggtgctacagagttcttgaagtgggtggcctaactacggctacactagaa ggacagtatttggatctgcgctctgctgaagccagttacctcggaaaaagag ttgtagctcttgatccggcaaaacaaaccacgctggtagcgggtgtttttgttt gcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatc ttttctacggggctgacgctcagtggaacgaaaactcacgttaagggaatttgg tcatgccgtctcagaagaactcgtcaagaaggcgatagaaggcgatgcgctg cgaatcgggagcggcgataccgtaaaagcacgaggaagcggtcagcccattc gccgccaagctcttcagcaatatcacgggtagccaacgctatgtctgatagc ggctccgccacaccagccggccacagtcgatgaatccagaaaagcggccat tttccaccatgatattcggcaagcaggcatcgccatgggtcacgacgagatcct cgccgtcgggcatgctcgcttgagcctggcgaaacagttcggctggcgcgag cccctgatgctcttcgtccagatcatcctgatcgacaagaccggcttccatccg agtacgtgctcgtcgtatgcgatgtttcgttgggtgctgaatgggcaggtagc cggatcaagcgtatgcagccgccgcatcagccatgatggatactttctc ggcaggagcaaggtgagatgacaggagatcctgccccggcacttcgccccaa tagcagccagtccttcccgttcagtgacaacgtcgagcacagctgcgcaa ggaacgcccgtcgtggccagccacgatagccgcgctgcctcgtcttcagtt cattcagggcaccggacaggtcggtcttgacaaaaagaaccgggcgcccct gcgctgacagccggaacacggcggcatcagagcagccgattgtctgttgtgc ccagtcatagccgaatagcctctccaccaagcggccggagaacctgcgtgc aatccatcttgttcaatcataatattattgaagcatttatcaggggtcgtctcgtccc	
2	atgcttctcctggtgacaagccttctgctctgtgagttaccacaccagcattcct cctgatecca	DNA sequence of GMCSFR-alpha (UniProtKB - P15509) signal peptide (aa 1- 22)
3	MLLLVTSLLLCELPHPAFLIP	Amino acid sequence of GMCSFR-alpha (UniProtKB - P15509) signal peptide (aa 1-

		22)
4	atgcttctcctggtgacaagccttctgctctgtgagttaccacaccagcattcctcctgatcca	DNA sequence of GMCSFR-alpha signal peptide with deduced aa
5	ttcaaccaagaagttcaaattcccttgaccgaaagtactgtggcccatgtcctataaaactggatatgttacaataaactgctaccaatttttgatgagagtaaaacttggtatgagagccaggcttctgtatgtctcaaaatgccagccttctgaaagtatacagcaaagaggaccaggatttacttaaactggtgaagtcatacattggatggactagtacacattccaacaaatggatcttggcagtggaagatggctccattcttcaccaacctaacaataattgaaatgcagaaggagactgtgcactctatgcctcgagctttaaggctatatagaaaactgttcaactccaatacgtacatctgtatgcaaaggactgtg	DNA sequence encoding the NKG2D extra-cellular domain
6	FNQEVQIPLTESYCGPCPNWICYKNNCYQFFDES KNWYESQASCMSQNASLLKVYSKEDQDLLKLVK SYHWMGLVHIPTNGSWQWEDGSILSPNLLTIEM QKGDICALYASSFKGYIENCSTPNTYICMQRTV	Amino acid sequence of the NKG2D extra-cellular domain peptide
7	gaatctaaatatggcccccttgcaccatgtccc	DNA sequence encoding the IgG4 (UniProtKB - P01861) Hinge domain (aa 99-110)
8	ESKYGPPCPSCP	Amino acid sequence of the IgG4 (UniProtKB - P01861) Hinge domain (aa 99-110) peptide
9	ttctgggttttggtagtgtcggcgggggtgcttgcttgctattcattgttggttacgtggcctttataatttctgggta	DNA sequence encoding the CD28 (UniProtKB - P10747) transmembrane domain (aa 153-179)
10	FWVLVVVGGVLACYSLLVTVAFIIFWV	Amino acid sequence of the CD28 (UniProtKB - P10747) transmembrane domain (aa 153-179) peptide
11	agattctctgtggtgaagaggggtagaaagaagctcctctatatctttaagcaac	DNA sequence

	cttttatgcgccagtacagactactcaagaggaggacggatgttcacgcatg tccccgaagaggaagaaggggggtgcgagttg	encoding the 4-1BB (UniProtKB - Q07011) co-stimulatory signaling domain: 4- 1BB (aa 209-255)
12	RFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCS CRFPEEEEEGGCEL	Amino acid sequence of the 4-1BB (UniProtKB - Q07011) co-stimulatory signaling domain: 4- 1BB (aa 209-255) peptide
13	tacttcctgggacgactgttctcggggggcggggtgctgcagaagctgcaac gcgaaaacaaagaattaccgagactgagagtcggtatcaagaactgcagggc caaagaagcgacgtttactccgatcctaataccaacggccttactacaaa	DNA sequence encoding the DAP12 (UniProtKB - O43914) intracellular domain (aa 62-113)
14	YFLGRLVPRGRGAAEAATRQKQRTETESPYQELQ GQRSDVYSDLNTQRPYYK	Amino acid sequence of the DAP12 (UniProtKB - O43914) intracellular domain (aa 62-113) peptide
15	ESPYQELQGQRSDVYSDL	ITAM motif with ESP sequence
16	MLLLVTSLLLCELPHPAFLIPGAHAFNQEVQIPLT ESYCGPCPKNWICYKNNCYQFFDESKNWWYESQA SCMSQNASLLKVYSKEDQDLLKLVKSYHWMGLV HIPTNGSWQWEDGSILSPNLLTIEMQKGDALYA SSFKGYIENCSTPNTYICMQRTVASFVPVFLPAKP TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVH TRGLDFACDFWVLVVVGGVLACYSLLVTVAFIIF WVRFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDG CSCRFPEEEEEGGCELYFLGRLVPRGRGAAEAATR KQRTETESPYQELQGQRSDVYSDLNTQRPYYK	Amino acid sequence of the NKG2Dbp CAR
17	gggtcctcccaataacgataatagatacggacggacgcagctagattaa ccctagaaagataatcatattgtgacgtacgttaaagataatcatgtgtaaaattg acgcatgaaaacttttatgaggacagccccccccaaagccccagggatg taattacgtccctccccgctagggggcagcagcagccgccccggggtcc gtccgggtccggcgtccccccgcatccccgagccggcagcgtgcgggga cagccccgggcacggggaaggtggcacgggatcgctttctctgaacgttct cgctgtctttgagcctgcagacacctggggggatacggggaaaaggcggg atccactgaggggtaccgctccggtgccgctcagtgggcagagcgcacatc gcccacagtccccgagaagttggggggaggggtcggcaattgaaccggtgc	DNA sequence of the plasmid for the construction of NKG2Dbz1 CAR

ctagagaaggtggcgcggggtaaactgggaaagtgatgctgtactggctc cgccttttcccgagggtgggggagaaccgtatataagtgcagtagtcgcgt gaacgttcttttcgaacgggttggccgcagaacacaggtaaagtccgtgtg tggttcccgccggcctggcctctttacgggttatggcccttgcgtgccttgaatt actccacctggctgcagtagctgattcttgatcccgagcttcgggttggaaagt gggtgggagagttcgaggccttgcgttaaggagccccctgcctcgtgcttga gttgaggcctggcctgggcgctggggccgcgcgtgcgaatctggtggcac cttcgcgcctgtctcgtgcttgcataagtctctagccatttaaaattttgatgac ctgctgcgacgcttttttctggcaagatagcttgtaaatcgggccaagatctg cacactggtatttcggttttggggccgcggcgggcgacggggcccgtgcgt cccagcgcacatgttcggcgaggcggggcctgcgagcgcggccaccgaga atcggacggggtagtctcaagctggccggcctgctctggtgcctggcctcg cgccgccgtgtatcgccccgccctgggcggcaaggctggcccgtcggcac cagttgcgtgagcggaaagtggccgcttcccggccctgctgcaggagctc aaaatggaggacgcggcgctcgggagagcggcggtgagtcaccacac aaaggaaaagggcctttccgtcctcagccgtcgttcatgtactccacggag taccgggcgccgtccaggcacctcgattgttctcgagcttttgagtagctcg tctttaggttgggggagggttttatgcgatggagttccccacactgagtgg gtggagactgaagttaggccagcttggcacttgatgaattctccttgaatttg cccttttgagtttgatcttgggtcatttcaagcctcagacagtggttcaaatgtt ttttctccatttcaggtgctgtgaaagcttagctgaattctaatacactactata gggaaataagagagaaaaagaagtaagaagaataataagagccaccatgc ttctcctggtgacaagccttctgctctgtgagttaccacacccagcattcctctg atcccaggcgcgcgtgccttcaaccaagaagttcaaatcccttgaccgaaagt tactgtggcccatgtcctaaaaactggatatgttcaaaaaataactgctaccaatt ttttgatgagagtaaaaaactggtatgagagccaggccttctgtatgtctcaaaatg ccagccttctgaaagtatacagcaagaggaccaggatttactttaaactggtga agtcataatcattggatgggactagtagacattccaacaaatggatcttggcagt ggaagatggctccattctctcaccacactactaacaataattgaaatgcagaa gggagactgtgcactctatgcctcgagctttaaaggctatatagaaaactgttca actccaatacgtacatctgtatgcaaaggactgtggctagcaactggtcacac ccccagtttgagaaaggtgggggagggtcaggtggtggggggtcaaattggt ctcatccccagttcgagaaggcgaggagggttcaggtgggggtgggagca actggagccaccctcaattgaaaaaggaggtggaggagggtggaggtggtg gatctgaatctaaatatggcccccttggccaccatgtcccttctgggttttgta gttgcggcggtgcttcttctattcattgttgggtacggtggttttataatttt ctgggtgaagattctctgtggtgaagaggggtagaagaagctcctctatatcttt aagcaaccttttatgcggccagtacagactactcaagaggaggacggatgttc atgccattccccgaagaggaagaaggggggtgcgagttgagagtgaagtt cagcaggagcgcagacgccccgcgtaccagcagggccagaaccagctct ataacgagctcaatctaggacgaagagaggagtagcatgttttgacaagag acgtggccgggaccctgagatggggggaaagccgagaaggaagaaccctc aggaaggcctgtgagtcgactgagtttgacaaaaccacaactagaatgcagt gaaaaaaatgctttattgtgaaattgtgatgctattgtttattgttaaccattata agctgcaataaacaagttaacaacaacaattgcattcattttatgtttcaggttca gggggagggtgtgggaggttttttaagcaagtaaaacctctacaaatgtggtac ttaagaatgatgctttttccccgtatccccccaggtgtctgcaggctcaaaagag cagcgagaagcgttcagaggaaagcgatcccgtgccaccttccccgtgcc	
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18	gccccgcgtaccagcagggccagaaccagctctataacgagctcaatctag gacgaagagaggagtacgatgttttgacaagaga	DNA sequence encoding the CD3 zeta ITAM-1 intracellular domain
27	APAYQQGQNQLYNELNLGRREEYDVLDKRRGRD PEMGGKPRRKNPQEGL	Amino acid sequence of the CD3 zeta ITAM-1 intracellular signaling domain
19	APAYQQGQNQLYNELNLGRREEYDVLDKR	Amino acid sequence of CD3 zeta ITAM-1
20	MLLLVTSLLLCELPHPAFLIPGAHAFNQEVQIPLT	Amino acid sequence

	ESYCGPCPKNWICYKNNCYQFFDESKNWYESQA SCMSQNASLLKVYSKEDQDLLKLVKSYHWMGLV HIPTNGSWQWEDGSILSPNLLTIEMQKGDCALYA SSFKGYIENCSTPNTYICMQRTVASNWSHPQFEKG GGGSGGGGSNWSHPQFEKGGGSGGGGSNWSHP QFEKGGGSGGGGSESKYGPPCPPCPFWVLVVVG GVLACYSLLVTVAFIIFWVRFSVVKRGRKKLLYIF KQPFMRPVQTTQEEDGCSCRFPEEEGGCELRVK FSRSADAPAYQQGQNQLYNELNLGRREEYDVLD KRRGRDPEMGGKPRRKNPQEGL	of the NKG2Dbz1 CAR
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CLAIMS:

1. A $\alpha\beta$ T cell genetically modified to express a recombinant chimeric antigen receptor (CAR), wherein the recombinant CAR comprises (a) an extracellular domain comprising an antigen binding region, (b) a transmembrane domain, (c) at least one co-stimulatory signaling domain, and (d) an intracellular signaling domain, wherein the extracellular domain comprises the extracellular domain of an NKG2D receptor, and wherein the intracellular signaling domain comprises one immunoreceptor tyrosine-based activation motif (ITAM).
2. The $\alpha\beta$ T cell of claim 1, wherein the ITAM is a DAP12 ITAM.
3. The $\alpha\beta$ T cell of claim 2, wherein the DAP12 ITAM is ESPYQELQGQRSDVYSDL (SEQ ID NO: 15).
4. The $\alpha\beta$ T cell of claim 1, wherein the ITAM is CD3zeta ITAM-1.
5. The $\alpha\beta$ T cell of claim 4, wherein the CD3zeta ITAM-1 is APAYQQGQNQLYNELNLGRREEYDVLDKR (SEQ ID NO: 19).
6. The $\alpha\beta$ T cell of any one of claims 1 to 3, wherein the intracellular signaling domain is the intracellular signaling domain of DAP12.
7. The $\alpha\beta$ T cell of claim 4 or 5, wherein the intracellular signaling domain comprises APAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGL (SEQ ID NO: 27).
8. The $\alpha\beta$ T cell of any one of claims 1 to 7, wherein the transmembrane domain is CD28 transmembrane domain.
9. The $\alpha\beta$ T cell of any one of claims 1 to 8, wherein the co-stimulatory signaling domain is selected from the group consisting of 4-1BB, CD28, CD2 and OX-40.

