

COMBINED EXPRESSION OF A CHIMERIC CD3 FUSION PROTEIN AND AN ANTI-CD3-BASED BISPECIFIC T CELL ACTIVATING ELEMENT

BACKGROUND

Technical Field

The present disclosure relates generally to immunotherapy, and in particular, the present disclosure relates to the combined expression of a chimeric CD3 fusion protein and an anti-CD3-based bispecific T cell activating element.

Related Art

In recent years, immunotherapy has achieved unprecedented success in the complete remission rate of hematological tumors. In 2017, two CAR-T products targeting CD19 have been successfully marketed and approved for the treatment of acute leukemia and Non-Hodgkin lymphomas in children and adolescents, respectively.

However, there are two major problems associated with immunotherapy for treating solid tumors: on the one hand, the serious and even fatal clinical side effects associated with CAR-T therapy, which mainly include cytokine release syndrome (CRS), macrophage activation syndrome, neurotoxicity, etc., remain a great risk for clinical application of CAR-T therapy. Furthermore, CAR-T therapy has not yet demonstrated significant clinical efficacy in the clinical treatment of solid tumors.

WO2016070061 to Zhao et al. describes engineered T cells expressing a bispecific antibody and a chimeric ligand engineered activation receptor (CLEAR). However, Zhao's disclosure is limited to receptor/ligand targets PD1/PD-L1 and CD27/CD70 and the expression of PD1 or CD27 CLEARs.

WO2016/054520 to Kim et al. describes effector cells expressing engineered cell surface protein and using the effector cell for the treatment of diseases. And in one of their embodiment, the combination therapy of effector cells with engineered CD3e expression and bispecific T cell engager (BiTE) was described. However, in the method of Kim, CD3e and the BiTEs are not co-expressed in a single effector cell, and continuous low dose infusion is required due to the low PK half-life and toxicity of BiTEs. Independent administration of these effector cells and BiTEs does not result in synergy in the solid tumor microenvironment.

Therefore, there is still a need to develop a therapeutic regimen for inhibiting solid tumors with good clinical efficacy and low clinical side effects.

SUMMARY

It is an object of the present disclosure to provide a treatment regimen for inhibiting solid tumors with good clinical efficacy and minimized clinical side-effects.

A first aspect of the present disclosure provides a DNA construct encoding a chimeric CD3 fusion protein (e.g., chimeric CD3e fusion protein), and an anti-CD3-based bispecific T cell activating element.

In a preferred embodiment, the chimeric CD3 is fusion protein that comprises one or more polypeptide that is recognizable by an anti-CD3 antibody, and optionally one or more of the following: a transmembrane domain (TM), a co-stimulatory domain and CD3 signal activation domains, such as a CD3 ζ domain.

In a preferred embodiment, the anti-CD3-based bispecific T cell activating element is a fusion protein that comprises one or more tumor antigen recognition domain and one or more anti-CD3 antibody fragment(s), including for example, a single domain antibody sequence (VHH), a single-chain antibody variable region sequence (scFv), and/or an antigen-binding fragment (Fab) that targets CD3. In a preferred embodiment, the chimeric CD3 fusion protein has a structure shown in the following formula I:



In the formula,

L is absent or a signal peptide sequence;

EC is a polypeptide binding domain from or derived from a CD3e protein, that is recognizable by an anti-CD3 antibody and binds to the anti-CD3 antibody;

the polypeptide binding domain is also referred to as a recognition binding domain of the anti-CD3 antibody;

the polypeptide binding domain is also referred to as a subunit of recognition binding domain of the anti-CD3 antibody;

H is absent or a linker or a hinge region;

TM is a transmembrane domain;

C is absent or a costimulatory signaling molecule;

CD3 ζ is absent or a cytoplasmic signaling sequence derived from CD3 ζ ;

each "-" is independently a linker peptide or a peptide bond.

In another preferred embodiment, the L is a signal peptide of a protein selected from the following group: CD8, GM-CSFR (DNA SEQ ID NO.: SEQ 1, AA SEQ ID NO.: SEQ 2), CD4, CD137, or a combination thereof.

In another preferred embodiment, the polypeptide binding domain is a CD3e extracellular region or a portion thereof recognizable by an anti-CD3 antibody.

In another preferred embodiment, the anti-CD3 antibody is selected from the following group: an scFV (single-chain antibody), a single domain antibody sequence (also known as a nanobody or VHH), a diabody, or a variant thereof, or a combination thereof.

In another preferred embodiment, a clone of the anti-CD3 antibody comprises L2K (DNA SEQ ID NO.: SEQ 19, AA SEQ ID NO.: SEQ 20), UCHT1, OKT3, F6A, I2C or a combination thereof. In another preferred embodiment, the polypeptide binding domain specifically recognizes and binds to an anti-CD3 antibody, which may be present as a segment of a bispecific antibody.

In another preferred embodiment, EC comprises or consists of position 1 to 104 of a wild type or mutant CD3e protein, and the amino acid sequence thereof is shown in SEQ ID NO.: SEQ 4.

In another preferred embodiment, H is a linker or a hinge region of a protein selected from the following group: CD8 (DNA SEQ ID NO.: SEQ 5, AA SEQ ID NO.: SEQ 6), CD28 (DNA SEQ ID NO.: SEQ 57, AA SEQ ID NO.: SEQ 58), CD137, or a combination thereof.

In another preferred embodiment, the TM is a transmembrane region of a protein selected from the following group: CD28 (DNA SEQ ID NO.: SEQ 59, AA SEQ ID NO.: SEQ 60), CD3 epsilon, CD45, CD4, CD5, CD8 (DNA SEQ ID NO.: SEQ 7, AA SEQ ID NO.: SEQ 8), CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, or a combination thereof.

In another preferred embodiment, C is a costimulatory signal molecule of a protein selected from the following group: OX40, CD2, CD7, CD27, CD28, CD30, CD40, CD70, CD134, 4-1BB (CD137), PD1, Dap10, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), NKG2D, GITR, TLR2, or a combination thereof.

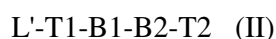
In another preferred embodiment, C comprises a 4-1BB-derived costimulatory signal molecule (DNA SEQ ID NO.: SEQ 9, AA SEQ ID NO.: SEQ 10), and/or a CD28-derived

costimulatory signal molecule (DNA SEQ ID NO.: SEQ 61, AA SEQ ID NO.: SEQ 62).

In another preferred embodiment, CD3 ζ is a cytoplasmic signaling sequence as represented by AA SEQ ID NO. NO.: 12.

In another preferred embodiment, the DNA construct is expressed in a cis or fusion form with a safety switch protein, and proteins capable of serving as a safety switch comprise: inducible Caspase 9 (iCasp9), CD19, CD20, EGFR, HER2, CD30, CD19, c-Met, Claudin 18.2, or a combination thereof.

In another preferred embodiment, the anti-CD3-based bispecific T cell activating element (BiTA) has a structure shown in the following formula II:



In the formula,

L' is absent or a signal peptide sequence;

T1 is absent or a tag element;

B1 is a tumor antigen recognition region or a CD3 antigen recognition region;

B2 is a CD3 antigen-binding antibody fragment or a tumor antigen recognition region;

T2 is absent or a tag element;

each "-" is independently a linker peptide or peptide bond.

In another preferred embodiment, L' is a signal peptide of a protein selected from the following group: CD8, GM-CSFR, CD4, CD137, or a combination thereof.

In another preferred embodiment, the tag element comprises a tag protein, a fluorescein labeled protein or an enzyme labeled protein.

In another preferred embodiment, the tag protein comprises a FLAG protein (DNA SEQ ID NO.: SEQ 13, AA SEQ ID NO.: SEQ 14), and His protein (DNA SEQ ID NO.: SEQ 35, AA SEQ ID NO.: SEQ 36).

In another preferred embodiment, B1 is a tumor antigen recognition region and B2 is a CD3 antigen recognition region.

In another preferred embodiment, the tumor antigen recognition region comprises of one or more receptor or ligand binding domains, an antibody fragment, including single domain antibody sequence (VHH), and/or single-chain antibody variable region sequence (scFv),

and/or a TCR sequence.

In another preferred embodiment, the tumor antigen is selected from the following group: TSHR, CD19, CD123, CD22, CD30, CD171, CS-1, CLL-1, CD33, EGFRvIII, GD2, GD3, BCMA, Tn Ag, PSMA, ROR1, FLT3, FAP, TAG72, CD38, CD44v6, CEA, EPCAM, B7H3, KIT, IL-13Ra2, Mesothelin, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, CD24, PDGFR- β , SSEA-4, CD20, folate receptor alpha, ERBB2 (Her2/neu), MUC1, EGFR, NCAM, Prostase, PAP, ELF2M, ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor β , TEM1/CD248, TEM7R, CLDN6, GPRC5D, CXORF61, CD97, CD179a, ALK, polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-1a, MAGE-A1, legumain, HPV E6, E7, MAGE A1, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-associated antigen 1, p53, p53 mutant, prostein, survivin and telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoint, ML-IAP, ERG (TMPRSS2ETS fusion gene), NA17, PAX3, androgen receptor, cyclin B1, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxylesterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, IGLL1, DLL3 or a combination thereof.

In another preferred embodiment, the tumor antigen recognition region targets CAIX and/or HER2.

In another preferred embodiment, the tumor antigen recognition region is a VHH antibody (DNA SEQ ID NO.: SEQ 17, AA SEQ ID NO.: SEQ 18) targeting CAIX.

In another preferred embodiment, the tumor antigen recognition region is a single-chain antibody (DNA SEQ ID NO.: SEQ 65, AA SEQ ID NO.: SEQ 66) targeting HER2.

In another preferred embodiment, the CD3 antigen-binding antibody fragment is a single domain antibody sequence (VHH), a single-chain antibody variable region sequence (scFv), or an antigen-binding fragment (Fab) that targets CD3.

In another preferred embodiment, the BiTA is a secreted BiTA.

In another preferred embodiment, the BiTA targets CAIX (DNA SEQ ID NO.: SEQ 21, AA SEQ ID NO.: SEQ 22), or targets HER2 (DNA SEQ ID NO.: SEQ 49, AA SEQ ID NO.:

SEQ 50).

In another preferred embodiment, the secreted BiTA may be autocrine, and/or paracrine.

In another preferred embodiment, the secretory cell type of the secreted BiTA may be: a T cell, an NK cell, a macrophage, a B cell, a red blood cell, or a combination thereof.

In another preferred embodiment, the secretory cell type of the secreted BiTA is a T cell.

In another preferred embodiment, the BiTA can bind to chimeric CD3e.

In another preferred embodiment, the BiTA can bind to the T cell receptor (TCR) complex.

In another preferred embodiment, the TCR is derived from a T cell according to the fifth aspect, and/or a T cell that has not been engineered.

In another preferred embodiment, the nucleic acid molecule encoding the chimeric CD3 fusion protein and the nucleic acid molecule encoding the bispecific T cell activating element are provided separately. Advantageously, the nucleic acid molecule encoding the chimeric CD3 fusion protein and the nucleic acid molecule encoding the bispecific T cell activating element are co-expressed in the same immune cell.

A second aspect of the invention provides a vector, characterized in that the vector comprises the nucleic acid molecule.

In another preferred embodiment, the vector is selected from the group consisting of lentiviral, adenoviral, and retroviral vectors.

A third aspect of the invention provides a genetically engineered immune cell (e.g. T cell), characterized in that the immune cell expresses the nucleic acid molecules as described above. In an optionally embodiment, the immune cell is engineered to express the chimeric CD3 fusion protein and the bispecific T cell activating element, whereby the nucleic acid molecule encoding the chimeric CD3 fusion protein and the nucleic acid molecule encoding the bispecific T cell activating element are not provided on the same DNA construct.

In another preferred embodiment, the T cell is derived from a person or a non-human mammal.

In another preferred embodiment, the T cell further comprises other chimeric antigens.

A fourth aspect of the present disclosure provides a composition, characterized in that the composition comprises the fusion protein and BiTA.

In another preferred embodiment, the fusion protein in the composition is located in the extracellular region of a T cell membrane.

In another preferred embodiment, the BiTA in the composition is autocrine, paracrine or exogenous BiTA.

In another preferred embodiment, the composition is expressed in the form of a fusion protein of the structural formula I and the structural formula II with a 2A protein, the structural formula of which is: I-2A-II or II-2A-I, and the sequence of 2A includes one of T2A (DNA SEQ ID NO.: SEQ 23, AA SEQ ID NO.: SEQ 24), P2A, F2A or E2A or a combination thereof.