10. The $\alpha\beta$ T cell of any one of claims 1 to 9, wherein the recombinant CAR further comprises, between the antigen binding region and the transmembrane region, a hinge region.

11. The $\alpha\beta$ T cell of claim 10, wherein the hinge region is an IgG4 hinge region.

12. A method of preparing the $\alpha\beta$ T cell of any one of claims 1 to 11, the method comprises:

- (i) obtaining or providing $\alpha\beta$ T cell;
- (ii) providing a recombinant nucleic acid encoding the recombinant chimeric antigen receptor (CAR); and
- (iii) transferring the recombinant nucleic acid encoding the recombinant CAR into the $\alpha\beta$ T cell.

13. A pharmaceutical composition comprising a pharmaceutically effective amount of the $\alpha\beta$ T cell of any one of claims 1 to 11 and a pharmaceutically acceptable excipient.

14. A method of treating cancer or tumor in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of the $\alpha\beta$ T cell of any one of claims 1 to 11, or the pharmaceutical composition of claim 13.

15. The method of claim 14, wherein the $\alpha\beta$ T cells are derived from allogeneic or autologous $\alpha\beta$ T cells.

16. A method of treating cancer or tumor in a subject in need thereof, the method comprises:

- (i) obtaining $\alpha\beta$ T cells from the subject, or from a $\alpha\beta$ T cell donor which is different from the subject to be treated;
- (ii) providing a recombinant nucleic acid encoding a recombinant chimeric antigen receptor (CAR), wherein the recombinant CAR comprises (a) an extracellular domain comprising an antigen binding region, (b) a transmembrane domain, (c) at least one co-stimulatory signaling domain, and (d) an intracellular signaling domain, wherein the extracellular domain comprises the extracellular domain of an NKG2D receptor, and wherein the intracellular signaling domain comprises one immunoreceptor tyrosine-based activation motif (ITAM);

- (iii) transferring the recombinant nucleic acid encoding the recombinant CAR into the $\alpha\beta$ T cell to obtain genetically modified $\alpha\beta$ T cells; and
- (iv) administering to the subject a pharmaceutically effective amount of the $\alpha\beta$ T cells obtained from (iii).

17. The method of any one of claims 14 to 16, wherein the cancer or tumor expresses NKG2D ligand(s).

18. The method of any one of claims 14 to 17, wherein the cancer or tumor is a solid cancer or tumor.

19. The method of any one of claims 14 to 18, wherein the cancer or tumor is colorectal cancer or colorectal tumor, or ovarian cancer or ovarian tumor.

20. The method of any one of claims 12 and 16-19, wherein transferring the recombinant nucleic acid in step (iii) is carried out using electroporation.

21. A recombinant chimeric antigen receptor (CAR) comprising (a) an extracellular domain comprising an antigen binding region, (b) a transmembrane domain, (c) at least one co-stimulatory signaling domain, and (d) an intracellular signaling domain, wherein the extracellular domain comprises the extracellular domain of an NKG2D receptor, and wherein the intracellular signaling domain comprises one immunoreceptor tyrosine-based activation motif (ITAM).

22. The recombinant CAR of claim 21, wherein the ITAM is a DAP12 ITAM.

23. The recombinant CAR of claim 22, wherein the DAP12 ITAM is ESPYQELQGQRSDVYSDL (SEQ ID NO: 15).

24. The recombinant CAR of any one of claims 21 to 23, wherein the intracellular signaling domain is the intracellular signaling domain of DAP12.

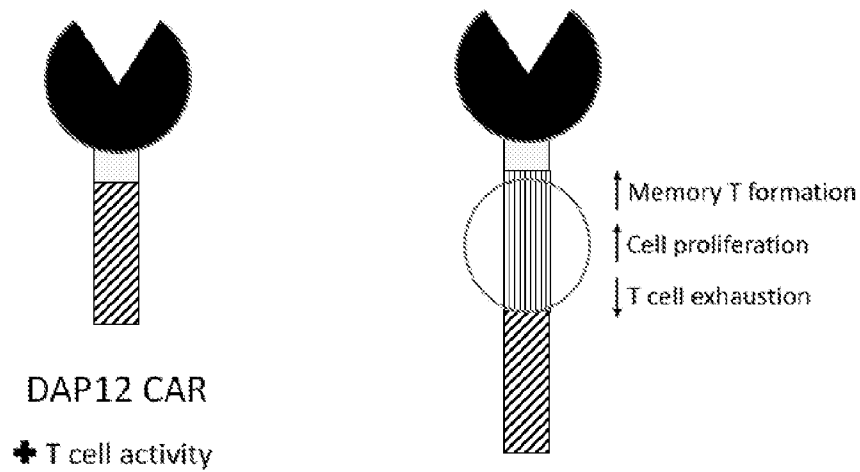
25. The recombinant CAR of any one of claims 21 to 24, wherein the transmembrane domain is CD28 transmembrane domain.

26. The recombinant CAR of any one of claims 21 to 25, wherein the at least one co-stimulatory signaling domain is selected from the group consisting of 4-1BB, CD28, CD2 and OX-40.

27. The recombinant CAR of any one of claims 21 to 26, wherein the recombinant CAR further comprises, between the antigen binding region and the transmembrane region, a hinge region.

28. The recombinant CAR of claim 27, wherein the hinge region is an IgG4 hinge region.

- A. A single ITAM is sufficient to activate $\alpha\beta$ -T cells
- B 4-1BB + DAP12 combination enhances CAR T functions



- c. Single ITAM mediates lower cytokine release by $\alpha\beta$ -T cells

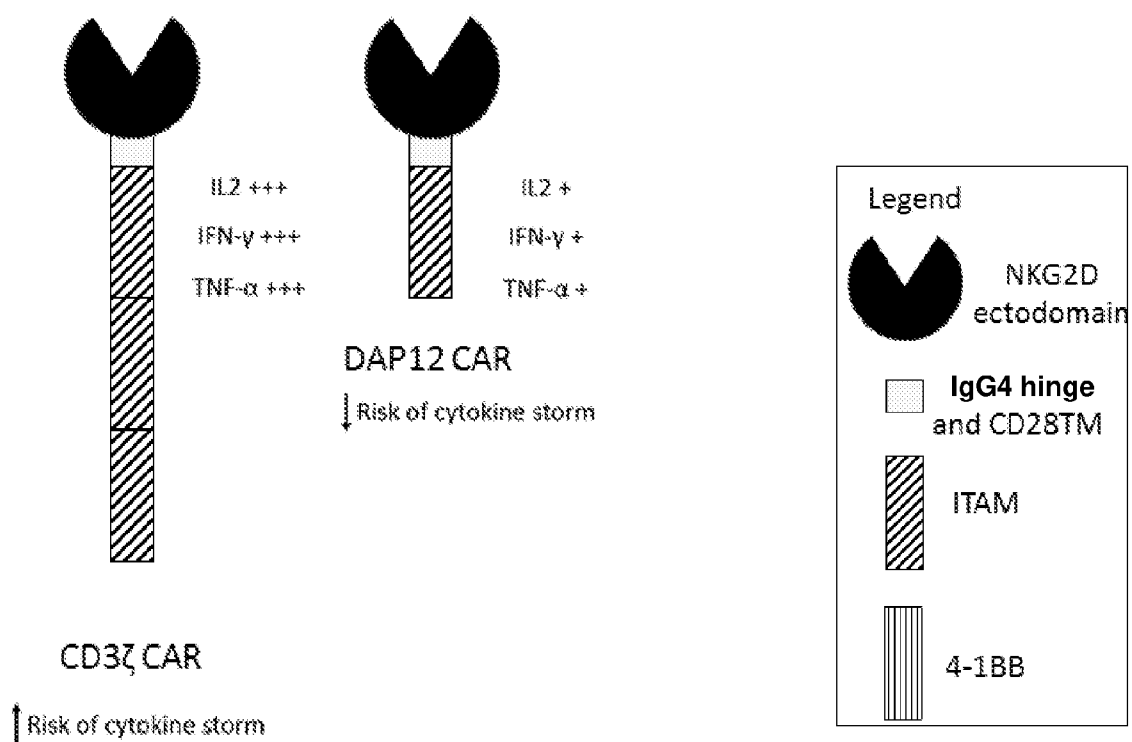
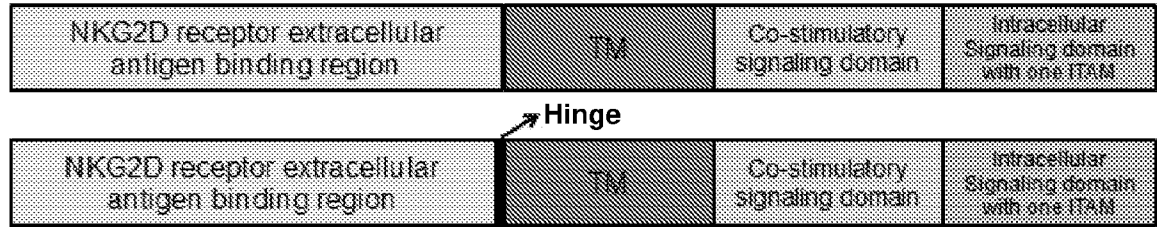
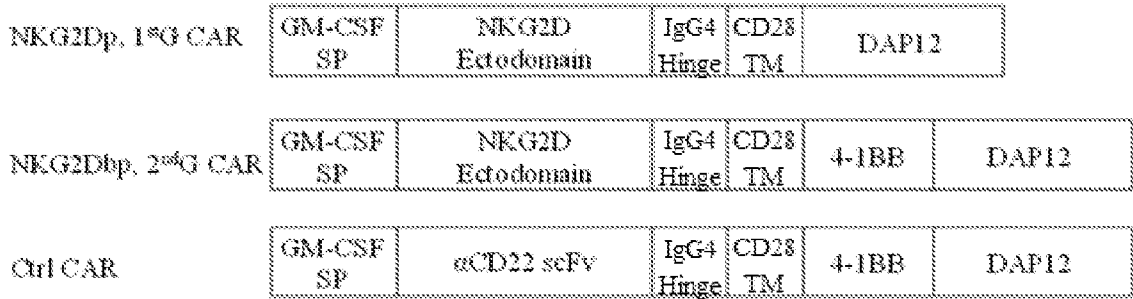