In another preferred embodiment, the I-2A-II structure is a sequence that targets CAIX (DNA SEQ ID NO.: SEQ 25, AA SEQ ID NO.: SEQ 26) or HER2 (DNA SEQ ID NO.: SEQ 49, AA SEQ ID NO.: SEQ 50).

In another preferred embodiment, the II-2A-I structure is a sequence that targets CAIX or HER2 (DNA SEQ ID NO.: SEQ 67, AA SEQ ID NO.: SEQ 68).

In another preferred embodiment, the I-2A-II or II-2A-I structure is expressed in a cis or fusion form with a safety switch protein, and proteins capable of serving as a safety switch comprise: inducible Caspase 9 (iCasp9), CD19, CD20, EGFR, HER2, CD30, CD19, c-Met, Claudin 18.2, or a combination thereof.

In another preferred embodiment, the composition is expressed in the form of a combination of a fusion protein of the structural formula I and the structural formula II with an IRES sequence, the structural formula of which is: I-IRES-II or II-IRES-I, IRES is a nucleotide sequence internal ribosome entry site.

In another preferred embodiment, the IRES functions as initiating amino acid translation of a downstream gene.

In another preferred embodiment, the I-IRES-II or II-IRES-I structure is expressed in a cis or fusion form with a safety switch protein, and proteins capable of serving as a safety switch comprise: inducible Caspase 9 (iCasp9), CD19, CD20, EGFR, HER2, CD30, CD19, c-Met, Claudin 18.2, or a combination thereof.

A fifth aspect of the present disclosure provides a non-naturally occurring T cell population. The T cells as described above are present in the T cell population at a ratio C1 of 10% or more, based on the total number of T cells in the T cell population.

In another preferred embodiment, the C1 is 10% or more, preferably $C1 \geq 20\%$, and more

preferably $C1 \geq 30\%$.

In another preferred embodiment, BiTA, and/or BiTA-secreting T cells C2 are also present in the T cell population.

A sixth aspect of the present disclosure provides a composition, comprises (a) the genetically engineered T cells as described above and/or the T cell population as described above, and (b) a pharmaceutically acceptable carrier, diluent and/or excipient.

A seventh aspect of the present disclosure relates to the use of the genetically engineered T cells, the T cell population, and/or the composition as described above in the preparation of a medicament for the prevention and/or treatment of cancer or tumors, or for use in a method as described below.

An eighth aspect of the present disclosure provides a method for preventing or treating a disease, comprising: administering an appropriate amount of the genetically engineered T cells, the T cell population, and/or the composition according as described above to a subject in need of treatment.

In another preferred embodiment, the disease is cancer or a tumor.

In another preferred embodiment, the tumor is selected from the following group: a hematological tumor, a solid tumor, and a combination thereof.

In another preferred embodiment, the hematological tumor is selected from the following group: acute myelocytic leukemia (AML), multiple myeloma (MM), chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), diffuse large B cell lymphoma (DLBCL), and a combination thereof.

In another preferred embodiment, the solid tumor is selected from the following group: gastric cancer, peritoneal metastasis in gastric cancer, liver cancer, leukemia, kidney tumor, lung cancer, small intestine cancer, bone cancer, prostate cancer, colorectal cancer, breast cancer, colon cancer, cervical cancer, ovarian cancer, lymphoma, nasopharyngeal carcinoma, adrenal tumor, bladder tumor, non-small cell lung cancer (NSCLC), glioma, endometrial cancer, testicular cancer, urinary tract tumor, thyroid cancer and a combination thereof.

In another preferred embodiment, the solid tumor is selected from the following group: ovarian cancer, mesothelioma, lung cancer, pancreatic cancer, breast cancer, liver cancer, endometrial cancer, or a combination thereof. In another preferred embodiment, the method further comprises administering an appropriate amount of a cytokine secreted by a stimulating

cell or drug compound and a composition thereof to enhance the responsiveness of an immune cell.

In an embodiment, the method further comprises the step of administering dasatinib to the subject.

In another preferred embodiment, the immune cell comprises the T cell, the T cell population, and/or the composition as described above, and an endogenous T cell, NK cell, macrophage and B cell.

A ninth aspect of the present disclosure provides a method of reducing the toxicity of an immune cell engineered with a chimeric CD3e fusion protein and a bispecific T cell activating element, comprising the administration of dasatinib; as well as the use of dasatinib in the preparation of a medicament for reducing the toxicity of an immune cell engineered with a chimeric CD3e fusion protein and a bispecific T cell activating element.

It is to be understood that within the scope of the present disclosure, the various technical features of the present disclosure as described above and the various technical features specifically described hereinafter (e.g. in the examples) may be combined with each other to constitute a new or preferred technical solution.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the structures of CAB-T according to a first group of experiments and its control group.

FIG. 2 shows the structures of the CAB-T according to a second group of experiments and its control group.

FIG. 3 shows the structures of the CAB-T according to a third group of experiments and its control group.

FIG. 4 shows the transduction frequency results of a T cell engineered by the first group of structures.

FIG. 5 shows the transduction frequency results of a T cell engineered by the second group of structures.

FIG. 6 shows the transduction frequency assay results of a T cell engineered by the third group of structures.

FIG. 7 shows a CAIX-CAB-T cytokine release assay (of the constructs from the first group

of experiments).

FIG. 8 shows a CAIX-CAB-T cytokine release assay (of the constructs from the second group of experiments).

FIG. 9 shows a CAIX-CAB-T cell activation level assay (of the constructs from the first group of experiments).

FIG. 10 shows a CAIX-CAB-T cell activation level assay (of the constructs from the second group of experiments).

FIG. 11 shows an HER2-CAB-T cell activation level assay (of the constructs from the third group of experiments).

FIG. 12 shows a CAIX-CAB-T paracrine activated T cell level assay (of the constructs from the second group).

FIG. 13 shows an HER2-CAB-T paracrine activated T cell level assay (of the constructs from the third group).

FIG. 14 shows CAIX-CAB-T and its control cellular immune checkpoint expression level and cell differentiation phenotype analysis (of the constructs from the second group).

FIG. 15 shows HER2-CAB-T and its control cellular immune checkpoint expression level and cell differentiation phenotype analysis (of the constructs from the third group of experiments).

FIG. 16 shows a CAIX-CAB-T and its control cell-mediated tumor killing ability assay (of the constructs from the first group of experiments).

FIG. 17 shows a CAIX-CAB-T and its control cell-mediated tumor killing ability assay (of the constructs from the second group of experiments).

FIG. 18 shows an HER2-CAB-T and its control cell-mediated tumor killing ability assay (of the constructs from the third group of experiments).

FIG. 19 shows the structures of the CAB-T according to the fourth group of experiments and its control group.

FIG. 20 shows the transduction frequency results of T cell engineered by the fourth group of structures.

FIG. 21 shows an HER2-CAB-T cell activation level assay of the constructs from (the

fourth group of experiments).

FIG. 22 shows an HER2-CAB-T and its control cell-mediated tumor killing ability assay (of the constructs from the fourth group of experiments).

FIG. 23A shows a CAIX⁺MDA-MB231 tumor growth inhibition assay in NCG humanized mice at different doses for CAIX-CAB-T and its control group cells.

FIG. 23B shows the body weight change of the mice in the assay as shown in FIG. 23A.

FIG. 23C shows the images of tumors from mice in the assay as shown in FIG. 23A.

FIG. 23D shows tumor weight of the mice in the assay as shown in FIG. 23A.

FIG. 24A shows a NCI-N87 tumor growth inhibition in M-NSG humanized mice treated with HER2-CAB-T and its control T cells.

FIG. 24B shows the images of tumors from mice in the assay as shown in FIG. 24A.

FIG. 24C shows a statistical tumor weight of the mice in the assay as shown in FIG. 24A.

FIG. 25A shows Dasatinib inhibits IL-2 secretion of activated CAIX CAB-T at low concentrations.

FIG. 25B shows Dasatinib inhibits IFN- γ secretion of activated CAIX CAB-T at low concentrations.

FIG. 25C shows Dasatinib inhibits the killing activity of CAIX CAB-T at low concentrations.

FIG. 26A shows Dasatinib inhibits IL-2 secretion of activated HER2 CAB-T at low concentrations.

FIG. 26B shows Dasatinib inhibits IFN- γ secretion of activated HER2 CAB-T at low dose concentrations.

FIG. 26C shows Dasatinib inhibits the killing activity of HER2 CAB-T at low concentrations.

FIG. 27 shows a schematic diagram of the mechanism of action of CAB-T.

DETAILED DESCRIPTION

The present disclosure relates to an immunotherapeutic regimen for inhibiting tumors, particularly solid tumors, involving the use of T cells expressing a chimeric CD3 fusion protein, preferably a chimeric CD3e fusion protein, with an anti-CD3 antibody-based bispecific T cell

activator (BiTA), similar to a bispecific T-cell engager (BiTE). The chimeric CD3 fusion protein and BiTA with a CD3 antigen recognition site bind to each other and function to activate T cells and target tumor cells expressing tumor associated antigens (TAA). The present disclosure also provides a CAB structure and CAB-engineered T cells (CAB-T) expressing chimeric CD3 and an anti-CD3 antibody-based bispecific T cell activator. BiTA secreted by the CAB-T cells can simultaneously activate CAB-T cells and the endogenous TCR complex of non-engineered and engineered T cells in tumor tissues, exert the anti-tumor effect of CAB-T itself and mobilize the anti-tumor effect of the non-engineered T cells, thereby ensuring the effectiveness of this CAB-T technology for clinical application. The chimeric CD3 construct that is expressed by CAB-T and BiTA act synergistically to exert its antitumor effects: activation of the chimeric CD3 element is dependent on BiTA secreted by CAB-T, and the secretion of BiTA further stimulates CAB-T to secrete BiTA by activating the chimeric CD3 element, so that immune cell activation and anti-tumor effects are localized to the tumor microenvironment and the safety advantage of this CAB-T technology for clinical application is ensured.

Description of terms

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

As used herein, the term "about" when used in reference to a specifically recited value means that the value may vary by no more than 1% from the recited value. For example, as used herein, the expression "about 100" includes 99 and 101 and all values there between (e.g., 99.1, 99.2, 99.3, 99.4, etc.).

As used herein, the term "contain" or "include (comprise)" can be open, semi-closed, and closed. In other words, the term also includes "consisting essentially of ..." or "consisting of".

The term "administering" refers to the physical introduction of a product of the present disclosure into a subject using any of a variety of methods and delivery systems known to those skilled in the art, including intravenous, intramuscular, subcutaneous, intraperitoneal, spinal cord or other parenteral routes of administration, e.g., by injection or infusion.

The term "antibody" (Ab) shall include, but not limited to, an immunoglobulin that specifically binds to an antigen and comprises at least two heavy (H) chains and two light (L)

chains interconnected by disulfide bonds, or an antigen binding fragment thereof. Each H chain comprises a heavy chain variable region (abbreviated as VH herein) and a heavy chain constant region. The heavy chain constant region comprises three constant domains: CH1, CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated as VL herein) and a light chain constant region. The light chain constant region comprises a constant domain CL. The VH and VL regions can be further subdivided into hypervariable regions called complementary determining regions (CDRs), and the hypervariable regions are interspersed with more conserved regions called framework regions (FRs). Each of VH and VL contains three CDRs and four FRs, arranged from the amino end to the carboxyl end in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with the antigen.

It should be understood that the amino acid names herein are given by the international single English letter designation, and three-English-letter abbreviations corresponding to the amino acid names respectively are: Ala (A), Arg (R), Asn (N), Asp (D), Cys (C), Gln (Q), Glu (E), Gly (G), His (H), Ile (I), Leu (L), Lys (K), Met (M), Phe (F), Pro (P), Ser (S), Thr (T), Trp (W), Tyr (Y) and Val (V).

Chimeric antigen receptor (CAR)

The structure of the chimeric antigen receptor (CAR) is a fusion protein based on the intracellular segment domain of a TCR complex CD3 ζ and the intracellular activator domain from costimulatory signals CD28 or 4-1BB. Advantageously, T cells modified to express CAR are able to bind to target antigens in a MHC-independent manner, such that the activation of T cells does not depend on the presentation of antigens by MHC. This type of CAR is known as a second generation CAR structure, and two CAR-T drugs approved in 2017 belong to this structure type.

T cell receptor (TCR)

The T cell receptor (TCR) is the most complex receptor in the human body, and the interaction of six different receptor subunits together determines its broad signal transduction within T cells. The two chains of TCR α and TCR β together recognize a complex composed of a polypeptide-histocompatibility complex, and the subunits that transmit TCR signals, collectively known as CD3, include: one heterodimer formed by CD3 ϵ and CD3 γ , one heterodimer formed by CD3 ϵ and CD3 δ , and one CD3 ζ homodimer. All subunits of the TCR are type I transmembrane proteins and have immunoglobulin domains except for CD3 ζ . The

four different CD3 subunits in the TCR receptor complex have 10 immune receptor tyrosine-based activation motifs (ITAMs) in total, and can receive 20 tyrosine phosphate groups in total when the TCR receptor complex is activated. In transgenic mouse experiments, it is shown that changes in the proline rich region of the intracellular segment of CD3 ϵ or the conformation of CD3 ϵ play a crucial regulatory role in the delivery of intact TCRs. It has been demonstrated that TCR activity can be modulated by binding the ligand to TCR $\alpha\beta$ and stabilizing the arrangement of the CD3 subunits, ligand-independent TCR oligomerization, and binding to cholesterol.

TRuC structure

A novel T cell therapy platform, TRuCTM, self-developed by TCR² is a chimeric antigen receptor consisting of an antibody-based target antigen recognition sequence and a TCR receptor subunit. The TRuC structure can reprogram a complete TCR complex that recognizes tumor antigens. Unlike the CAR structure, the TRuC structure can be integrated into the TCR complex to exert its function. TRuC-T has the same tumor killing activity as the second generation CAR-T; at the same time, TRuC-T cells release cytokine at a level significantly lower than that of CAR-T cells because of the absence of additional costimulatory signal domains (CD28 or 4-1BB). TRuC-T shows antitumor activity in both hematological and solid tumor transplantation models. At the same time, TRuC-T shows more potent anti-tumor activity compared to CAR-T in multiple tumor models.

T cell antigen coupler (TAC)

A TAC (T cell antigen coupler) technology platform by Triumvira can induce a more potent anti-tumor response than CAR-T with lower toxicity by regulating endogenous TCR of the T cell. The TAC structure consists of three parts: 1. an extracellular antigen binding region; 2. a TCR-recruitment region of a CD3 single-chain antibody; 3. a CD4/CD8 co-receptor binding region. Preclinical experiments have shown that TAC-T technology can specifically bind tumor cells and produce cytotoxicity, and the activation of TAC-T cells is similar to the activation of normal T cells, avoiding the production of a large number of cytokines. In a mouse tumor transplant model, TAC-T shows a better activity than CAR-T against either a solid tumor or a hematological tumor. In addition, TAC-T is more capable of infiltrating into a tumor microenvironment in solid tumors.

Bispecific T cell engager (BiTE)

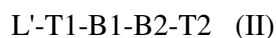
Blinatumomab, a bispecific T cell engager (BiTE) drug targeting CD19 and developed by

Amgen, USA, was approved by FDA in 2014 for clinical treatment of acute leukemia. This antibody consists of two moieties: scFv that recognizes the CD19 antigen and scFv that recognizes the TCR complex (CD3e). After recognizing the target antigen CD19 in the tumor cell, the BiTE antibody can utilize the anti-CD3 scFv moiety to induce endogenous TCR oligomerization of T cells, thereby activating T cells and triggering tumor killing. The way by which BiTE treats tumors is similar to those of TRuC and TAC techniques, all of which cause endogenous TCR activation in T cells. Theoretically, the three have comparable abilities of activating endogenous TCR signals, and all have potential great value for mobilizing and redirecting T cells to treat solid tumors. However, due to the poor safety resulting from the systemic administration of BiTE drugs, the extremely short half-life of BiTE drugs in vivo, and the like, the use of BiTE in achieving a desirable efficacy in the clinical treatment of solid tumors has yet to be shown.

Bispecific T cell activator (BiTA) structure

"Bispecific T cell activator structure", "bispecific T cell activating element", "BiTA", "bispecific T cell activator", and "-BiTA" stated herein refer to an anti-CD3-based bispecific T cell activator structure, which comprises of two moieties: (i) one or more tumor antigen recognition region(s), such as receptor or ligand binding domains, an antibody fragment, including single domain antibody sequence (VHH), a single-chain antibody variable region sequence (scFv), a antigen-binding fragment (Fab) and/or a T-cell receptor (TCR) sequence that recognizes a tumor antigen, and (ii) one or more CD3 antigen recognition regions, such as, an antibody fragment, including a single domain antibody sequence (VHH) that targets CD3, a single-chain antibody variable region sequence (scFv), or antigen-binding fragment (Fab).

In another preferred embodiment, the anti-CD3-based bispecific T cell activating element has a structure shown in the following formula II:



In the formula,

L' is absent or a signal peptide sequence derived of a protein selected from the following group: CD8, GM-CSFR, CD4, CD137, or a combination thereof.

T1 is absent or a tag element, optionally comprising a tag protein, a fluorescein labeled protein or an enzyme labeled protein. Preferably, tag protein comprises a FLAG protein (DNA SEQ ID NO.: SEQ 13, AA SEQ ID NO.: SEQ 14), and His protein (DNA SEQ ID NO.: SEQ 35, AA SEQ ID NO.: SEQ 36).

B1 is a tumor antigen recognition region, optionally comprising receptor or ligand binding domains, a single domain antibody sequence (VHH), and/or a single-chain antibody variable region sequence (scFv), and/or an antibody Fab, and/or a T-cell receptor (TCR) sequence, which is/are able to recognize a tumor antigen selected from the following group: TSHR, CD19, CD123, CD22, CD30, CD171, CS-1, CLL-1, CD33, EGFRvIII, GD2, GD3, BCMA, Tn Ag, PSMA, ROR1, FLT3, FAP, TAG72, CD38, CD44v6, CEA, EPCAM, B7H3, KIT, IL-13Ra2, Mesothelin, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, CD24, PDGFR- β , SSEA-4, CD20, folate receptor alpha, ERBB2 (Her2/neu), MUC1, EGFR, NCAM, Prostase, PAP, ELF2M, ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor β , TEM1/CD248, TEM7R, CLDN6, GPRC5D, CXORF61, CD97, CD179a, ALK, polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-1a, MAGE-A1, legumain, HPV E6, E7, MAGE A1, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-associated antigen 1, p53, p53 mutant, prostein, survivin and telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoint, ML-IAP, ERG (TMPRSS2ETS fusion gene), NA17, PAX3, androgen receptor, cyclin B1, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxylesterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, IGLL1, DLL3 or a combination thereof. Preferably, the tumor antigen recognition region B1 targets tumor antigen CAIX and/or HER2.

In another preferred embodiment, the tumor antigen recognition region B1 is a VHH antibody (DNA SEQ ID NO.: SEQ 17, AA SEQ ID NO.: SEQ 18) targeting CAIX.

In another preferred embodiment, the tumor antigen recognition region B1 is a single-chain antibody derived from Trastuzumab (DNA SEQ ID NO.: SEQ 65, AA SEQ ID NO.: SEQ 66) targeting HER2.

B2 is a CD3 antigen recognition region, optionally, is a single domain antibody sequence (VHH), and/or an antibody Fab, and/or a single-chain antibody variable region sequence (scFv) that targets CD3. In a preferred embodiment, the CD3 antigen-binding antibody fragment could derived from CD3 Ab clones of L2K, UCHT, OKT3, F6A, SP34 etc.

Optionally, the position of B1 and B2 may be reversed.

T2 is absent or a tag element, optionally comprising a tag protein, a fluorescein labeled protein or an enzyme labeled protein. Preferably, tag protein comprises a FLAG protein (DNA SEQ ID NO.: SEQ 13, AA SEQ ID NO.: SEQ 14), and His protein (DNA SEQ ID NO.: SEQ 35, AA SEQ ID NO.: SEQ 36).

each "-" is independently a linker peptide or peptide bond.

CD3e protein

"CD3e protein" and "CD3e" stated herein both refer to human CD3e protein.

"Extracellular region of CD3e protein" stated herein refers to amino acids 1-104 of the CD3e protein sequence, as illustrated by SEQ ID NO. NO.: 4.

The protein sequence comprises an amino acid sequence having a homology with the amino acid sequence of not less than 60%, for example, at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%.

Chimeric CD3e fusion protein

"Chimeric CD3e fusion protein", "CD3e fusion protein", and "chimeric CD3e protein" stated herein refer to a fusion protein expressed in T cells having a structure shown in the following formula I:



In the formula,

each "-" is independently a linker peptide or peptide bond;

L is an optional signal peptide sequence;

EC is a polypeptide binding domain from or derived from a CD3e protein, that is recognizable by an anti-CD3 antibody and binds to the anti-CD3 antibody;

the polypeptide binding domain is also referred to as a recognition binding domain of the anti-CD3 antibody;

the polypeptide binding domain is also referred to as a subunit of recognition binding domain of the anti-CD3 antibody;

H is an optional linker or hinge region;

TM is a transmembrane domain;

C is absent or a costimulatory signal molecule;

CD3 ζ is absent or a cytoplasmic signaling sequence derived from CD3 ζ .

In another preferred embodiment, the polypeptide binding domain is a CD3e extracellular region as illustrated by SEQ ID NO. NO.: 4, or a portion thereof recognizable by an anti-CD3 antibody.

In another preferred embodiment, the anti-CD3 antibody is selected from the following group: an scFV (single-chain antibody), a single domain antibody sequence (also known as a nanobody), a diabody, an antibody Fab or a variant thereof, or a combination thereof.

In another preferred embodiment, the polypeptide binding domain specifically recognizes and binds to an anti-CD3 antibody, which may be present as a segment of a bispecific antibody.

In another preferred embodiment, the hinge region is CD8 Hinge, and the amino acid sequence thereof is SEQ ID NO. NO.: 3.

In another preferred embodiment, the transmembrane region is CD8 TM, and the amino acid sequence thereof is SEQ ID NO. NO.: 8.

Chimeric CD3 and anti-CD3 based Bispecific T cell activator engineered T cells, CAB-T

"Chimeric CD3 and anti-CD3 based Bispecific T cell activator engineered T cells", "CAB-T cells", "CAB-T technology", "CAB structure", "CAB-T", "-CAB-T" and "-CAB" stated herein refer to T cells engineered to co-express both chimeric CD3 fusion protein and anti-CD3 based bispecific T cell activator. Preferably, the engineered T cells comprises a construct with the following structure: (i) a chimeric CD3e in the CAB structure comprising at least the following four components: a CD3e extracellular region, a CD8 hinge region and transmembrane region, 4-1BB intracellular region and a CD3 intracellular region; (ii) an anti-CD3 based bispecific T cell activator (BiTA) structure comprises at least the following two moieties: receptor or ligand binding domains, an antibody fragment, including single domain antibody sequence (VHH), an antibody Fab fragment, a single-chain antibody variable region sequence (scFv), or a T-cell receptor (TCR) sequence that recognizes and binds a tumor antigen, and a variable region sequence that recognizes CD3 antigen in TCR complexes.

As noted above, the amino acid sequence of the CD3e extracellular region is shown in SEQ ID NO. NO.: 4, the amino acid sequence of the 4-1BB is shown in SEQ ID NO. NO.: 10, and the amino acid sequence of the CD3 ζ is shown in SEQ ID NO. NO.: 12.