Fig. 1

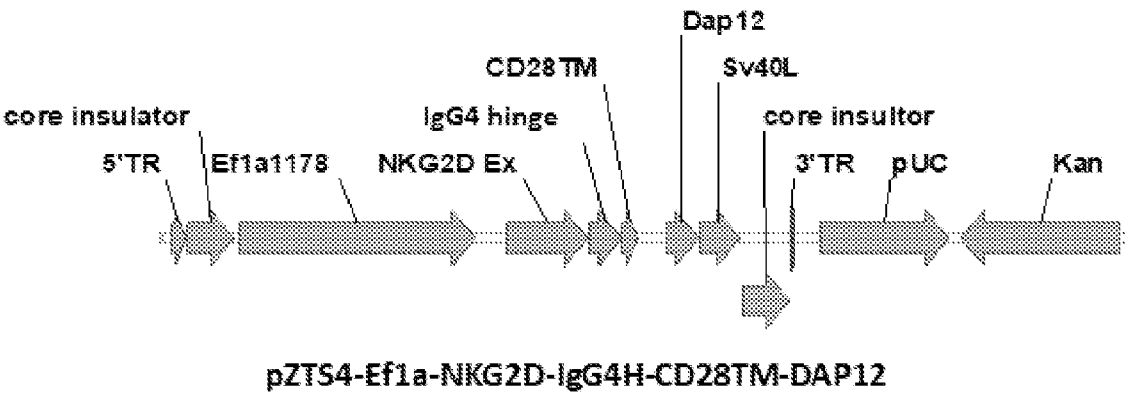
A



B



C



D

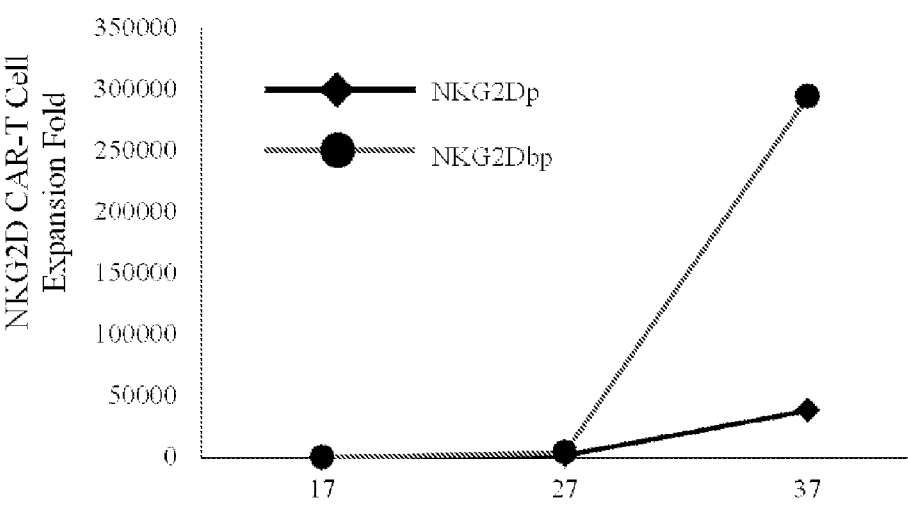
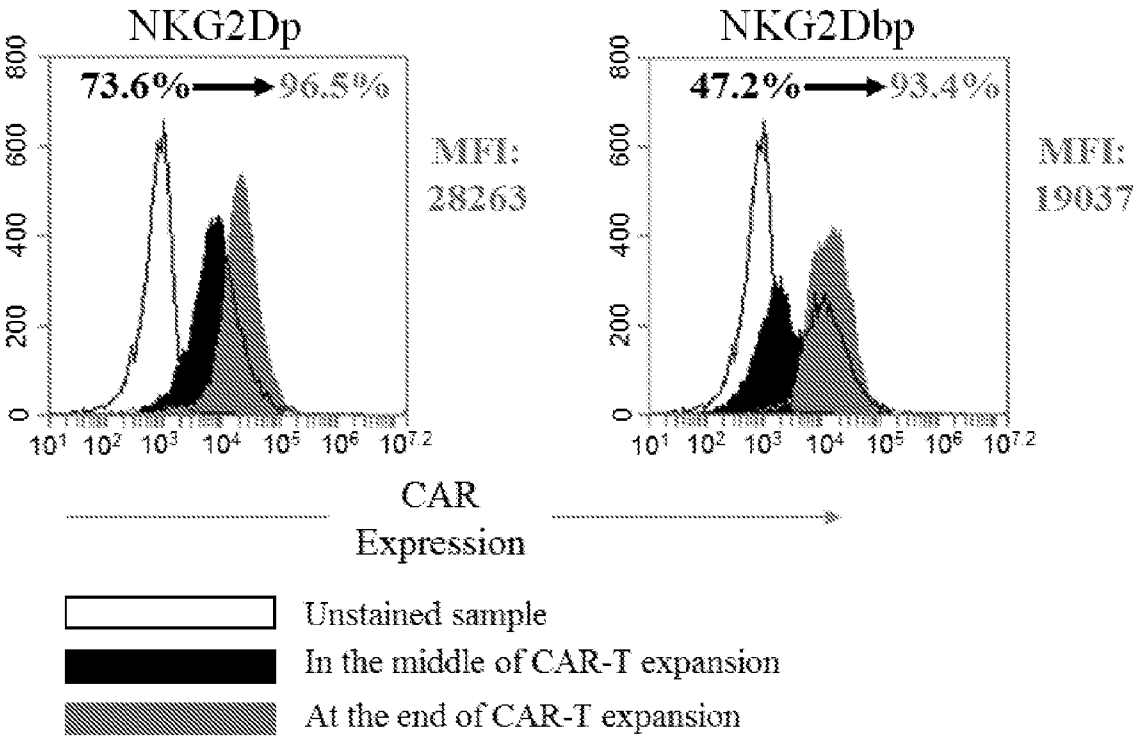


Fig. 2

E



F

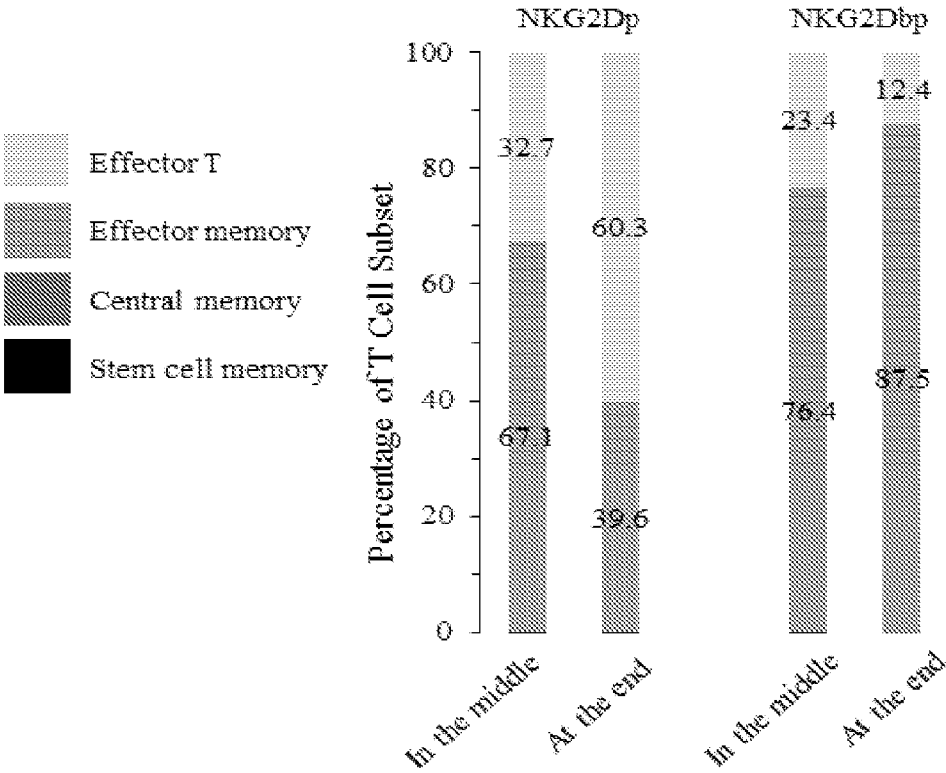


Fig. 2 (cont)

A

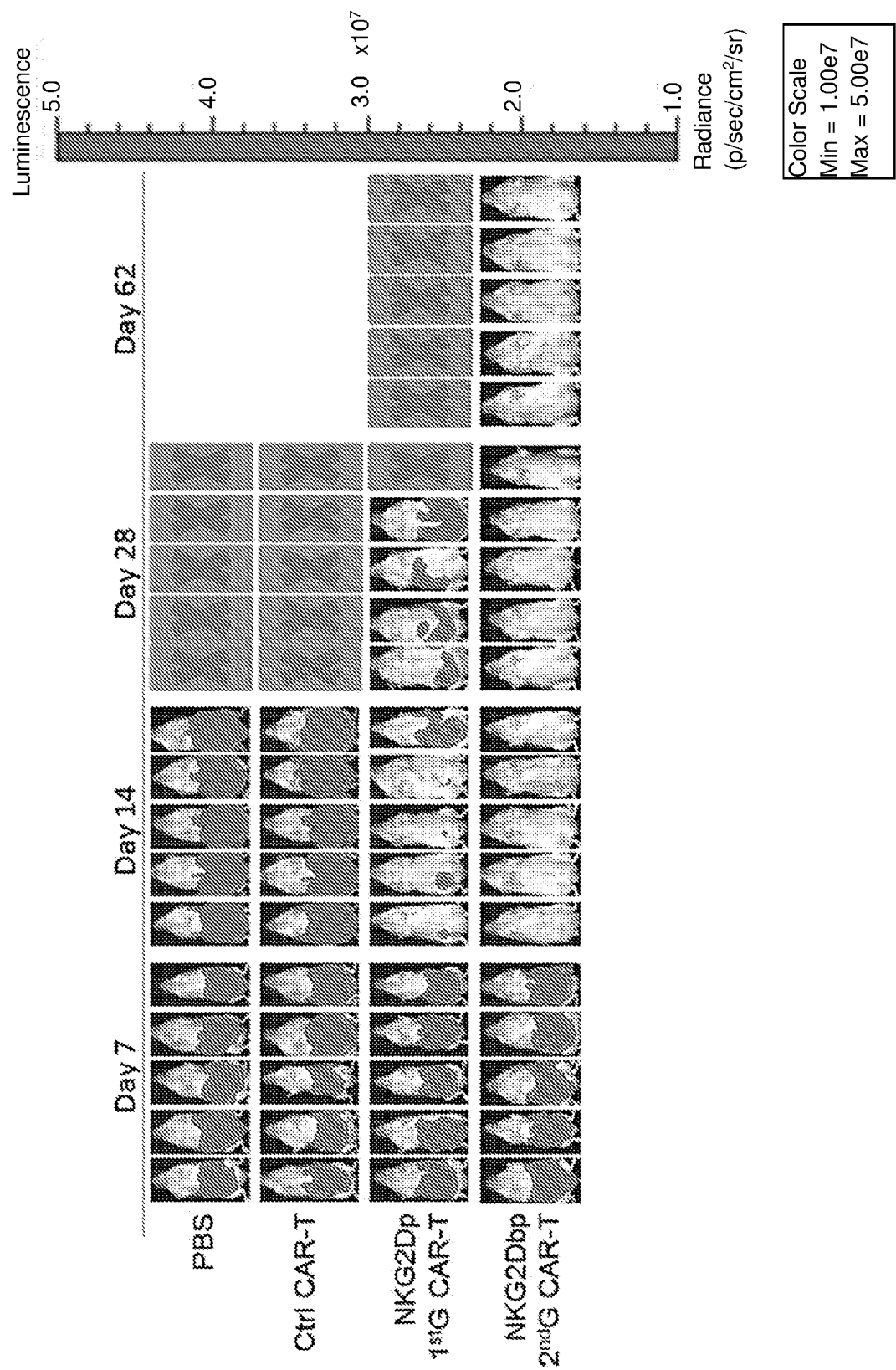


Fig. 3

5/22

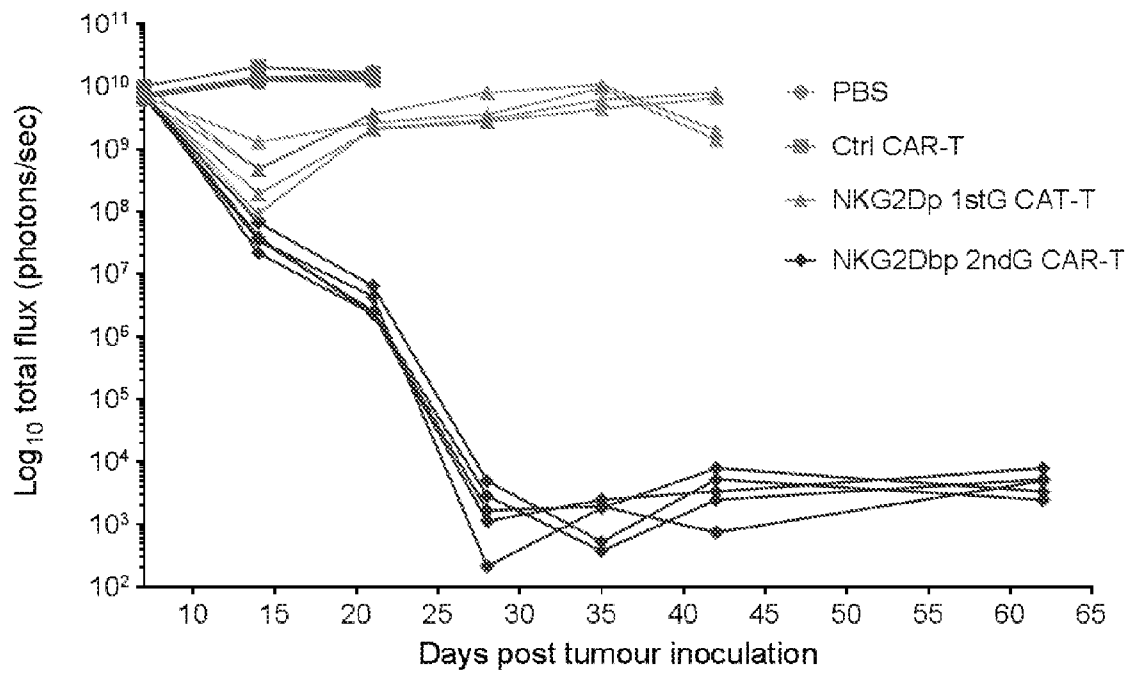
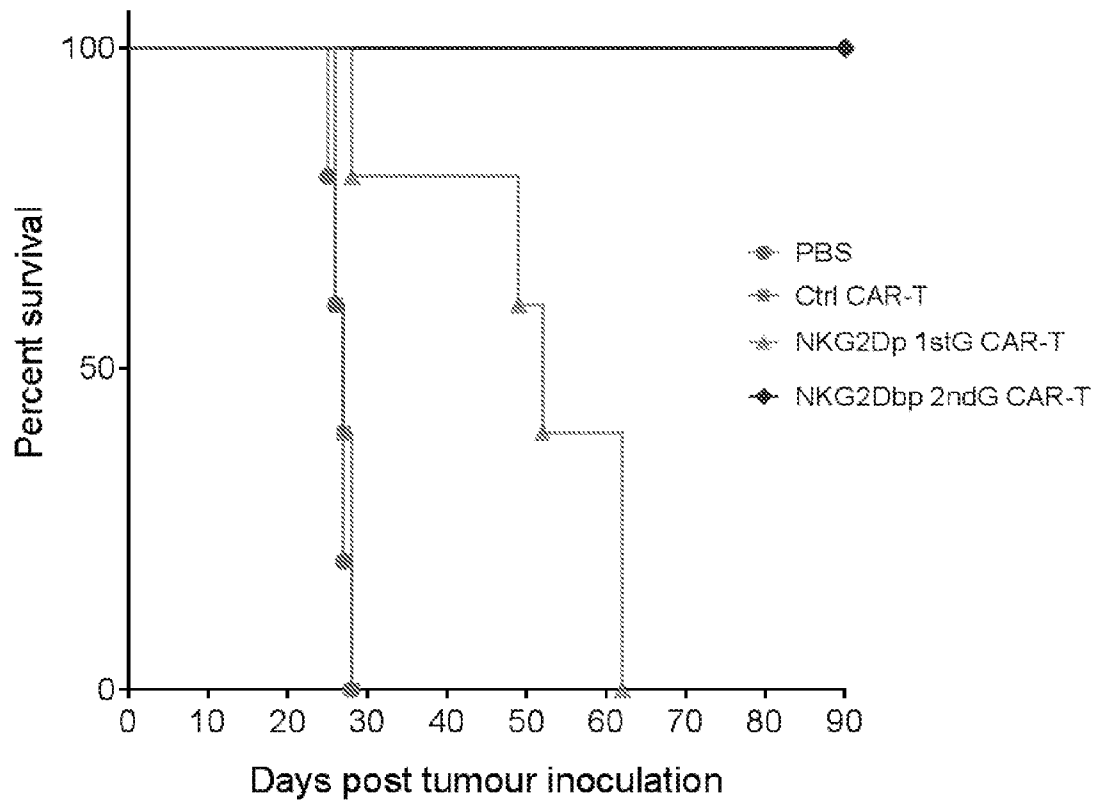
B**C**