Other preferred synthetic gene sequences are shown in Table 1 below:

Table 1. Synthetic gene names and nucleotide and amino acid sequences thereof

Synthetic Gene Name	DNA SEQ ID NO.	AA SEQ ID NO.
GM-CSFRsp (signal peptide)	SEQ 1	SEQ 2
CD3e ECD (extracellular region)	SEQ 3	SEQ 4
CD8 Hinge (hinge region)	SEQ 5	SEQ 6
CD8 TM (transmembrane region)	SEQ 7	SEQ 8
4-1BB costimulatory domain	SEQ 9	SEQ 10
CD3 ζ (TCR activation domain)	SEQ 11	SEQ 12
FLAG (tag sequence)	SEQ 13	SEQ 14
CD3e-BB ζ	SEQ 15	SEQ 16
CAIX VHH	SEQ 17	SEQ 18
L2K scFv	SEQ 19	SEQ 20
1 st -CAIX-BiTA	SEQ 21	SEQ 22
T2A	SEQ 23	SEQ 24
1 st -CAIX-CAB	SEQ 25	SEQ 26
Linker	SEQ 27	SEQ 28
CD3e full length	SEQ 29	SEQ 30
CAIX-TRuC	SEQ 31	SEQ 32
Truncated ERBB2 (tERBB2)	SEQ 33	SEQ 34
6 $\times$ His	SEQ 35	SEQ 36
CAIX-BiTA	SEQ 37	SEQ 38
CAIX-CAB	SEQ 39	SEQ 40
CAIX- ζ	SEQ 41	SEQ 42
CD8L (CD8 signal peptide)	SEQ 43	SEQ 44
CAIX-BB ζ	SEQ 45	SEQ 46
CAIX-28 ζ	SEQ 47	SEQ 48
HER2-CAB	SEQ 49	SEQ 50
HER2- ζ	SEQ 51	SEQ 52
HER2-BB ζ	SEQ 53	SEQ 54
HER2-28 ζ	SEQ 55	SEQ 56
CD28 Hinge	SEQ 57	SEQ 58
CD28 TM	SEQ 59	SEQ 60
CD28 costimulatory domain	SEQ 61	SEQ 62
HER2-BiTA	SEQ 63	SEQ 64

Trastuzumab scFv	SEQ 65	SEQ 66
HER2-CAB ^R	SEQ 67	SEQ 68
Humanized CAIX VHH	SEQ 69	SEQ 70
hCAIX-BiTA	SEQ 71	SEQ 72
hCAIX-CAB	SEQ 73	SEQ 74
hCAIX-BB ζ	SEQ 75	SEQ 76

CAIX

CAIX is a transmembrane protein expressed in a variety of solid tumor cells. The primary function of CAIX is to maintain the homeostasis of intracellular pH under hypoxic conditions, which is common in solid tumors. The expression of CAIX in tumor cells is considered to be a marker protein for hypoxia in a tumor environment and poor prognosis in patients. Common types of tumors that express CAIX include cervical cancer, kidney cancer, brain cancer, head and neck cancer, esophageal cancer, intestinal cancer, breast cancer, ovarian cancer, endometrial cancer, bladder cancer, and the like. In normal tissues, CAIX is mainly expressed in epithelial cells in the bile duct and small intestine, as well as gastric epithelial cells, etc., but unlike tumor cells, CAIX expressed in normal tissues is mainly localized in the cytoplasm. Therefore, CAIX is an ideal therapeutic target for targeted therapy including cell therapy.

HER2

HER2 is one of the most studied targets in tumor immunotherapy, and is commonly expressed in tissues such as breast cancer, gastric cancer, colorectal cancer, cervical cancer, endometrial cancer, urothelial cancer, ovarian cancer, and lung cancer. Although trastuzumab, a monoclonal antibody drug targeting HER2, has significantly improved the quality of life and prolonged the lifetime of patients with HER2-positive breast cancer, there are still a large number of patients with HER2-positive tumors that do not respond or develop resistance to the trastuzumab. Therefore, there is still a large market demand for the development of new therapeutic approaches targeting HER2. Currently, there are reports on CAR-T drugs targeting HER2. Among them, Steven A Rosenberg reports serious toxic and side effects in clinical trials of a third generation CAR-T drug targeting HER2, and such drug causes respiratory distress and severe immune cell infiltration in lung and thus causes the patient to die. Therefore, during the development of drugs targeting HER2 cells, it must be designed to highlight the safety of the drug.

Composition

The present disclosure provides a composition or a formulation comprising T cells engineered to co-express both chimeric CD3 fusion protein and anti-CD3 based bispecific T cell activator (i.e. the CAB-T cells), together with a pharmaceutically acceptable carrier, diluent or excipient. In one embodiment, the composition is a liquid formulation. Preferably, the composition is an injection. Preferably, the concentration of the CAB-T cells in the composition is 1×10^3 - 1×10^8 cells/ml, more preferably 1×10^4 - 1×10^7 cells/ml.

In one embodiment, the composition may include a buffer such as neutral buffered saline, sulfate buffered saline, and the like; a carbohydrate such as glucose, mannose, sucrose or dextran, and mannitol; a protein; a polypeptide or an amino acid such as glycine; an antioxidant; a chelating agent such as EDTA or glutathione; an adjuvant (for example, aluminum hydroxide); and a preservative. The composition of the present disclosure is preferably formulated for intravenous administration.

Therapeutic application

The present disclosure encompasses the therapeutic application of the T cells engineered to co-express both chimeric CD3 fusion protein and anti-CD3 based bispecific T cell activator (i.e. the CAB-T cells). T cells transduced with vectors comprising the nucleic acid construct of the present disclosure can target tumor cell markers, while autocrine or paracrine BiTA (secreted by the engineered T cells) can synergistically activate T cells and cause T cell immune responses, thereby significantly increasing their killing efficiency against tumor cells.

Accordingly, the present disclosure also provides a method for stimulating a T cell-mediated immune response of a target cell population or tissue in a mammal, comprising the step of administering the CAB-T cells.

Cancers that can be treated include tumor that has not been vascularized or has not been substantially vascularized, as well as vascularized tumors. Cancers can include non-solid tumors (for example, hematological tumor such as leukemias and lymphoma) or solid tumors. Types of cancer that can be treated with the nucleic acid construct and engineered T cells of the present disclosure include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancy, benign and malignant tumor, and malignancy, such as sarcoma, carcinoma, and melanoma. Adult tumor/cancer and pediatric tumor/cancer are also included.

Hematological cancer is a cancer of the blood or bone marrow. Examples of hematological (or hematogenous) cancer include leukemia, including acute leukemia (such as acute

lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia, and myeloblastic leukemia, promyelocytic leukemia, granulocyte-monocyte leukemia, monocyte leukemia and erythroleukemia), chronic leukemia (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (painless and high-grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia, and myelodysplasia.

A solid tumor is an abnormal mass of tissue that usually does not contain a cyst or fluid area. Solid tumors can be benign or malignant. Different types of solid tumors are named after the cell types that form them (such as sarcoma, carcinoma, and lymphoma). Examples of solid tumor such as sarcoma and carcinoma include fibrosarcoma, mucinous sarcoma, liposarcoma, mesothelioma, lymphoid malignancy, pancreatic cancer, and ovarian cancer.

The CAB-modified T cells of the present disclosure can also be used as a vaccine for ex vivo immunization and/or in vivo therapy in a mammal. Preferably, the mammal is human.

For ex vivo immunization, at least one of the following occurs in vitro prior to administration of the cells into the mammal: i) amplifying the cells, ii) introducing a CAB-encoding nucleic acid into the cells, and/or iii) cryopreserving the cells.

Ex vivo procedures are well known in the art and are discussed in more details below. Briefly, cells are isolated from a mammal, preferably human, and genetically modified (i.e., transduced or transfected in vitro) with a vector that expresses the CABs disclosed herein. CAB-modified cells can be administered to a mammalian recipient to provide a therapeutic benefit. The mammalian recipient can be human, and the CAB-modified cells can be autologous relative to the recipient. Alternatively, the cells may be allogeneic, syngeneic or xenogeneic relative to the recipient.

In addition to the use of cell-based vaccines for ex vivo immunization, the present disclosure also provides a composition and a method for in vivo immunization to elicit an immune response against antigens in a patient.

The present disclosure provides a method for treating a tumor, comprising administering a therapeutically effective amount of a CAB-modified T cell of the present disclosure to a subject.

The CAB-modified T cells of the present disclosure can be administered alone or in combination with a diluent and/or with other components such as IL-2, IL-17 or other cytokines

or cell populations in the form of a pharmaceutical composition. Briefly, the pharmaceutical composition of the present disclosure may comprise a population of target cells as described herein in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may include a buffer such as neutral buffered saline, sulfate buffered saline, and the like; a carbohydrate such as glucose, mannose, sucrose or dextran, and mannitol; a protein; a polypeptide or an amino acid such as glycine; an antioxidant; a chelating agent such as EDTA or glutathione; an adjuvant (for example, aluminum hydroxide); and a preservative. The composition of the present disclosure is preferably formulated for intravenous administration.

The pharmaceutical composition of the present disclosure can be administered in a manner suitable for the disease to be treated (or prevented). The amount and frequency of administration will be determined by factors such as the condition of a patient, and the type and severity of the patient's disease - although appropriate dosages may be determined by clinical trials.

When referring to "immunologically effective amount", "anti-tumor effective amount", "tumor-suppressing effective amount" or "therapeutic amount", the precise amount of the composition of the present disclosure to be administered can be determined by a physician, taking into account the age, weight, tumor size, and degree of infection or metastasis of the patient (subject), and individual differences in the condition. It is generally indicated that a pharmaceutical composition comprising a T cell as described herein can be administered at a dose of 10^4 to 10^9 cells/kg body weight, preferably a dose of 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. T cell composition can also be administered for multiple times at these doses. Cells can be administered by using injection techniques well known in immunotherapy (see, for example, Rosenberg et al, NewEng.J. of Med. 319: 1676, 1988). Optimal dosages and treatment regimens for a particular patient can be readily determined by a person skilled in the medical field by monitoring the patient's signs of disease and thus regulating the treatment.

The composition can be administered to a subject in any convenient manner, including by nebulization, injection, oral administration, infusion, implantation or transplantation. The composition described herein can be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intraspinally, intramuscularly, by intravenous (i.v.) injection or intraperitoneally. In one embodiment, a T cell composition of the present disclosure is administered to a patient by intradermal or subcutaneous injection. In another embodiment, the

T cell composition of the present disclosure is preferably administered by i.v. injection. The T cell composition can be injected directly into tumor, a lymph node or an infected site.

In certain embodiments of the present disclosure, cells activated and expanded using the methods described herein, or other methods known in the art to expand T cells to therapeutic levels are administered to a patient in conjunction with (e.g., prior to, simultaneously or following) any number of related therapeutic modalities, including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, cytarabine (also known as ARA-C) or natalizumab treatment for patients with MS or efalizumab treatment for patients with psoriasis or other treatments for patients with PML. In a further embodiment, the T cells of the present disclosure can be used in combination with chemotherapy, radiation, immunosuppressive agents such as cyclosporin, azathioprine, methotrexate, mycophenolate and FK506, antibodies or other immunotherapeutic agents. In a further embodiment, the cell composition of the present disclosure is administered to a patient in conjunction with (e.g., prior to, simultaneously or following) bone marrow transplantation, a chemotherapeutic agent such as fludarabine, external beam radiation therapy (XRT), or cyclophosphamide. For example, in one embodiment, the subject may undergo a standard treatment by high dose chemotherapy, followed by peripheral blood stem cell transplantation. In some embodiments, the subject receives an injection of the expanded immune cells of the present disclosure after transplantation. In one additional embodiment, the expanded cells are administered prior to or after surgery.

The dosage of the above treatment administered to the patient will vary with the precise nature of the condition being treated and the recipient being treated. The dosage ratios administered to human can be carried out according to practices accepted in the art. Typically, for each treatment or each course of treatment, 1×10^6 to 1×10^{10} modified T cells of the present disclosure can be administered to a patient by, for example, intravenous reinfusion.

The technical solution of the present disclosure has the following beneficial effects:

1. The present disclosure provides an immunotherapeutic regimen for inhibiting tumors, particularly solid tumors, namely combining T cells expressing a chimeric CD3 fusion protein with BiTA. The chimeric CD3 fusion protein and BiTA bind to each other and function to activate T cells and target tumor cells.

2. The present disclosure also provides a CAB technology for CAB-T cells to target tumor tissues by chimeric expression of CD3 and BiTA in T cells, and BiTA secreted by CAB-T cells

can simultaneously achieve the activation of the CAB-T cells and the activation of the endogenous TCR complexes of the non-engineered T cells in tumor tissues, exert the anti-tumor effect of CAB-T itself and mobilize the anti-tumor effect of the non-engineered T cells, thereby ensuring the effectiveness of CAB-T clinical application.

3. Since the activation of chimeric CD3 is dependent on BiTA secreted by CAB-T, a small amount of BiTA released by CAB-T in tumor tissues can stimulate CAB-T to release more BiTA in the local tumor microenvironment, which advantageously ensures the safety of CAB-T clinical application.

4. CAB-T can achieve better activation in solid tumor tissues, achieve the maximum anti-tumor effect at a tumor site, achieve safety and effectiveness similar to those of local administration of tumors, and has great advantages and potential in the clinical treatment of solid tumors compared with second generation CAR-T.

The present disclosure is further illustrated below with specific examples. It is to be understood that these examples are for illustrative purposes only and not intended to limit the scope of the present disclosure. Experimental methods in which specific conditions are not indicated in the following examples are generally carried out according to the conventional conditions described by, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989), or according to the conditions recommended by a manufacturer. Percentages and parts are by weight unless otherwise stated.