Fig. 3 (cont)

6/22

A

NKG2Dbz**NKG2Dbz-1****NKG2Dbp**

B

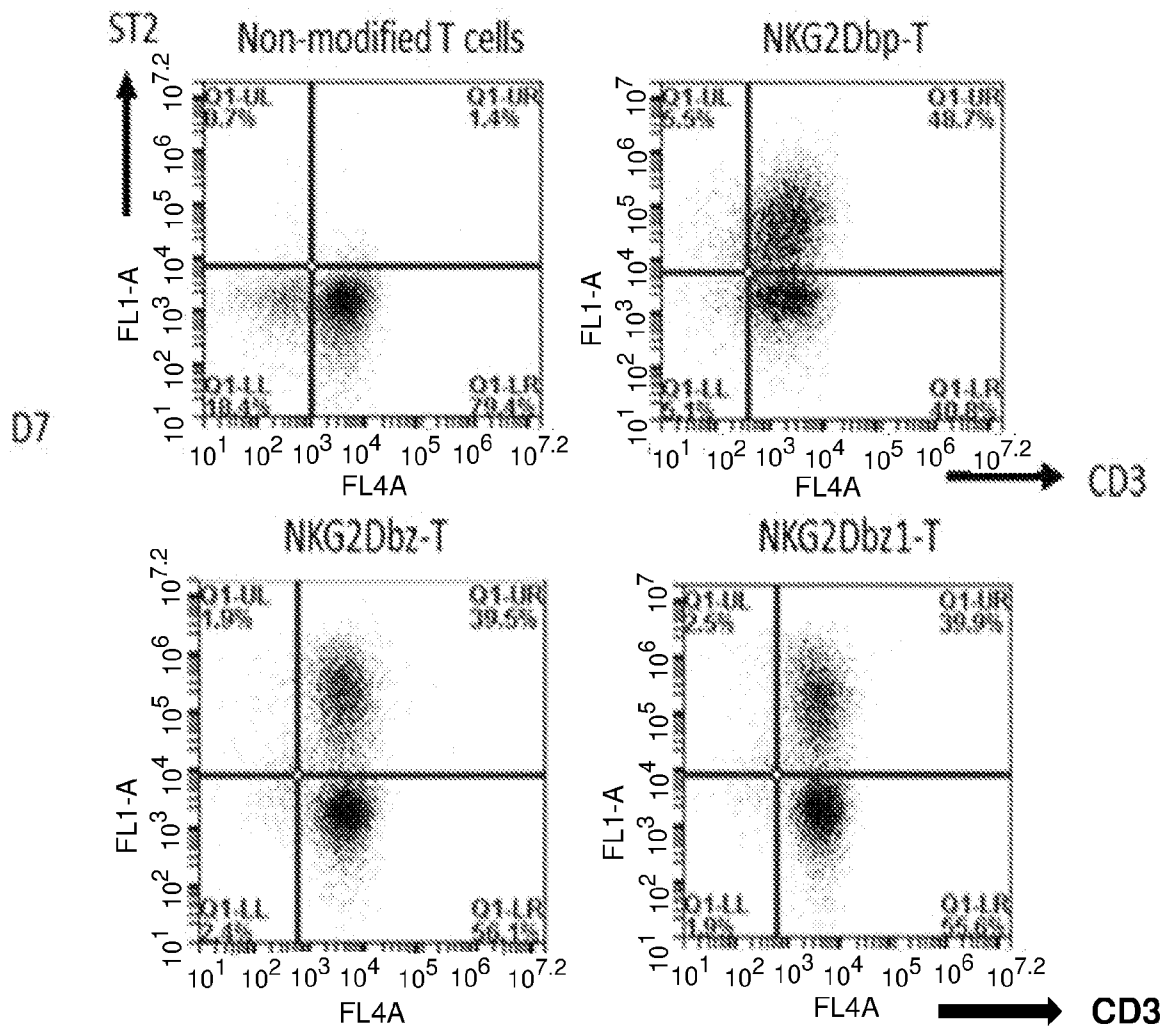


Fig. 4

C

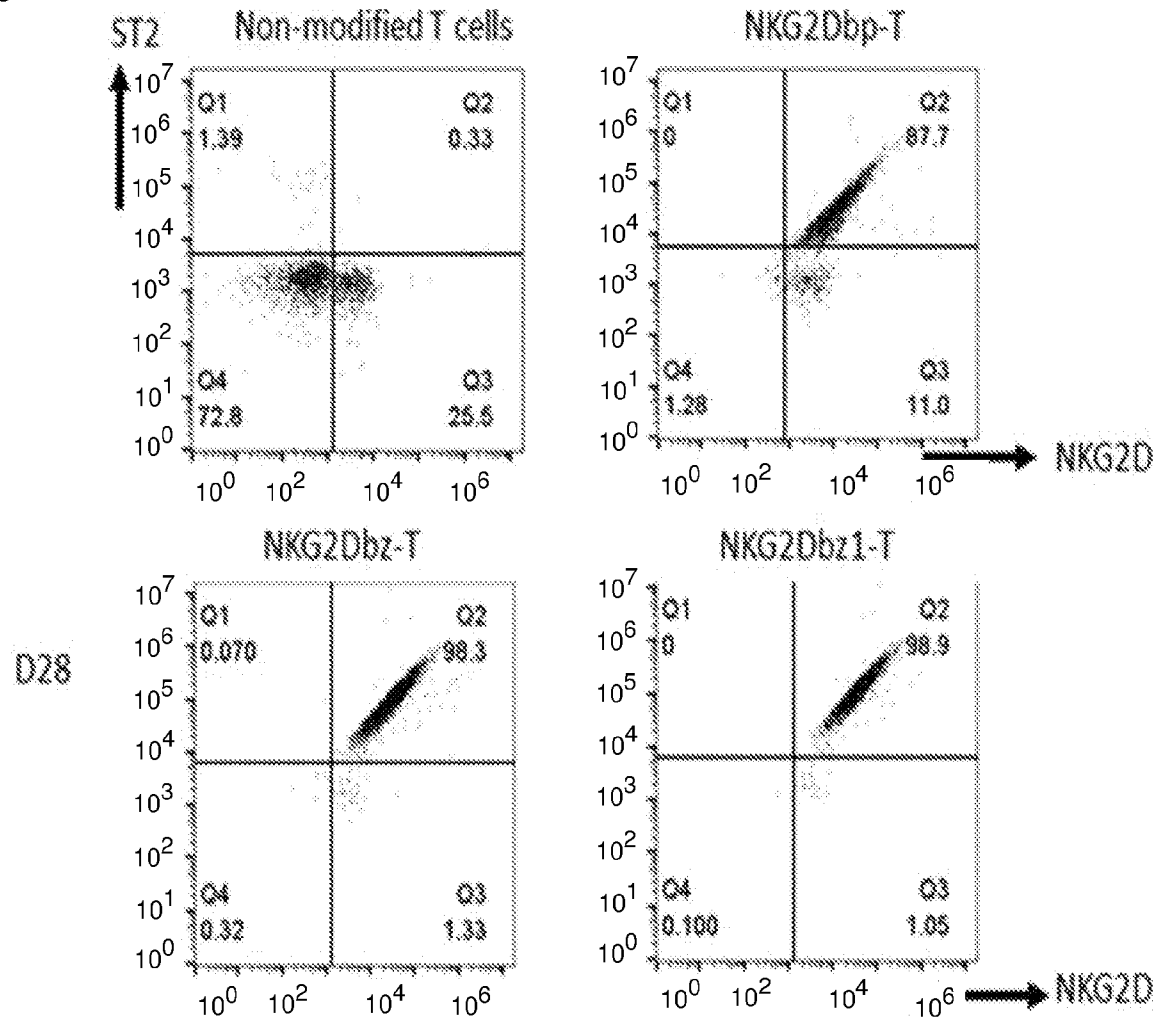


Fig. 4 (cont)

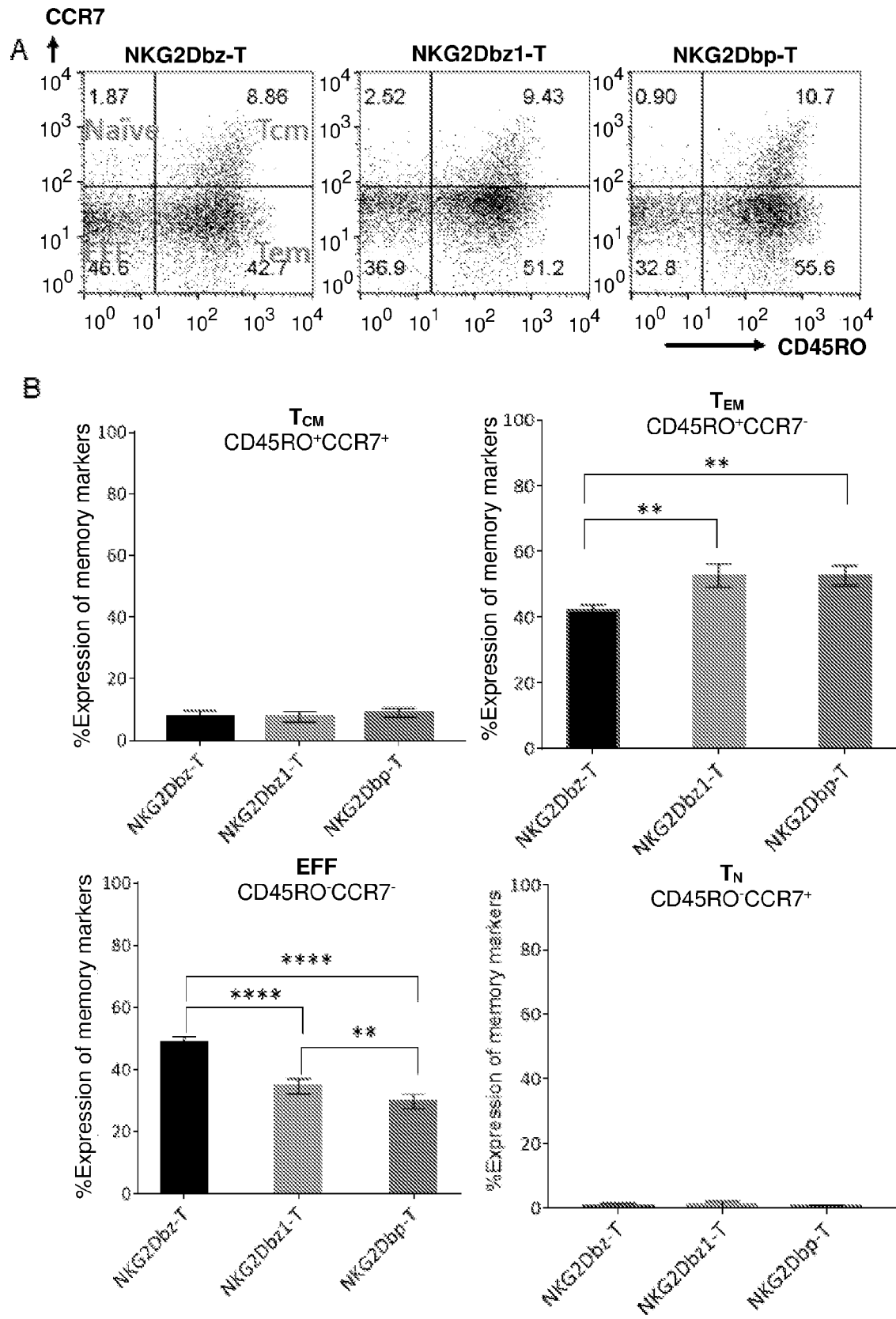


Fig. 5

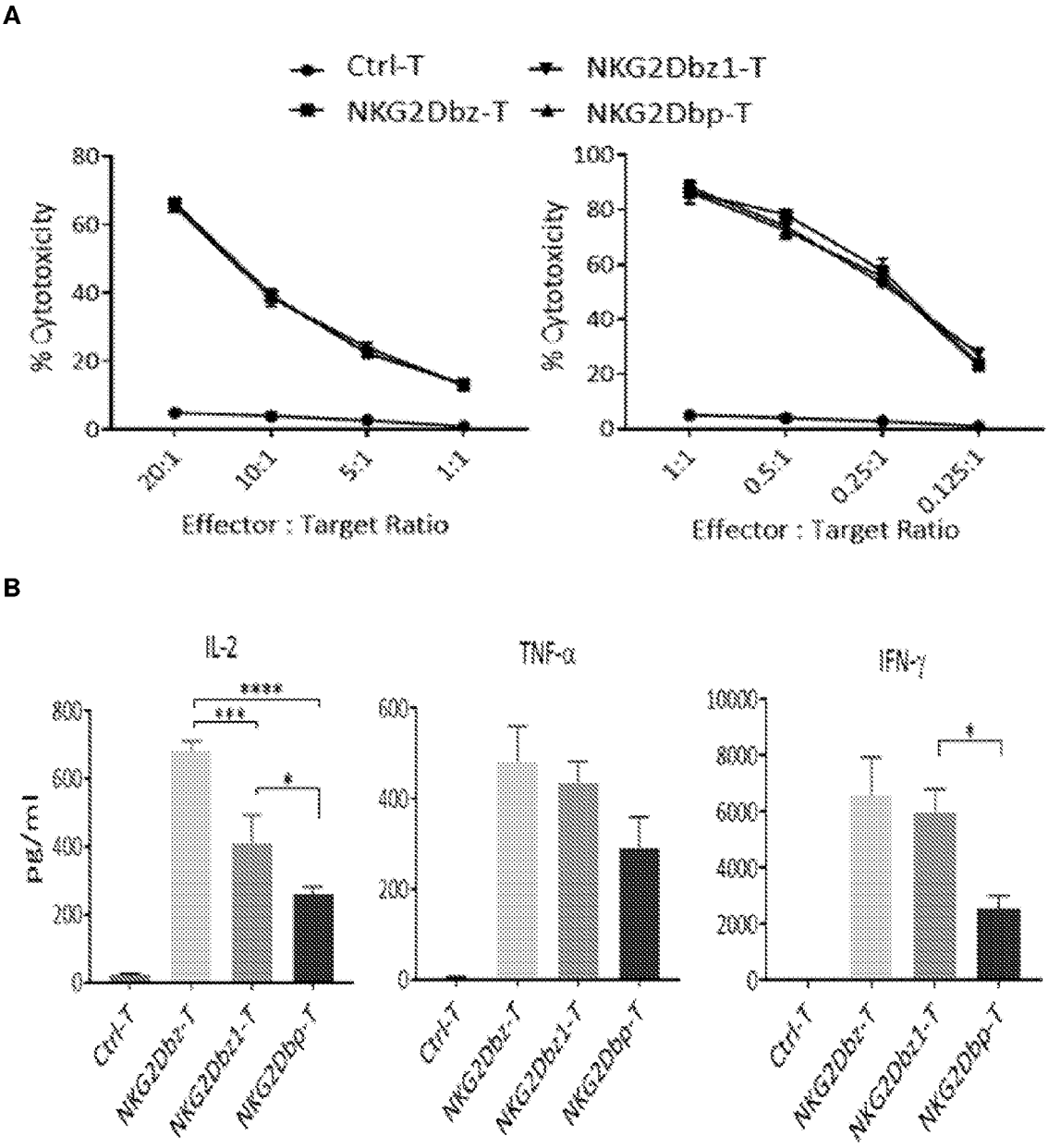


Fig. 6

10/22

C

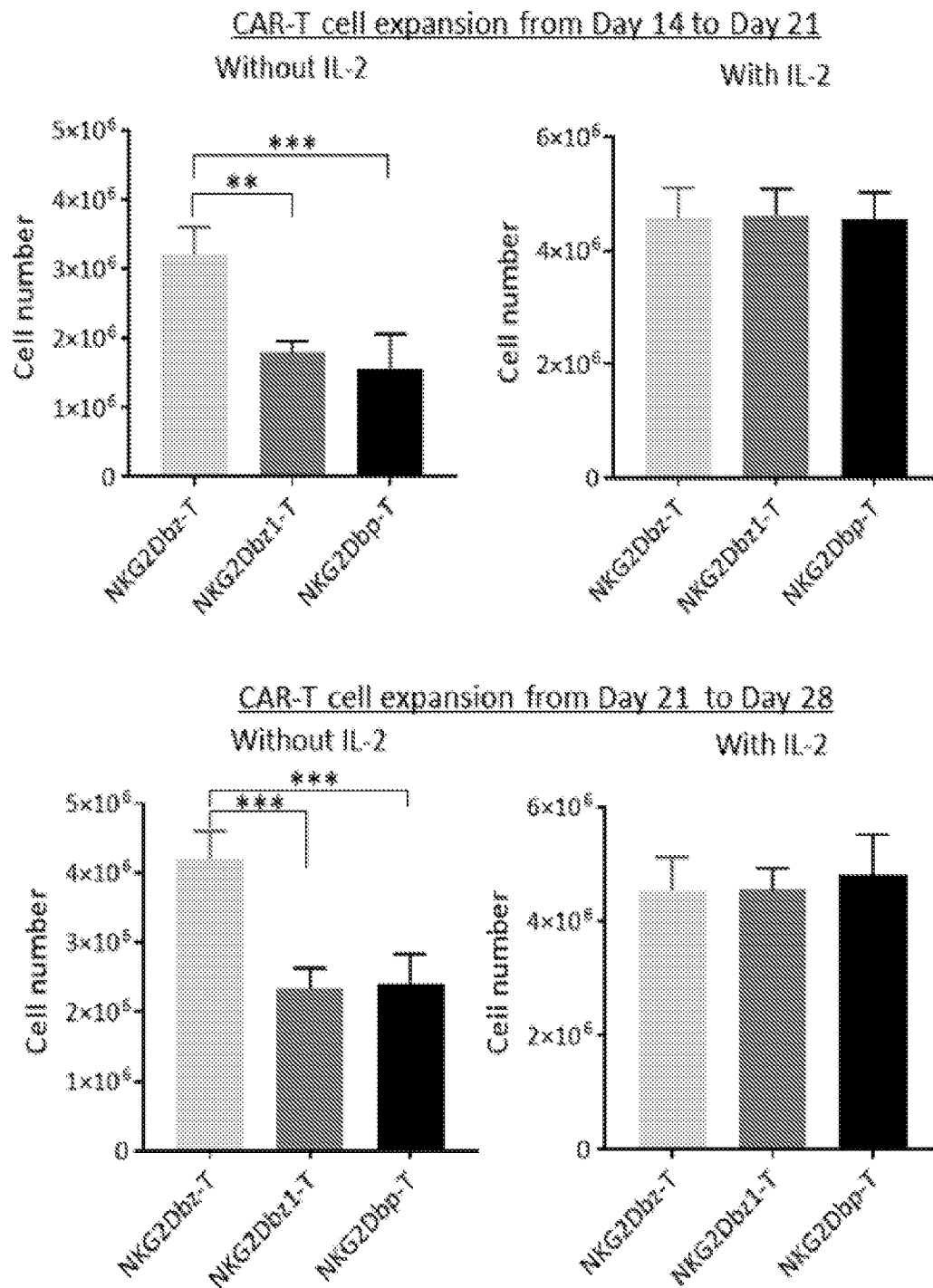


Fig. 6 (cont)

11/22

A

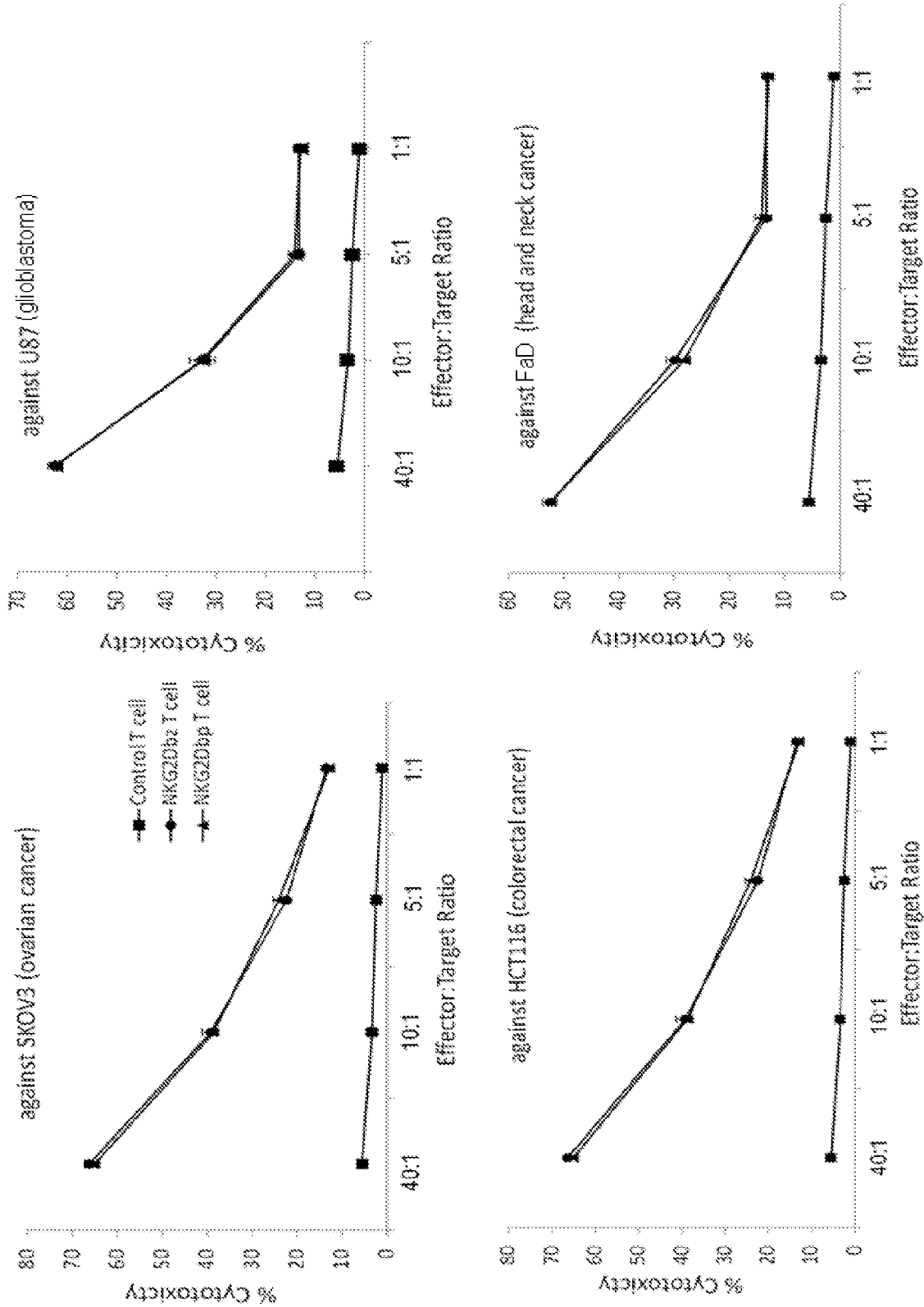


Fig. 7

12/22

B

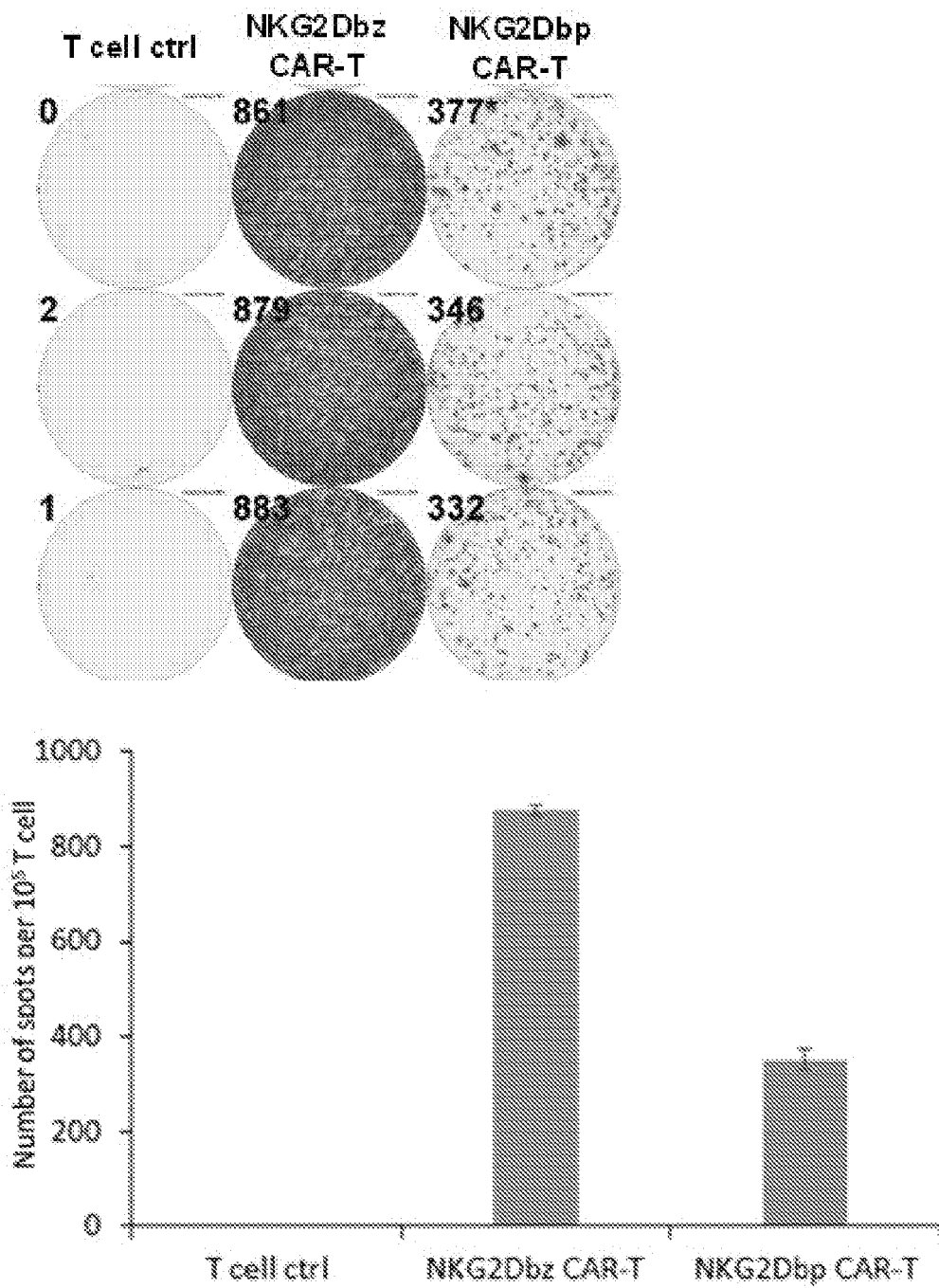


Fig. 7 (cont)