Example 1 Design of CAB and its Control Structures

1.1 Structural design of CD3e-BB ζ , 1st-CAIX-BiTA, 1st-CAIX-CAB and CAIX-TRuC in a control group

To validate the anti-tumor activity of CAB-T, we first designed a first group of four structures using a nanobody (VHH) targeting CAIX in a group of experiments: CD3e-BB ζ (DNA SEQ ID NO.: SEQ 15, AA SEQ ID NO.: SEQ 16), CAIX-BiTA (DNA SEQ ID NO.: SEQ 21, AA SEQ ID NO.: SEQ 22), CAIX-CAB (DNA SEQ ID NO.: SEQ 25, AA SEQ ID NO.: SEQ 26) and CAIX-TRuC (DNA SEQ ID NO.: SEQ 31, AA SEQ ID NO.: SEQ 32). In order to distinguish from the second group of CAIX-CAB structures described later, we named CAIX-BiTA and CAIX-CAB of this group as 1st-CAIX-BiTA and 1st-CAIX-CAB, respectively. Among them, 1st-CAIX-BiTA is a BiTA without tags, 1st-CAIX-CAB comprises CD3e-BB ζ and a CAB structure where BiTA is not labeled, and CAIX-TRuC is a control

structure using a platform technology of TCR² Therapeutics. The specific structure the above constructs are shown in FIG. 1.

1.2 Structural design of tERBB2, CD3e-BB ζ , CAIX-BiTA, CAIX-CAB, CAIX- ζ , CAIX-BB ζ and CAIX-28 ζ in an experimental group of nanobody (VHH) targeting CAIX

In another group of experiments, we designed a second group of structures using VHH targeting CAIX, including: truncated ERBB2 (tERBB2 used as a negative control, including the fourth extracellular domain of ERBB2, transmembrane region, and FLAG tag) (DNA SEQ ID NO.: SEQ 33, AA SEQ ID NO.: SEQ 34), CD3e-BB ζ (DNA SEQ ID NO.: SEQ 15, AA SEQ ID NO.: SEQ 16), CAIX-BiTA (DNA SEQ ID NO.: SEQ 37, AA SEQ ID NO.: SEQ 38), CAIX-CAB (DNA SEQ ID NO.: SEQ 39, AA SEQ ID NO.: SEQ 40), CAIX- ζ (a first generation CAR structure targeting CAIX) (DNA SEQ ID NO.: SEQ 41, AA SEQ ID NO.: SEQ 42), CAIX-BB ζ (a second generation CAR structure containing the 4-1BB costimulatory domain) (DNA SEQ ID NO.: SEQ 45, AA SEQ ID NO.: SEQ 46) and CAIX-28 ζ (a second generation CAR structure containing the CD28 costimulatory domain) (DNA SEQ ID NO.: SEQ 47, AA SEQ ID NO.: SEQ 48), 7 structures in total. In this group of structures, all the structures carry a FLAG tag, and all of the secretory BiTA antibodies have a His tag. The inclusion of His tag facilitates the subsequent detection of BiTA secretion levels. The details of these constructs are shown in FIG. 2.

1.3 Structural design of tERBB2, CD3e-BB ζ , HER2-BiTA, HER2-CAB, HER2- ζ , HER2-BB ζ and HER2-28 ζ in the experimental group of single-chain antibody targeting HER2

In the third groups of experiments, we tested the anti-tumor activity of a CAB platform using a single-chain antibody (scFv) that targets HER2. The single-chain antibody variable region sequence is derived from an antibody drug Herceptin (trastuzumab). In this example, we designed a third group of structures including: truncated ERBB2, CD3e-BB ζ , HER2-CAB (DNA SEQ ID NO.: SEQ 49, AA SEQ ID NO.: SEQ 50), HER2- ζ (DNA SEQ ID NO.: SEQ 51, AA SEQ ID NO.: SEQ 52), HER2-BB ζ (DNA SEQ ID NO.: SEQ 53, AA SEQ ID NO.: SEQ 54) and HER2-28 ζ , second generation CAR structure (DNA SEQ ID NO.: SEQ 55, AA SEQ ID NO. : SEQ 56), 7 structures in total. In this group of structures, all structures except HER2- ζ carry the FLAG tag, and all secretory BiTA antibodies also carry the His tag. The details are shown in FIG. 3.

Example 2 Lentivirus packaging of CAB and its control structures

In the present disclosure, we prepared CAB-T cells using lentivirus as a vector. First, we prepared a lentiviral vector carrying a gene encoding CAB and its control structures. A specific procedure for packing lentivirus is as follows.

1) 1×10^7 HEK 293T cells were seeded in a 10 cm culture plate, 10 mL of DMEM (Hyclone, SH30243.01) medium containing 10% FBS (Gibco, 10099-141C) was added, and the cells were mixed fully and incubated at 37 °C overnight.

2) On the second day, the medium was replaced with a serum-free DMEM when the cell confluence of HEK 293T (ATCC, CRL-3216) reached about 90%.

3) A plasmid complex was prepared, in which the amounts of various plasmids were 8 μ g plasmid DNA, 4 μ g psPAX2 and 2 μ g pMD2g, respectively, and dissolved in 1mL opti-MEM (Gibco, 31985-070), and 42 μ L PEI (Polysciences, 24765-2) was added; shaking was performed under vortex for 20 s. After standing at room temperature for 15 min, the mixture was gently added to the HEK293T medium along the side, and culture was kept at 37 °C.

4) Following the culture of cells for 4 h, the medium was removed, and washed once with PBS (Hyclone, SH30256.01), and 2% FBS pre-warmed fresh DMEM medium was added again.

5) After transfection for 48 h and 72 h, supernatants were collected respectively, and centrifuged at 2000 g for 5 min, and the precipitate was discarded. The supernatant was filtered with a 0.25 μ m filter (Sartorius, 16541-K) and then PEG 8000 (Sigma, 89510-1KG- F) with a final concentration of 5% and NaCl (Sigma, S5150-1L) with a final concentration of 0.15 M were added, vigorously mixed and allowed to stand overnight at 4 °C.

6) The virus supernatant was centrifuged at 2000 g, 4 °C for 20 min, the supernatant was removed, and virus pellets were dissolved in 50-100 μ L of PBS and frozen at -80 °C.

Example 3 Preparation of engineered T cells with CAB and its control structures

Following the completion of the preparation of the lentiviral vector carrying the CAB structure, the lentiviral vector can be used to infect immune cells to complete the preparation of CAB-T cells. A specific procedure for preparing CAB-T cells is as follows.

1) Commercialized PBMC (Saily Bio, SLB-HP050B) cells were cultured with X-VIVO 15 (LONZA, 04-418Q) containing 5% human blood albumin (GRIFOLS, 20% human blood

albumin) at an initial cell density of 1×10^6 /mL.

2) Anti-CD3/CD28 beads (Miltenyi biotec, 130-091-441) were added in a ratio of cells: Beads=3:1, and 1000 IU/mL of IL-2 (Si Huan Sheng Wu, SFDA approval number: S10970016) was added to activate T cell expansion.

3) After cell activation for 48 h, T cells were infected by adding appropriate amount of virus and 12 μ g/mL protamine (Sigma, P4005).

4) After lentivirus infection for 24 h, the cell suspension was aspirated, and a completely fresh X-VIVO 15 medium was added at a concentration of 1×10^6 cells/mL.

5) The density of cells was observed every day, T cell culture solution containing 1000 IU/ml of IL-2 was added in good time to maintain the density of T cells at about 1×10^6 cells/mL, and expansion was continued for 5-10 days to complete the preparation of CAR-T cells.

Example 4 Positive Frequency assay of CAB-T cells

Upon the completion of the preparation of CAB-T and its control group cells, the infection efficiency was determined for subsequent activity analysis. Specifically, a method for detecting the CAB-T positive frequency using a FLAG antibody is as follows.

1) $3-5 \times 10^5$ cells were taken, 200 μ L FACS buffer (PBS containing 1% FBS) was added into each flow cytometry tube, and the mixture was centrifuged at 300 g for 5 min; biotin-CAIX (sino biological, 10107-H02H) at a final concentration of 100 nM was added to a CAIX-Truc sample, and the mixture was incubated at 4 °C for 20 min; biotin-HER-2 (ACRO, HE2-H82E2) at a final concentration of 100 nM was added to an Her2-Truc sample, and the mixture was incubated at 4 °C for 20 min; the supernatant was discarded, 200 μ L Fixation/Permeabilization solution (BD bioscience, 554715) was added, and the mixture was incubated at 4 °C for 20 min.

2) The mixture was centrifuged at 300g for 5min; the supernatant centrifuged was removed, 200 μ L of $1 \times$ Perm/Wash buffer (BD bioscience, 554715) was added, and the mixture was resuspended, centrifuged at 400g for 5 min, and washed twice;

3) the supernatant centrifuged was removed, 100 μ L of anti-Flag antibody diluted with 1:1000 FACS buffer was added into each sample, and the cells were mixed well and incubated at 4 °C for 30 min.

4) After incubation, 1 mL of FACS buffer was added to each flow cytometry tube, and the mixture was centrifuged at 400 g for 5 min.

5) The supernatant centrifuged was removed, 1 mL FACS buffer was added, and the mixture was resuspended and centrifuged at 400 g for 5 min.

6) The supernatant centrifuged was removed, 100 μ L of SA-PE (Invitrogen, S866) diluted with 1:250 FACS buffer was added to each sample, the cells were mixed well and incubated at 4 °C for 30 min in the dark; after incubation, 1 mL of FACS buffer was added to each flow cytometry tube, and the mixture was centrifuged at 300g for 5min; the supernatant centrifuged was removed, and washing was repeated twice.

7) The sample was placed on a flow cytometer for detection.

Results:

In the first group of experiments, the FLAG antibody can be used to detect the positive frequencies of T cells engineered by the corresponding structure as the FLAG tag was carried in the CD3e-BB ζ structure and the 1st-CAIX-CAB structure, while biotin-labeled CAIX protein can be used to detect the positive frequencies of T cell transduction for T cells engineered by CAIX-TRuC. However, since the 1st-CAIX-BiTA structure does not have a suitable tag, the positive frequencies of T cells engineered by this structure cannot be detected. However, it can be judged from the subsequent results that the positive frequency of T cells engineered by 1st-CAIX-BiTA can meet the requirement of experimental analysis. The results of the detection are shown in FIG. 4.

In the second and third groups of experiments, we designed a FLAG tag for each structure, and the positive frequencies of the corresponding engineered T cells can be carried out the labelling and detection of the FLAG antibody (for first generation structure HER2- ζ without a FLAG tag, biotinylated HER2 was used for transduction efficiency detection). NT represents non-transduced T cells and were used as a negative control group. The test results are shown in FIG. 5 and FIG. 6. Differences in infection efficiency between different samples are within acceptable limits.

It can be seen from the above that the positive frequency of transduction for each group of engineered T cells designed by using the structures disclosed in the present disclosure and the prior art meet the requirement of experimental analysis.

Example 5 Antigen dependent cytokine release assay of CAB-T

When CAB-T cells are co-cultured with tumor cells, CAB-T can recognize and activate target antigens on the surface of tumor cells, thereby releasing a large number of inflammatory

cytokines. Based on this, the level of cytokines released by activated CAB-T cells was detected by enzyme-linked immunosorbent assay (ELISA) in the present example.

Detection procedure for ELISA is as follows.

1) 1×10^5 effector cells and 1×10^5 target cells were seeded at 200 μ L/well. A 96-well cell culture plate was co-cultured overnight, and centrifuged at 300 g for 5 min, and 150 μ L of the supernatant/well was pipetted into a new 96-well cell culture plate using a multichannel pipette, and cytokine detection was performed using IFN- γ (Invitrogen, 88-7316-88)/IL-2 (Invitrogen, 88-7025-88)/TNF- α (Invitrogen, 88-7346-88) assay kit, respectively.

2) The ELISA plate was coated with Human anti-IFN- γ /IL-2/TNF- α antibodies one day in advance. Human anti-IFN- γ /IL-2/TNF- α antibodies were diluted with PBS (1:250), 100 μ L of the antibody was added to each well, and the ELISA plate was sealed with a microplate sealer at 4 $^{\circ}$ C overnight.

3) Plate washing: liquid in the plate was quickly removed, Wash Buffer was added with a multichannel pipette at 200 μ L/well, and the plate washing was repeated for five times.