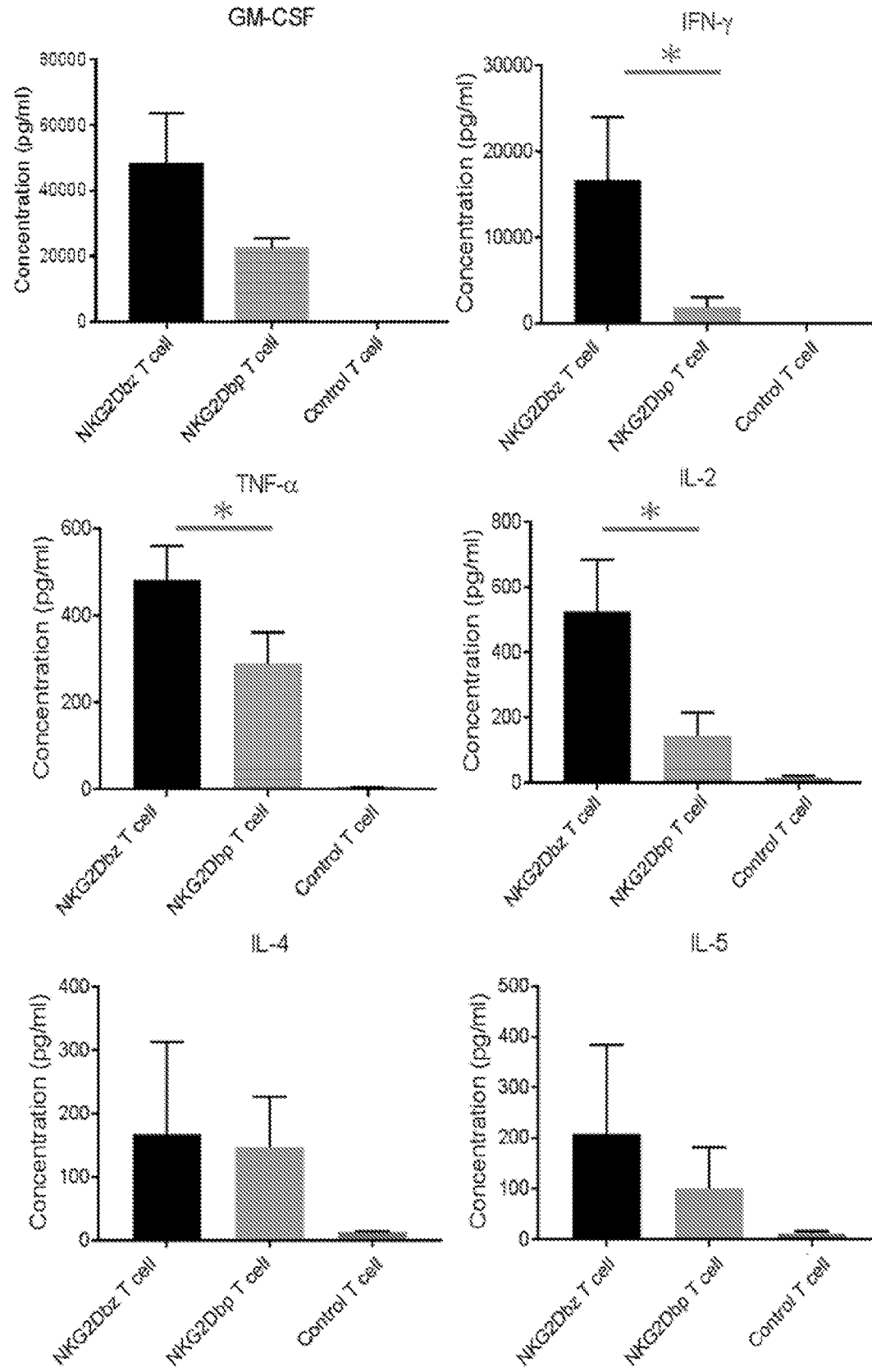


Fig. 8

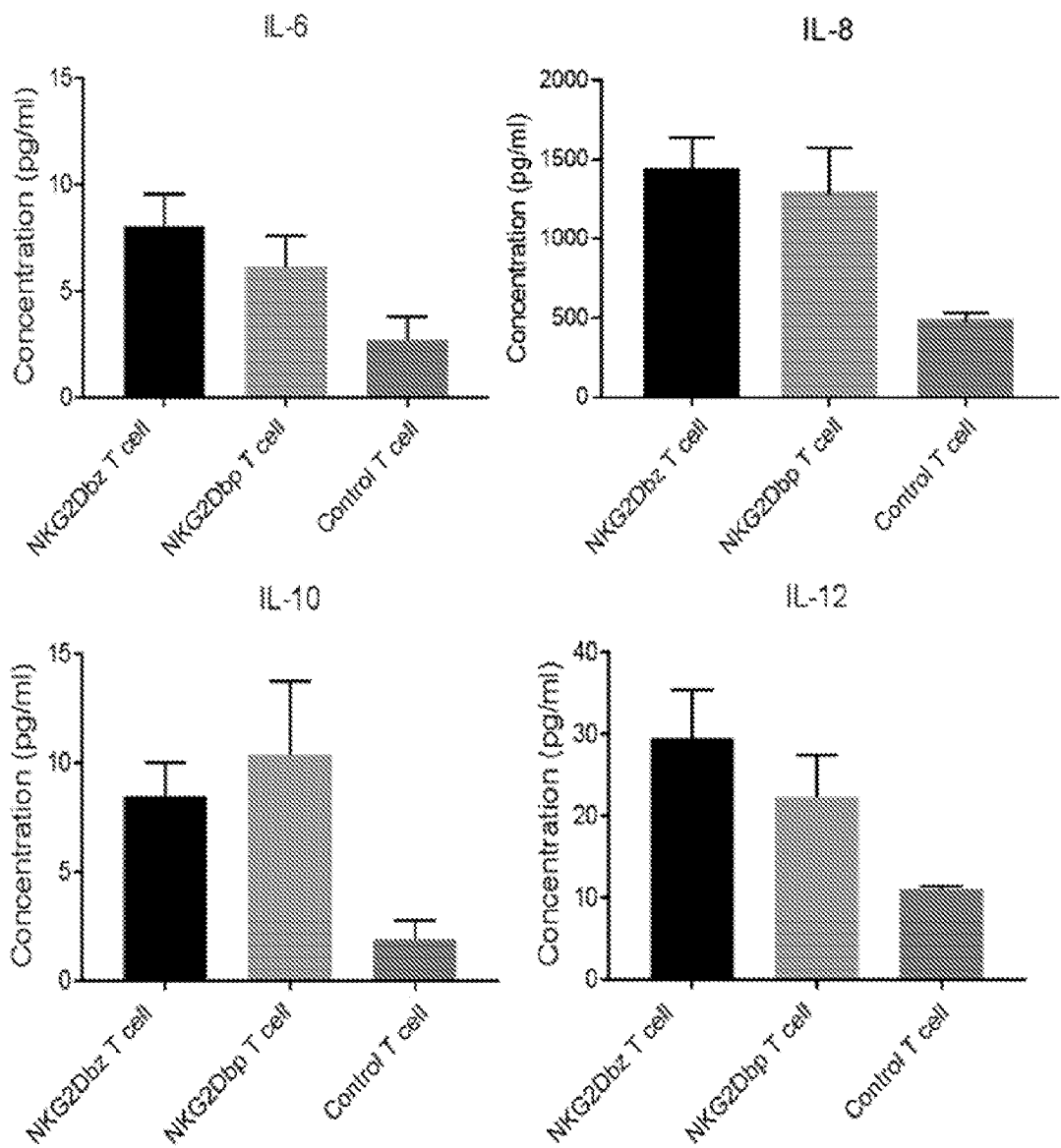


Fig. 8 (cont)

15/22

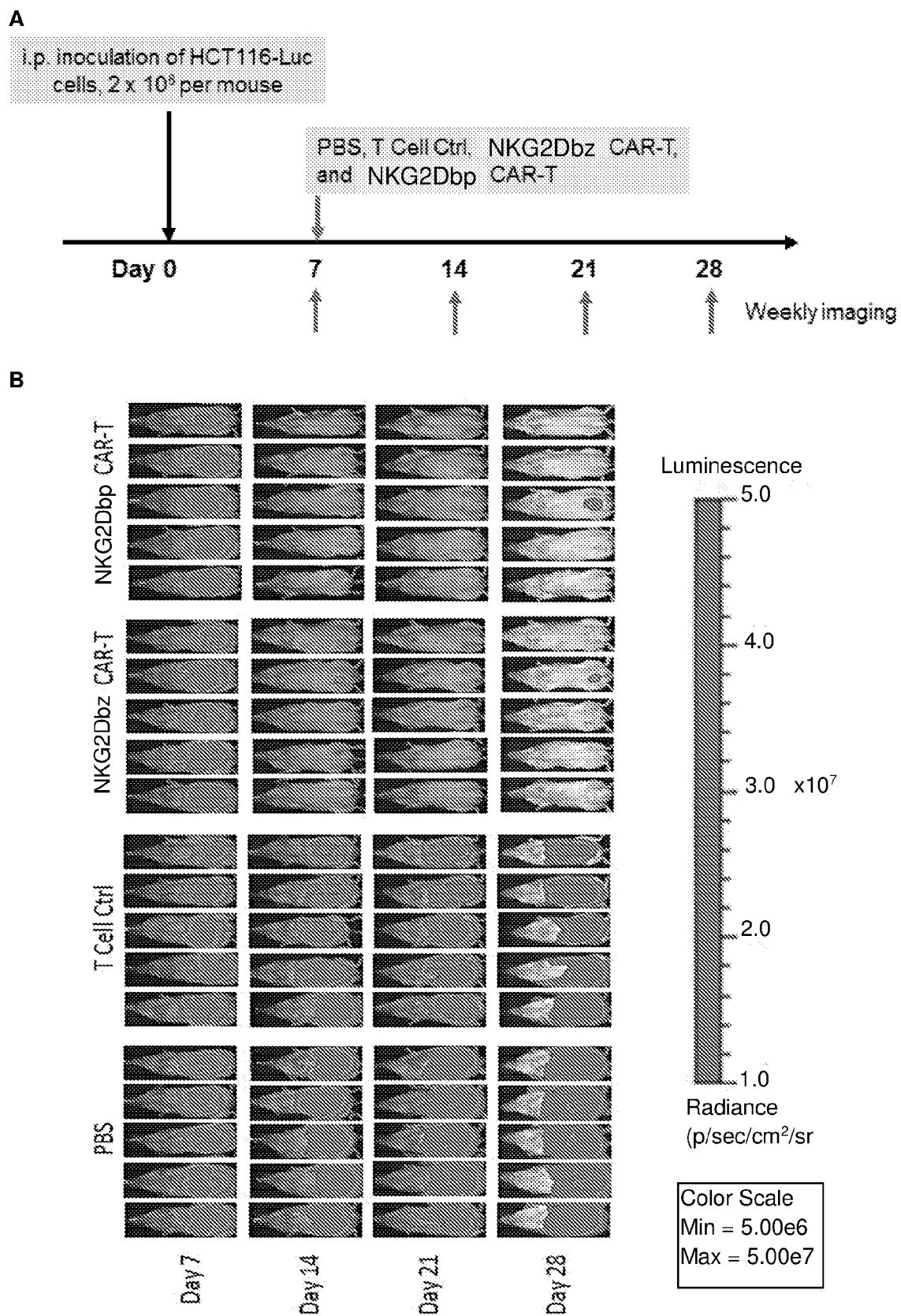


Fig. 9

C

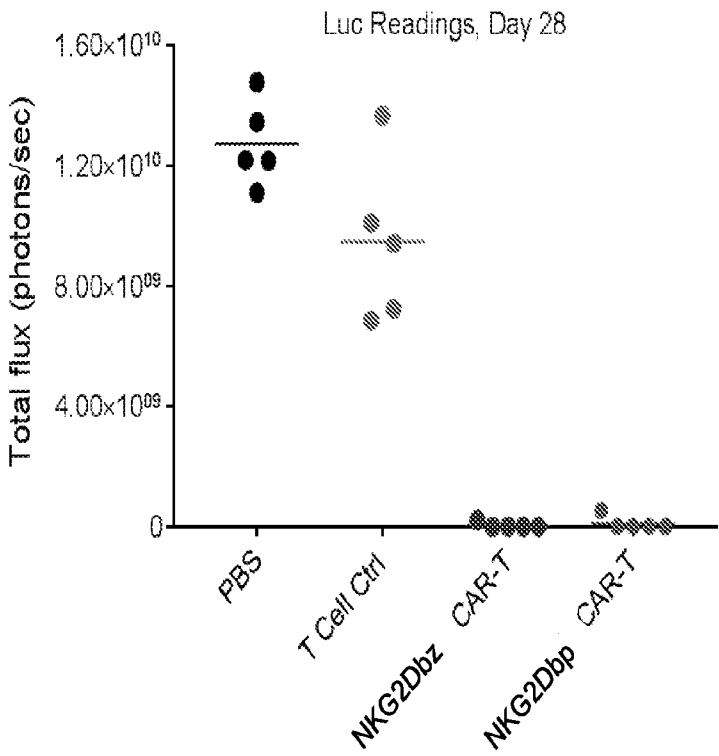
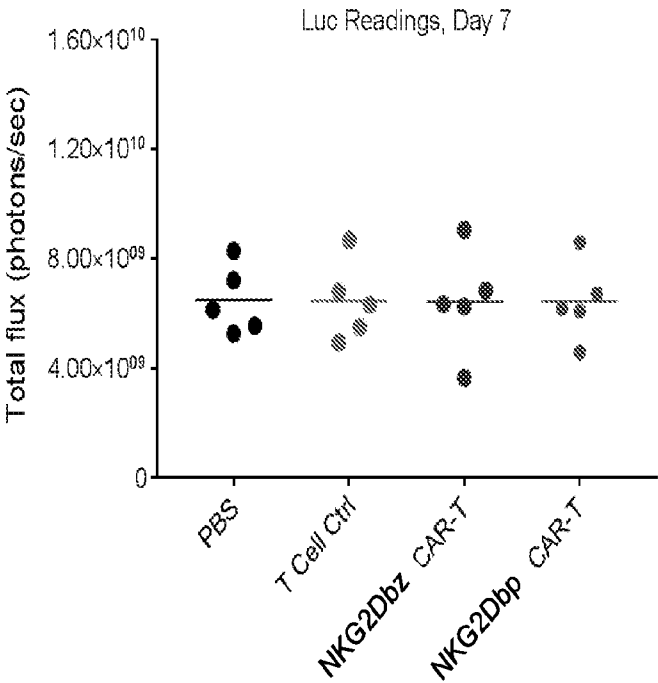


Fig. 9 (cont)

A

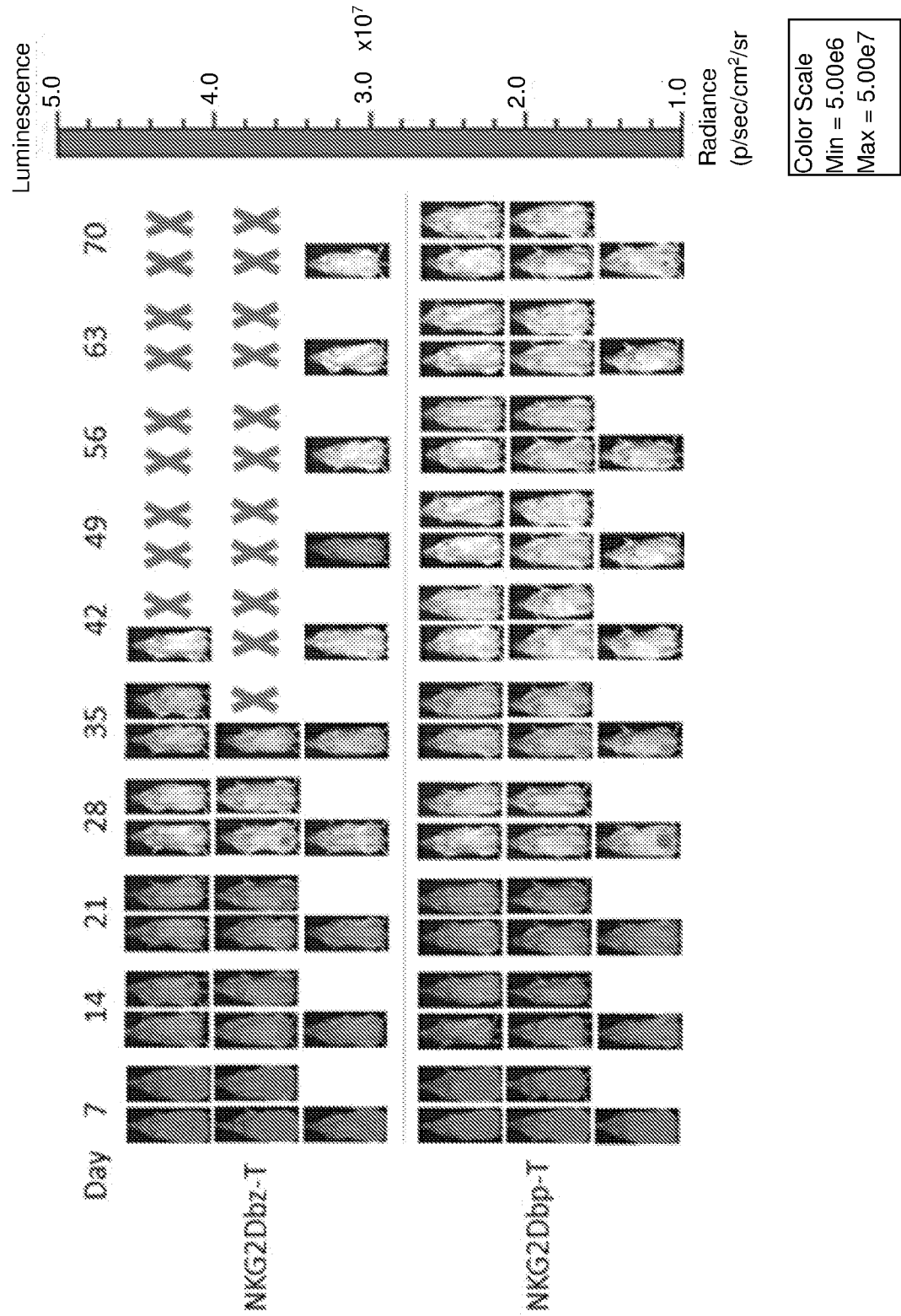


Fig. 10

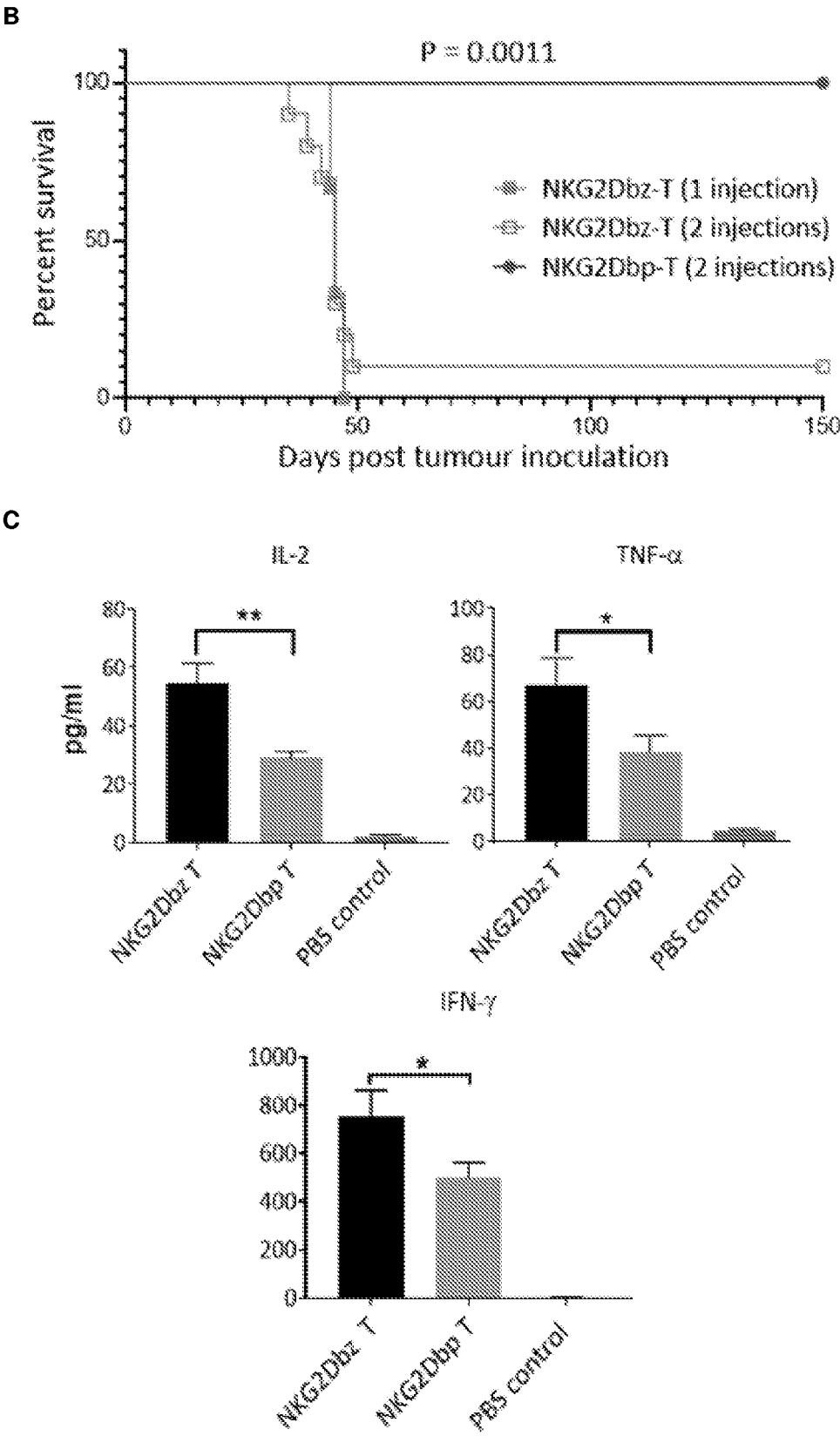
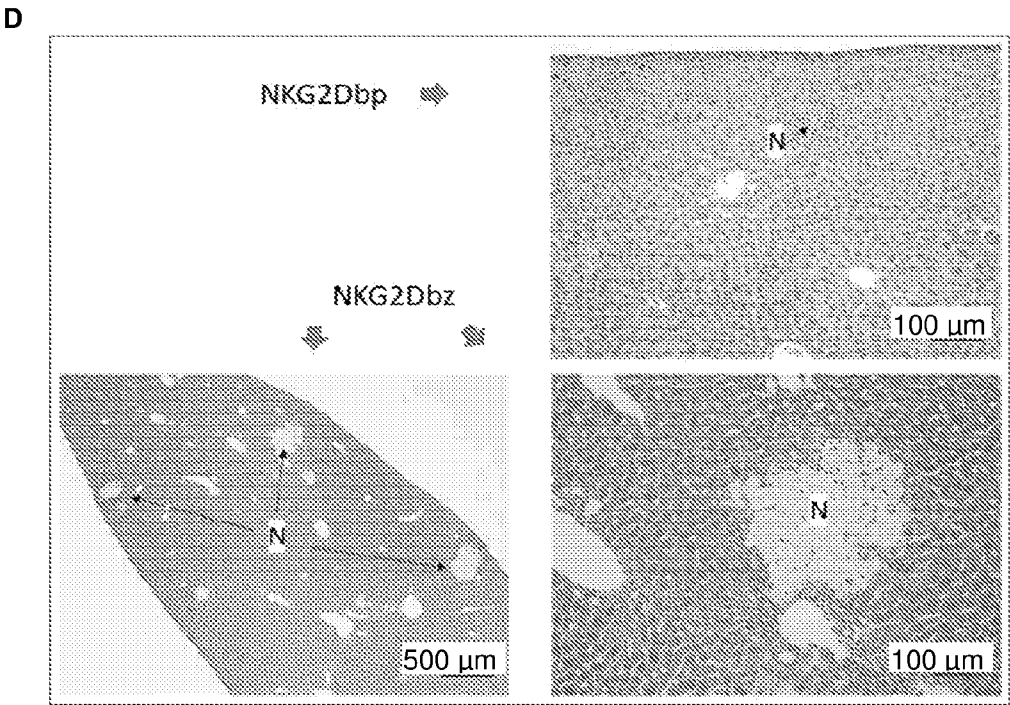


Fig. 10 (cont)



E

	# mice with hair loss / # mice examined	Representative images
PBS	0/3	
NKG2Dbz-T (1 injection)	1/3	
NKG2Dbz-T (2 injections)	3/5	
NKG2Dbp-T	0/3	