4) 200 μ L of 1 $\times$ ELISA/ELISASPOT diluent was added to each well, covered with a microplate sealer, and blocked at room temperature for 60 min.

5) Plate washing: liquid in the plate was quickly removed, Wash Buffer was added with a multichannel pipette at 200 μ L/well, and the plate washing was repeated for five times.

6) Human IFN- γ ELISA Standard was prepared, and 8 gradients (in pg/mL) were set: 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125.

7) The standard and the sample were added to the ELISA plate at 100 μ L/well, and the sample and the standard were both diluted to the desired concentration with 1 $\times$ ELISA/ELISASPOT diluent, covered with a microplate sealer and incubated for 2 h at room temperature.

8) Plate washing: liquid in the plate was quickly removed, Wash Buffer was added with a multichannel pipette at 200 μ L/well, and the plate washing was repeated for four times.

9) The Human IFN- γ /IL-2/TNF- α detection antibody was diluted with PBS (1:250), 100 μ L of the antibody was added to each well, and the ELISA plate was sealed with a microplate sealer and incubated for 1 h at room temperature.

10) HRP-conjugated Streptavidin was prepared by diluting with PBS (1:250), and added

at 100 μ L/well to the ELISA plate, which was covered with a microplate sealer and incubated for 30 min at room temperature.

11) Plate washing: liquid in the plate was quickly removed, Wash Buffer was added with a multichannel pipette at 200 μ L/well, and the plate washing was repeated for five times.

12) TMB Substrate was warmed to room temperature 30 minutes in advance, and added at 100 μ L/well to the ELISA plate. After reaction for 5-10 min at room temperature, 50 μ L/well of Stop solution was added.

13) The absorbance was read in a microplate reader at the detection wavelength of OD=450 nm.

14) A standard curve was calculated according to the concentration and OD value of the standard, and the concentration of the sample to be tested was calculated according to the standard curve. The plot was made by GraphPad Prism mapping software.

Results:

In the first group of experiments, after the CAIX-CAB-T cells or their control group cells were co-cultured with CAIX+ HEK 293T cells or CAIX-HEK293T, respectively, the release levels of inflammatory cytokines IL-2 and IFN- γ in the supernatant were detected. The results were shown in FIG. 7. When CAIX-CAB-T and its control groups were co-cultured with CAIX-HEK 293T cells, respectively, none of the immune cells showed significant cytokine release. When CAIX-CAB-T and its control were co-cultured with CAIX+ HEK 293T cells, T cells engineered by 1st-CAIX-BiTA, 1st-CAIX-CAB and CAIX-TRuc all exhibited co-culture time-dependent cytokine release levels. The cytokines accumulated in co-culture for 48 h were significantly higher than the cytokine levels accumulated in co-culture for 24 h. When non-engineered T cells and T cells engineered by CD3e-BB ζ were co-cultured with CAIX+ HEK 293T cells, respectively, no significant release of IL-2 and IFN- γ cytokines was detected. At the same time, it was unexpectedly found that the level of cytokine released by 1st-CAIX-CAB-T cells was significantly higher than that of 1st-CAIX-BiTA-T cells after 48 hours of co-culture; after T cells engineered by CD3e-BB ζ and 1st-CAIX-BiTA were mixed in a ratio of 1:1 and then co-cultured with CAIX+ HEK 293T cells, the levels of cytokines released were found to be comparable to those of 1st-CAIX-CAB-T cells. The above results indicated that CAIX-CAB-T cell activation was dependent on CAIX antigen, and the BiTA and CD3e-BB ζ synergistically promoted T cell activation; it was also demonstrated that CAB-T cells had comparable in vitro activation ability as compared to control group TRuC-T cells.

In the second group of experiments, after CAIX-CAB-T and its control group cells were co-cultured with CAIX+ MB-231 or CAIX-MB-231 cells (CAIX expression levels were shown in FIG. 8. A), respectively, the release levels of the inflammatory cytokines IL-2, IFN- γ , and TNF- α in the supernatant were detected. The results were shown in FIG. 8. When CAIX-CAB-T cells and their controls were co-cultured with CAIX-MB-231 cells, respectively, none of the cells showed significant cytokine release. After CAIX-CAB-T and its control groups were co-cultured with CAIX+ MB-231 cells for 48 h, respectively, the T cells engineered by CAIX-BiTA, CAIX-CAB, CAIX-BB ζ , CAIX-28 ζ and CAIX- ζ had varying degrees of activation levels. From the data, it can be found that CAIX-CAB-T and CAIX-BiTA, and T cells structurally modified by first and second generation CAR had almost the same release ability in IFN- γ and TNF- α ; while in terms of IL-2 release, CAIX-CAB-T was weaker than second generation CAR cells, but slightly higher than CAIX-BiTA and first generation CAR-modified T cells. The results of the second group of experiments indicated the dependence of CAIX-CAB-T cell activation on CAIX antigen and the difference in cytokine release compared to second generation CAR, that is, IFN- γ and TNF- α released by CAIX-BiTA-T and CAIX-CAB-T were substantially equivalent to the release by the second generation CAR, and CAIX-BiTA-T and CAIX-CAB-T were weaker than the second generation CAR in IL-2 release.

Example 6 Antigen dependent T cell activation marker upregulation of CAB-T

When CAB-T cells are co-cultured with tumor cells, CAB-T can recognize and activate target antigens on the surface of tumor cells. The expression levels of T cell activation marker proteins including CD137, CD25, CD27 and the like on membrane surface are significantly up-regulated. The cell proliferation ability represented by the expression level of Ki67 is increased, and the killing ability of T cells represented by CD107a is also enhanced. Based on this, the above-described staining method and flow cytometry were used to detect changes in the expression level of the above-mentioned membrane surface proteins in activated CAB-T cells in the present example.

A specific cell staining procedure was as follows:

- 1) 1×10^5 effector cells and 1×10^5 target cells were seeded at 200 μ L/well. A 96-well cell culture plate was co-cultured overnight and centrifuged at 300 g for 5 min, and each well was added with 200 μ L FACS buffer, and centrifuged at 300 g for 5 min.
- 2) The supernatant centrifuged was removed, and the cells were resuspended by adding 200 μ L FACS buffer and centrifuged at 300 g for 5 min.

- 3) The antibody was diluted with FACS buffer (100 μ L/well)
 BV421 Mouse Anti-Human CD3 (BD Bioscience, 562426) 1:500 dilution
 PE Mouse Anti-Human CD137 (BD Bioscience, 555956) 1:200 dilution
 APC Mouse Anti-Human CD27 (BD Bioscience, 561786) 1:200 dilution
 PE-cy7 Mouse Anti-Human CD25 (BD Bioscience, 557741) 1:200 dilution
- 4) The supernatant centrifuged was removed, and 100 μ L of the antibody was added to each well, mixed, and incubated at 4 °C for 30 min in the dark.
- 5) 200 μ L FACS buffer was added to each well and centrifuged at 300 g for 5 min, and the supernatant was removed.
- 6) The supernatant centrifuged was removed and step 2.5 was repeated.
- 7) The supernatant was discarded, and 200 μ L Fixation/Permeabilization solution (BD bioscience, 554715) was added and incubated at 4 °C for 20 min.
- 8) Centrifugation was performed at 300g for 5min. The supernatant centrifuged was removed, 200 μ L 1 $\times$ Perm/Wash buffer (BD bioscience, 554715) was added for resuspending, and centrifugation was performed at 400g for 5min.
- 9) Washing was performed twice and the antibody FITC Mouse Anti-Flag (Biolegend, 637318) was diluted with FACS buffer by 1:1000 dilution.
- 10) Centrifugation was performed at 400g for 5min. the supernatant centrifuged was removed, and washing was performed twice.
- 11) Flow cytometry detection was performed using FSC/SSC gating to obtain a desired lymphocyte population (PBMCs), CD3 BV421+ and Flag FITC+ cell populations were selected to obtain live CAR-T cells, and then gated by using PBMCs not subjected to virus transduction as standard to obtain the percentage of CAR-T cells CD137 PE+ cells.

Results:

In the first group of experiments, after CAIX-CAB-T cells or their control group cells were co-cultured with CAIX+ HEK 293T cells or CAIX-HEK293T, respectively, changes in the expression levels of CD137 and CD107a on the surface of T cell membranes were detected. The results were shown in FIG. 9. When CAIX-CAB-T and its control groups were co-cultured with CAIX-HEK 293T cells, the expression levels of CD137 and CD107a in CAB-T and its

control group immune cells were not significantly changed. After CAIX-CAB-T and its control groups were co-cultured with CAIX+ HEK 293T cells for 24 h, respectively, the expression levels of CD137 and CD107a in T cells engineered by 1st-CAIX-BiTA, 1st-CAIX-CAB and CAIX-TRuC were significantly up-regulated, and 1st-CAIX-CAB-T demonstrated a much higher up-regulation level as compared to 1st-CAIX-BiTA. When non-engineered T cells and T cells engineered by CD3e-BB ζ were co-cultured with CAIX+ HEK 293T cells, respectively, no significant up-regulation of CD137 and CD107a was detected. After T cells engineered by CD3e-BB ζ and 1st-CAIX-BiTA were mixed in a ratio of 1:1 and then co-cultured with CAIX+ HEK 293T cells for 24 h, the expression levels of CD137 and CD107a were found to be significantly higher than those of CD3e-BB ζ -T and 1st-CAIX-BiTA-T individually co-cultured with CAIX+ HEK 293T. The above results indicated that CAIX-CAB-T cell activation was dependent on CAIX antigen, and the BiTA and CD3e-BB ζ synergistically promoted T cell activation; it was also demonstrated that CAB-T cells had comparable in vitro activation ability to control group TRuC-T cells.

In the second group of experiments, after CAIX-CAB-T cells and their control group cells were co-cultured with CAIX+ MB-231 cells or CAIX- MB-231 for 48 h, respectively, the changes in expression levels of CD137, CD25, CD27 and Ki67 on the surface of a T cell membrane were detected. The results were shown in FIG. 10. When CAIX-CAB-T and its control groups were co-cultured with CAIX- MB-231 cells, respectively, the expression levels of CD137, CD25, CD27 and Ki67 in CAIX-CAB-T and its control group immune cells were not significantly changed. After CAIX-CAB-T and its control groups were co-cultured with CAIX+ MB-231 cells, the expression levels of CD137, CD25, CD27 and Ki67 in T cells engineered by CAIX-BiTA, CAIX-CAB, first generation CAR and second generation CAR were significantly up-regulated. When T cells engineered by tERBB2 and CD3e-BB ζ were co-cultured with CAIX+ MB-231 cells, respectively, no significant up-regulation of CD137, CD25, CD27 and Ki67 was detected. The above results indicated that the CAIX-CAB-T cell activation was dependent on CAIX antigen, and it was also demonstrated that CAB-T cells had comparable in vitro activation ability to the control group BiTA-T, the first generation CAR and the second generation CAR cells.

In a third group of experiments, we detected the in vitro activation ability of the HER2-CAB structure constructed using the Trastuzumab-derived scFv sequence. The results were shown in FIG. 11. After HER2-CAB-T cells and their control group cells were co-cultured with HER2-positive SKBR3 cell line or HER2-negative RAJI cell line for 48 h, respectively, the

changes in the expression levels of CD137, CD25, CD27 and Ki67 on the surface of a T cell membrane were detected. The results were shown in FIG.12. When HER2-CAB-T and its control groups were co-cultured with RAJI cells, respectively, the expression levels of CD137, CD25, CD27 and Ki67 in HER2-CAB-T and its control group immune cells were not significantly changed. After HER2-CAB-T and its control groups were co-cultured with HER2-positive SKBR3 cells, the expression levels of CD137, CD25, CD27 and Ki67 in T cells engineered by HER2-CAB, first generation CAR and second generation CAR were significantly up-regulated. When T cells engineered by tERBB2 and CD3e-BB ζ were co-cultured with SKBR3 cells, respectively, no significant up-regulation of CD137, CD25, CD27 and Ki67 was detected. The above results indicated that HER2-CAB-T cell activation was dependent on HER2 antigen and it was also demonstrated that CAB-T cells had comparable in vitro activation ability to the control group BiTA-T, first generation CAR and second generation CAR cells.