Fig. 10 (cont)

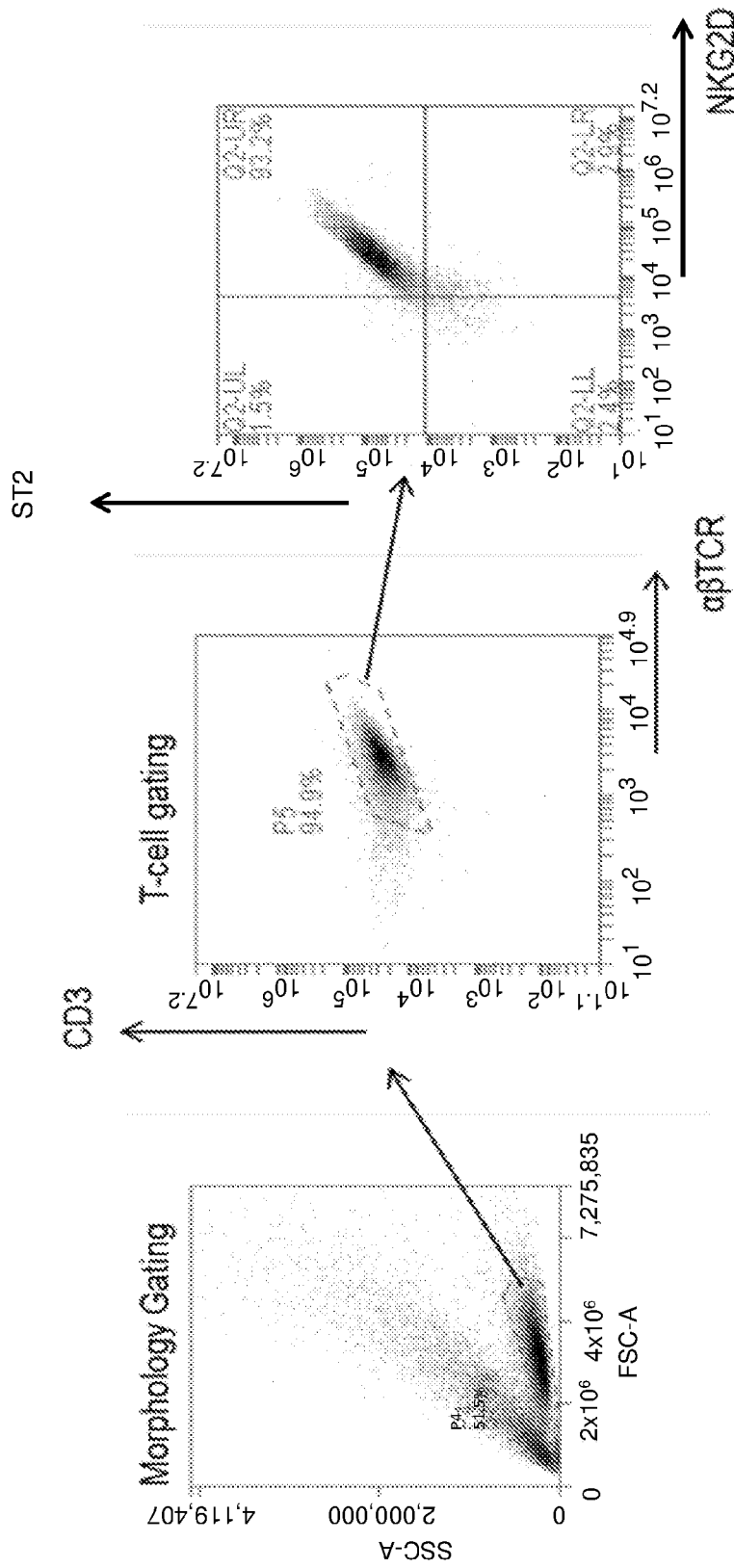


Fig. 11

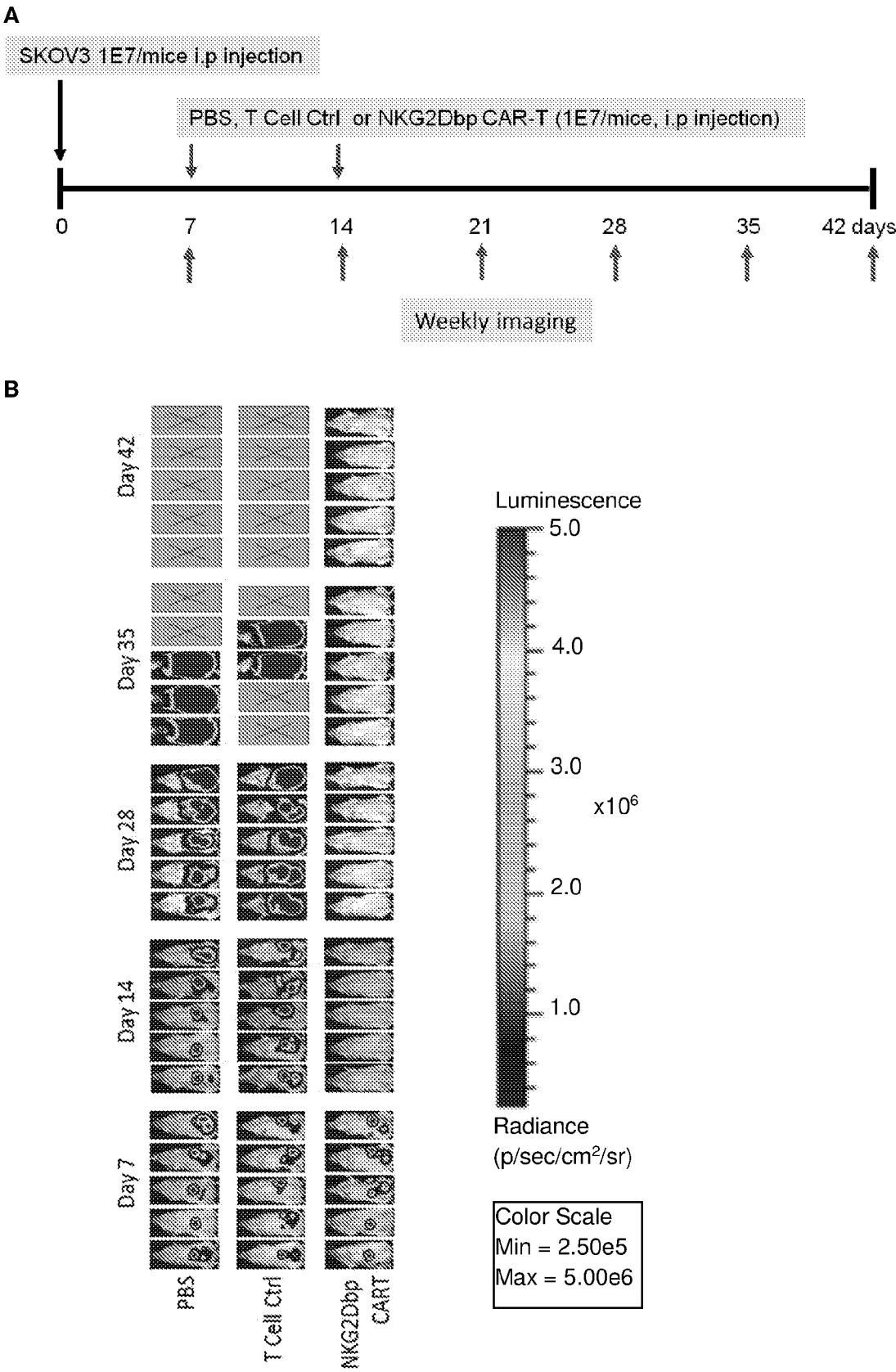


Fig. 12

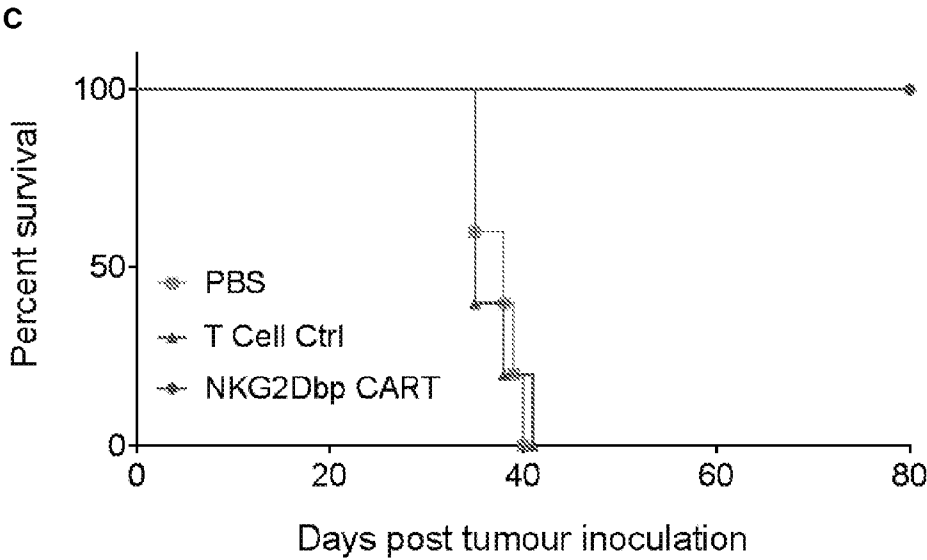


Fig. 12 (cont)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2020/050520

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/0783 (2010.01) C07K 14/725 (2006.01) A61K 35/17 (2015.01) A61P 35/00 (2006.01)

According to International Patent Classification (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)

DATABASES: WPI, EPODOC, FAMPAT, MEDLINE, EMBASE, BIOSIS

SEARCH TERMS: Chimeric antigen receptor, CAR, CART, DAP12, CD3 zeta, FcR gamma, ITAM, NKG2D, CD314 and related terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2019/133969 A2 (MEMORIAL SLOAN-KETTERING CANCER CENTRE) 4 July 2019 Page 5, Lines 23-25; Page 8, Lines 15-29; Page 9, Line 9 to Page 11, Line 20; Results; Figure 1A, 3-5; SEQ ID NO: 23	1-28
X	WO 2018/140725 A1 (NOVARTIS AG ET AL.) 2 August 2018 Claims 1-68; Page 1, Lines 21-25; Page 6, Lines 16-22; Page 7, Lines 6-8; Page 10, Line 9; Page 10, Lines 24-26; Page 11, Lines 16-19	1-3, 6, 8-28
X	WO 2018/147805 A1 (AGENCY FOR SCIENCE, TECHNOLOGY AND RESEARCH) 16 August 2018 Claims 1-55; Paragraphs [0006], [0022], [0025]-[0029], [0108]-[0111], [0122]-[0123]; SEQ ID NO: 7	1-3, 6, 8-28

☒ Further documents are listed in the continuation of Box C.

☒ 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

30/11/2020

(day/month/year)

Date of mailing of the international search report

01/12/2020

(day/month/year)

Name and mailing address of the ISA/SG



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2020/050520

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FERNÁNDEZ L. ET AL., Memory T Cells Expressing an NKG2D-CAR Efficiently Target Osteosarcoma Cells. <i>Clin Cancer Res.</i> , 28 June 2017, Vol. 23, No. 19, pages 5824-5835 [Retrieved on 2020-11-30] <DOI: 10.1158/1078-0432.CCR-17-0075> Whole document	-
A	YANG D. ET AL., T cells expressing NKG2D chimeric antigen receptors efficiently eliminate glioblastoma and cancer stem cells. <i>J Immunother Cancer.</i> , 9 July 2019, Vol. 7, No. 1, pages 171: 1-13 [Retrieved on 2020-11-30] <DOI: 10.1186/S40425-019-0642-9> Whole document	-
A	LANIER L.L., NKG2D Receptor and Its Ligands in Host Defense. <i>Cancer Immunol Res.</i> , June 2015, Vol. 3, No. 6, pages 575-582 [Retrieved on 2020-11-30] <DOI: 10.1158/2326-6066.CIR-15-0098> Whole document	-
P,X	NG Y.Y. ET AL., T Cells Expressing NKG2D CAR with a DAP12 Signaling Domain Stimulate Lower Cytokine Production While Effective in Tumor Eradication. <i>Mol Ther.</i> , 5 September 2020, S1525-0016(20)30426-30423 [Retrieved on 2020-11-30] <DOI: 10.1016/J.YMTHE.2020.08.016> Abstract	1-28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2020/050520

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. ☐ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Although a sequence listing has been filed, it was not used for the purpose of this search.

Since only version or copy of a sequence listing has been filed or furnished, the statements under item 2 are not required.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2020/050520

Note: This Annex lists known patent family members relating to the patent documents cited in this International Search Report. This Authority is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2019/133969 A2	04/07/2019	SG 11202006050X A KR 20200106051 A AU 2018394353 A1 US 2020/0317777 A1 CA 3085606 A1	29/07/2020 10/09/2020 09/07/2020 08/10/2020 04/07/2019
WO 2018/140725 A1	02/08/2018	EP 3574005 A1 US 2019/0389928 A1	04/12/2019 26/12/2019
WO 2018/147805 A1	16/08/2018	US 2020/0032207 A1 SG 11201907319Q A EP 3580331 A1 CN 110494558 A	30/01/2020 27/09/2019 18/12/2019 22/11/2019