Example 7 Analysis of non-engineered T cells activated by paracrine CAB-T

The original intention for designing the CAB structure is to achieve the following effects: when encountering tumor cells, CAB-T activates its own anti-tumor activity, and meanwhile non-engineered T cells around CAB-T are activated by BiTA drugs secreted by CAB-T to achieve paracrine activation. In order to detect the paracrine activation function of CAB-T in T cells, we made a verification using a transwell experiment. Specifically, the experiment was performed by separating the CAB-T and non-engineered T cells using a physical barrier through a 0.4 μ m Transwell system, CAB-T cells were placed in an upper chamber, and non-engineered T cells and tumor cells were placed in a lower chamber. Soluble BiTA secreted by CAB-T can enter the lower chamber freely through a 0.4 μ m grid to activate the ability of the underlying non-engineered T cells to activate by recognizing tumor cells.

A specific experimental procedure was as follows:

- 1) 1×10^6 BiTA-T cells and 1×10^6 CAB-T were resuspended in 200 μ L of X-VIVO 15 medium, respectively, and then added to the upper chamber of 0.4- μ m Transwell (Coring, 3413).
- 2) 1×10^6 non-engineered T cells and 1×10^6 target cells were resuspended in 500 μ L of X-VIVO-15 medium and added to the lower chamber of Transwell.
- 3) The upper chamber was carefully placed into the lower chamber and incubated in an incubator for 48 hours.

4) A culture plate co-cultured overnight was taken out, and 500 μ L of cell supernatant in the lower chamber was taken out. After centrifugation at 300 g for 5 min, 150 μ L of supernatant/well was pipetted using a multichannel pipette and transferred to a new 96-well cell culture plate for ELISA detection of IFN- γ /IL-2/TNF- α .

Results:

In the second group of experiments (FIG. 12), both BiTAs secreted by CAIX-BiTA-T and CAIX-CAB-T were able to activate the ability of the underlying non-engineered T cells to recognize CAIX-positive MB-231 tumor cells by Transwell pores, which exhibited high release levels of IFN- γ and TNF- α . At the same time, we found no significant change in IL-2 release levels, which was consistent with the results of the study in Example 9.

It can be seen from the results shown in the third group of experiments (FIG. 13) that HER2-CAB-T also showed the same paracrine activation for the ability of non-engineered T cells to recognize tumor antigens. HER2-CAB-T cells, but not control tERBB2-T cells, can activate the underlying non-engineered T cells in Transwell to recognize HER2-positive SKBR3 tumor cells. HER2-CAB-T did not help the non-engineered T cells to recognize HER2-negative RAJI cells.

Both the second and third groups of experiments demonstrated that the CAB-T structure can activate the ability of non-engineered T cells to recognize tumor cells via paracrine BiTA.

Example 8 Analysis of immune checkpoint expression levels and cell differentiation phenotypes of CAB-T cell

The expression level of immune checkpoint proteins on immune cells and the differentiation phenotype of immune cells have a great relationship with the therapeutic effect of adoptive T cells. Therefore, lower expression levels of immune checkpoints and higher proportions of memory T cells both predict better clinical response rates. We used flow cytometry to detect the effect of the CAB structure on the immune checkpoint expression levels and cell phenotypes of T cells engineered by the CAB structure.

A specific analysis procedure was as follows:

1) 96-well cell culture plates co-cultured overnight was centrifuged at 300 g for 5 min, 200 μ L FACS buffer was added to each well, and centrifuged at 300 g for 5 min.

2) The supernatant centrifuged was removed, and the cells were resuspended with 200 μ L FACS buffer, and centrifuged at 300 g for 5 min.

3) The antibody was diluted with FACS buffer to prepare antibody mix (100 μ L/well)

BV421 Mouse Anti-Human CD3 (BD Bioscience, 562426)	1:500 dilution
APC Mouse Anti-Human TIM-3 (Biolegend, 345012)	1:200 dilution
Alexa Fluor 647 Mouse Anti-Human LAG-3 (Biolegend, 369304)	1:200 dilution
APC Mouse Anti-Human CD279 (BD Bioscience, 558694)	1:200 dilution
PE-cy5 Mouse Anti-Human CD45RA (BD Bioscience, 555490)	1:200 dilution
BV605 Mouse Anti-Human CCR7 (BD Bioscience, 563711)	1:200 dilution

4) The supernatant centrifuged was removed, and 100 μ L of the antibody mix was added to each well, and incubated at 4 °C for 30 min in the dark.

5) 200 μ L FACS buffer was added to each well, and centrifuged at 300 g for 5 min, and the supernatant was discarded.

6) The supernatant centrifuged was removed and step 2.5 was repeated.

7) 200 μ L of Fixation/Permeabilization solution (BD bioscience, 554715) was added and incubated at 4 °C for 20 min.

8) Centrifugation was performed at 300g for 5min; the supernatant centrifuged was removed, 200 μ L of 1 $\times$ Perm/Wash buffer (BD bioscience, 554715) was added, resuspended, and centrifuged at 400g for 5min; and washing was performed twice.

9) The antibody FITC Mouse Anti-Flag (Biolegend, 637318) was diluted with FACS buffer by 1:1000 dilution.

10) Centrifugation was performed at 400g for 5min; the supernatant centrifuged was removed, and washing was performed twice.

11) Flow cytometry detection was performed using FSC/SSC gating to obtain desired lymphocyte populations, and CD3 BV421+ and Flag FITC+ cell populations were selected to obtain live CAR-T cells.

Results:

It can be seen from the results of the second group of experiments (FIG. 14) that after co-culture with CAIX-positive MB-231 cells, the expression levels of immune checkpoint protein on the surface of CAIX-CAB-T cells, including LAG-3, PD-1 and TIM-3, were at lower levels than second generation CAR-T engineered by CAIX-28 ζ , the expression levels of PD-1 and TIM-3 on CAB-T cells were basically the same as the CAIX-BB ζ second generation CAR-T,

and the expression level of LAG-3 in CAB-T cells was slightly lower than that in second generation CAR-T engineered by CAIX-BBζ. Lower expression levels of immune checkpoint indicated the clinical advantage of CAB-T cells compared to second generation CAR-T.

In addition, in the second group of experiments, we also used CD45RA and CCR7 for differentiation phenotype analysis of immune cells. Differentiation marker proteins were: initial T cells (CD45RA⁺, CCR7⁺), central memory T cells (CD45RA⁻, CCR7⁺), effector memory T cells (CD45RA⁻, CCR7⁻), and effector T cells for terminal differentiation (CD45RA⁺, CCR7⁻), respectively. It can be seen from the results shown in FIG. 14.D that compared with the second generation CAR-T, the cell proportion of the initial differentiated T cell phenotype and the central memory T cell phenotype of CAIX-CAB-T was significantly higher than that of the second generation CAR-T cells, while the proportion of effector memory T cells of second generation CAR-T cells was significantly higher than that of CAB-T cells. The more central memory T cell phenotype predicted better clinical efficacy, showing the advantage of CAB-T in the differentiated state compared to the second generation CAR-T.

It can be seen from the results shown in the third group of experiments (FIG. 15) that the expression status of the immune checkpoint and the differentiation result of the cell phenotype for HER2-CAB-T were basically consistent with the analysis result of CAIX-CAB-T. Namely, the expression level of the immune checkpoint of HER2-CAB-T was lower than that of second generation CAR-T engineered by CAIX-28ζ, and was basically equal to or slightly lower than that of second generation CAR-T engineered by CAIX-BBζ. The differentiation state of HER2-CAB-T also had a higher proportion of central memory T cell phenotype than the second generation CAR-T.

Example 9 Antigen dependent killing activity of CAB-T

Whether CAB-T cells have in vitro killing activity is the key basis for judging the potential clinical efficacy of CAB-T. To verify the tumor killing activity of CAB-T, we used an LDH method for detection.

A specific experimental procedure was as follows:

1) Experimental wells, effector cell control wells, target cell control wells, target cell maximum release wells, medium control wells, and volume control wells were arranged, respectively; the experimental procedure was performed according to the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, G1781) standard procedure.

2) Different target-effector ratios were set, i.e. number of effector cells: number of target cells = 0: 1, 1:1, 5: 1, 10: 1, and 20: 1

3) Number of cells: 1×10^4 target cells, 50 μL /well.

4) For experimental wells, 100ul of cells at different dilution ratios of effector cells: target cells = 0:1, 1:1, 5:1, 10:1, and 20:1 (effector cells 50 μL + target cells 50 μL) were added to cell culture plate in triplicate;

5) For effector cell control wells, effector cells: target cells = 0:0, 1:0, 5:0, 10:0, and 20:0, in duplicate;

6) For target cell control wells, 50 μL of target cells at 1×10^4 /well and 50 μL of medium were added;

7) For target cell maximum release wells, 50 μL of target cells at 1×10^4 and 50 μL of medium were added, and 10 μL of lysate was added 1 h before the sample was collected;

8) For medium control well, 100 μL of medium was added;

9) For volume control wells, 100 μL of medium was added, 10 μL of the lysate was added to the target cell maximum release wells 1 h before the sample was collected, meanwhile 10 μL of lysate was added, and incubation was performed at 37 °C.

10) According to the designed layout, the samples were added into a plate, and incubated at 37 °C with 5% CO_2 for 24h, or 36h, or 48h;

11) Assay buffer was taken from a refrigerator at -20 °C and dissolved in a refrigerator at 4 °C in the dark. During use, 12 ml of assay buffer was added to a flask of substrate mix and mixed.

12) The culture plate was centrifuged at 250g for 4min, and 50 μL /well of cellular supernatant was transferred to a new ELISA plate.

13) 50 μL /well of substrate mix was added to the new ELISA plate (12 mL of assay buffer was added to a flask of mix and mixed in the dark).

14) Incubation was performed at room temperature for 30 min in the dark, and 50 μL /well stop solution was added.

15) The absorbance was read in a microplate reader at the detection wavelength of OD=490 nm in 1 h.

16) The cell killing ratio (%) was calculated based on an OD value

Experimental well = effector-target ratio - medium control (mean)

Spontaneous release of target cell = target cell control - medium control (mean)

Spontaneous release of effector cell = effector cell control - medium control (mean)

Maximum release of target cell = maximum release of target cells (mean) - volume control (mean)

Cell killing ratio (%) = (experiment - spontaneous release of target cell - spontaneous release of effector cell)/(maximal release of target cell - spontaneous release of target cell).

Results:

BiTA secreted by CAB-T can activate CAB-T cells themselves and their surrounding non-engineered T cells in a target antigen-dependent manner, and kill target antigen-positive tumor cells; meanwhile, CAB-T cells express CD3e-BB ζ , so that CAB-T can rely on endogenous TCR activation, furthermore CD3e-BB ζ can enhance the activation level of CAB-T cells, and in turn promotes CAB-T to release more BiTA, and these effects reinforce each other. Therefore, in theory, CAB-T should have a stronger killing effect on tumor cells than BiTA-T.

In the first group of experiments, in order to detect the killing effect of CAIX-CAB-T cells on CAIX⁺ HEK 293T target cells, we chose CAIX-CAB-T cells as effector cells of the experimental group for killing effect detection. The effector cells were co-cultured with the target cells for 24 and 48 h at an effector-target ratio of 0:1, 1:1, 5:1, 10:1, and 20:1, respectively, and the supernatant was taken to determine the ability of T cells to kill target cells at different effector-target ratios. It can be seen from the results of FIG. 16 that 1st-CAIX-CAB-T cells and their control group T cells had no killing effect on CAIX- HEK293T; while 1st-BiTA-T cells, 1st-CAIX-CAB-T, CAIX-TRuC-T, and mixed T cells of CD3e-BB ζ -T and 1st-BiTA-T showed different degrees of killing ability on CAIX⁺ HEK 293T cells, and this killing ability increased with the increase of the effector-target ratio. Moreover, the killing on target cells by CAIX-CAB-T and its control group T cells became more significant with the prolongation of co-culture time, and the killing effect of co-culture for 48 h was significantly stronger than that of co-culture for 24 h. The CD3e-BB ζ -T and non-engineered T cell control groups showed no killing effect on CAIX⁺ HEK 293T cells. In addition, 1st-CAIX-CAB-T, CAIX-TRuC-T, and mixed T cells of CD3e-BB ζ -T and 1st-BiTA-T had comparable killing ability on CAIX⁺ HEK 293T cells and were superior to 1st-BiTA-T in killing ability. From this, we can determine that

the killing ability of CAIX-CAB-T on tumor cells depended on the expression of its target antigen, and its killing ability was comparable to that of the control group CAIX-TRuC-T cells. In addition, BiTA-T and CD3e-BBζ-T also showed synergistic effects on target cells killing.

The method for the second group of experiments was identical to that of the first group of experiments. We detected the killing ability of CAIX-CAB-T and its control cells on CAIX⁺MB-231 or CAIX-MB-231 tumor cells. The effector cells were co-cultured with the target cells for 36 h at an effector-target ratio of 0:1, 1:1, 5:1, 10:1, and 20:1, respectively, and the supernatant was taken to determine the ability of T cells to kill target cells at different effector-target ratios. It can be seen from the results shown in FIG. 17 that CAIX-CAB-T and its control cells had no killing effect on CAIX-MB-231 control tumor cells, and CAIX-CAB-T and first and second generation CAR-T cells targeting CAIX exhibited comparable killing ability on CAIX⁺MB-231 cells. At the same time, tERBB2-T and CD3e-BBζ-T control group cells had no killing ability on CAIX⁺MB-231 cells. CAIX-CAB-T was shown to have comparable killing ability on tumor cells as first and second generation CAR-T, and this killing ability was target antigen-dependent. It should be noted that CAIX-CAB-T and CAIX-BiTA-T did not show differences in killing ability on target cells in this group of experiments due to higher transduction or different donor cell sources or the like.

The third group of experiments was identical to the first and second groups of experiments. We detected the ability of HER2-CAB-T and its control cells to kill HER2 positive tumor cells SKBR3 or HER2 negative tumor cells RAJI. The effector cells were co-cultured with the target cells for 36 h at an effector-target ratio of 0:1, 1:1, 5:1, 10:1, and 20:1, respectively, and the supernatant was taken to determine the ability of T cells to kill target cells at different effector-target ratios. It can be seen from the results shown in FIG. 18 that HER2-CAB-T and its control cells had no killing effect on HER2-negative RAJI cells; and HER2-CAB-T and HER2-targeted first and second generation CAR-T cells exhibited comparable killing ability on SKBR3. At the same time, tERBB2-T and CD3e-BBζ-T control cells had no killing ability on SKBR3 cells. HER2-CAB-T was shown to have comparable killing ability on tumor cells as first and second generation CAR-T, and this killing ability was target antigen-dependent.

Example 10 *In vitro* activity comparison of different CAB structures with BiTA and second generation CAR

Due to the different expression levels of CD3e-BBζ and BiTA in cells, we attempted to analyze the differences between different structures of CAB and BiTA and second generation

CAR. We first designed a group of CAB and its control structures as shown in FIG. 19, including: tERBB2, HER2-BiTA, HER2-CAB, HER2-CAB^R (DNA SEQ ID NO.: SEQ 67, AA SEQ ID NO.: SEQ 68) and HER2-BBζ. The lentivirus was packaged according to the method described in Example 2 using the vectors carrying the above structures and the T cells were infected according to the method described in Example 3. The positive frequency of the infected cells was detected according to the method described in Example 4, and the detection results were shown in FIG 20. The positive frequencies of engineered T cells were basically equal.

To identify the in vitro activation ability of the engineered T cells, according to the method described in Example 5, after the engineered T cells were co-cultured with HER2-negative RAJI cells and HER2-positive SKBR3 cells, respectively, release levels of cytokines IL-2 and IFNγ were detected. The results were shown in FIG. 21. The levels of cytokine release after activation of HER2-CAB-T and HER2-CAB^R-T cells were basically equal.

To identify differences in the in vitro killing ability of the engineered T cells, we detected the in vitro killing ability of each of the engineered T cells on RAJI and SKBR3 cells according to the method described in Example 9. Compared with the HER2-CAB structure, since the HER2-CAB^R structure had BiTA in the front and the CD3e-BBζ structure in the back, theoretically, HER2-CAB^R-T can secrete higher levels of BiTA. We speculated that HER2-CAB^R-T had better in vitro killing ability than HER2-CAB-T cells. As shown in FIG. 22, the results were consistent with our hypothesis, namely, HER2-CAB^R-T demonstrated superior in vitro killing ability over HER2-CAB-T cells.

Example 11 *In vivo* efficacy of CAIX CAB-T

The anti-tumor activity of CAB-T in mouse tumor models is the key basis for judging the potential clinical efficacy of CAB-T. To verify the anti-tumor activity of CAB-T in a mouse tumor model, the following validation experiments were performed.

A specific experimental procedure was as follows.

CAIX⁺MDA-MB-231 cell expansion culture and inoculation

1) Enough CAIX⁺MDA-MB-231 cells were cultured and expanded in vitro, the cells were collected after trypsin digestion, washed with PBS for 3 times and counted, the cell density was adjusted to 15x10⁶ cells/ml with a 80% RPMI-1640 basic medium containing 20% Matrigel, the cells were placed into a 50ml centrifuge tube, the opening of the centrifuge tube

was tightly covered and sealed with a sealing film, and the centrifuge tube was passed into an SPF grade animal room through a transfer window.

2) 68 female 8-week-old NCG severe immunodeficient mice purchased in advance were fed adaptively for 1 week, and then the hair at right abdomen of each mouse was removed with a razor. CAIX+MDA-MB-231 cells with a density of 15×10^6 cells/ml were dissociated and thoroughly mixed with a 1 ml pipette, and 0.2 ml of cells were subcutaneously inoculated with a 1 ml syringe to the right abdomen of each NCG mouse, namely, each NCG mouse was inoculated with 3×10^6 CAIX+MDA-MB-231 cells, and the cells were observed daily for subcutaneous tumor formation in NCG mice, and each NCG mouse was numbered 6 days after inoculation using an ear tag with a number;

Grouping, administration, and measurement of CAIX+MDA-MB-231 tumor-bearing mice

3) 10 days after inoculation, the maximum broad axis W and the maximum long axis L of the subcutaneous tumor at the right abdomen of each NCG mouse were measured using a vernier caliper, and the body weight of each mouse was weighed using an electronic balance. The subcutaneous tumor volume at the right abdomen of each NCG mouse was calculated according to the tumor volume $T = 1/2 \times W \times W \times L$. The mice with oversized and undersized tumors were excluded, and NCG mice were averagely divided into 11 groups according to the average tumor volume with 6 mice in each group;

4) The grouping was performed according to the following administration scheme for each group and the corresponding reagents or cells were injected. In hCAIX-BiTA (DNA SEQ ID NO.: SEQ 71, AA SEQ ID NO.: SEQ 72), hCAIX-CAB (DNA SEQ ID NO.: SEQ 73, AA SEQ ID NO.: SEQ 74), and hCAIX-BB ζ (DNA SEQ ID NO.: SEQ 75, AA SEQ ID NO.: SEQ 76) structures for engineering T cells, the antibody sequences for recognizing CAIX are all derived from the humanized sequence of the VHH amino acid sequence SEQ ID NO. 18.

Administration scheme for each group

Group	Testing sample	Dosage of administration	Times of administration	Mode of administration
PBS	PBS	N/A	Once on Day 10 and Once on Day 13	Tail vein injection

NT cell 2.5x10 ⁶ /mouse	NT	2.5x10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection
hCAIX-BiTA-T cell 2.5x10 ⁶ /mouse	hCAIX-BiTA-T	2.5x10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection
hCAIX -BiTA-T cell 0.75x10 ⁶ /mouse	hCAIX -BiTA-T	0.75x10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection
hCAIX -BiTA-T cell 0.25x10 ⁶ /mouse	hCAIX -BiTA-T	0.25 x 10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection
hCAIX -CAB-T cell 2.5x10 ⁶ /mouse	hCAIX -CAB-T	2.5 x 10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection
hCAIX -CAB-T cell 0.75x10 ⁶ /mouse	hCAIX -CAB-T	0.75 x 10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection
hCAIX -CAB-T cell 0.25x10 ⁶ /mouse	hCAIX -CAB-T	0.25 x 10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection
hCAIX -BBζ-T cell 2.5x10 ⁶ /mouse	hCAIX -BBζ-T	2.5 x 10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection
hCAIX -BBζ-T cell 0.75x10 ⁶ /mouse	hCAIX -BBζ-T	0.75 x 10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection
hCAIX -BBζ-T cell 0.25x10 ⁶ /mouse	hCAIX -BBζ-T	0.25 x 10 ⁶ /mouse	Once on Day 10 and Once on Day 13	Tail vein injection

5) Tumor volume and body weight of the mice were measured twice a week. The body weight and tumor volume of the mice were measured for the last time 37 days after inoculation of the tumor cells. After the mice were euthanized, the tumors of each mouse were dissected, the tumor weight was weighed, and the tumors were photographed.

Results and discussion

The tumor growth curve, tumor picture and tumor weight of each group of mice are shown in the figures, the tumors of mice in the PBS group and the NT group increased rapidly with the prolongation of the inoculation time, indicating that the CDX transplantation model was successfully established in this experiment; compared with the PBS group and NT group, the

hCAIX-BiTA-T cell group and the hCAIX-BBζ-T cell group showed the inhibition effect on tumor growth only at 2.5×10^6 /mouse, while the hCAIX-CAB-T cell group had certain effects for three dose groups and these effects were dose-dependent, in which all tumors in the 2.5×10^6 /mouse group regressed, and 5 mice in the 0.75×10^6 /mouse group had tumor regression; the hCAIX-CAB-T cell group was significantly superior to the hCAIX-BiTA-T cell group and the hCAIX-BBζ-T cell group at the same dose. The result trends of tumor growth curve, tumor picture, and tumor weights of each group were consistent (FIG. 23A, C, and D). There was no significant decrease in body weight of the mice in each group throughout the experimental period (FIG. 23B), indicating the safety of each test sample.

Example 12 *In vivo* efficacy of HER2 CAB-T

The anti-tumor activity of HER2 CAB-T was verified in M-NSG immune-deficient mice tumor model by the following experiments.

NCI-N87 cell expansion culture and inoculation

1) NCI-N87 cells were cultured and expanded in vitro, the cells were collected after trypsin digestion, washed with PBS for 3 times and counted, the cell density was adjusted to 10×10^6 cells/ml with a 80% RPMI-1640 basic medium containing 20% Matrigel, the cells were placed into a 50ml centrifuge tube, the opening of the centrifuge tube was tightly covered and sealed with a sealing film, and the centrifuge tube was passed into an SPF grade animal room through a transfer window.

2) 32 female 8-week-old NSG severe immuno-deficient mice were fed adaptively, and the hair at right abdomen of each mouse was removed with a razor. NCI-N87 cells with a density of 10×10^6 cells/ml were dissociated and thoroughly mixed with a 1 ml pipette, and 0.2 ml of cells were subcutaneously inoculated with a 1 ml syringe to the right abdomen of each NSG mouse, namely, each NSG mouse was inoculated with 3×10^6 NCI-N87 cells, and the cells were observed daily for subcutaneous tumor formation in NSG mice, and each NSG mouse was numbered 6 days after inoculation using an ear tag with a number.

Grouping, administration, and measurement of NCI-N87 tumor-bearing mice

3) 6 days after inoculation, the maximum broad axis W and the maximum long axis L of the subcutaneous tumor at the right abdomen of each NSG mouse were measured using a vernier caliper, and the body weight of each mouse was weighed using an electronic balance. The subcutaneous tumor volume at the right abdomen of each NCG mouse was calculated

according to the tumor volume $T = 1/2 \times W \times W \times L$. The mice with oversized and undersized tumors were excluded, and NSG mice were averagely divided into 4 groups according to the average tumor volume with 6 mice in each group;

4) The grouping was performed according to the following administration scheme for each group and the corresponding reagents or cells were injected. In NT (DNA SEQ ID NO.: SEQ 33, AA SEQ ID NO.: SEQ 34), HER2 CAB^R-T (DNA SEQ ID NO.: SEQ 67, AA SEQ ID NO.: SEQ 68), HER2 CAB-T (DNA SEQ ID NO.: SEQ 49, AA SEQ ID NO.: SEQ 50), and HER2 CAR-T structures for engineering T cells, the scFv antibody sequences for recognizing HER2 are all derived from Trastuzumab amino acid sequence SEQ ID NO. 66.

Administration scheme for each group

Group	Day 0 Dosage of administration	Day 2 Dosage of administration	Mode of administration
NT cell	5.0x10 ⁶ /mouse	3.0 x10 ⁶ /mouse	Tail vein injection
HER2 CAB ^R -T	5.0x10 ⁶ /mouse	3.0 x 10 ⁶ /mouse	Tail vein injection
HER2 CAB-T	5.0x10 ⁶ /mouse	3.0 x 10 ⁶ /mouse	Tail vein injection
HER2 CAR-T	5.0x10 ⁶ /mouse	3.0 x 10 ⁶ /mouse	Tail vein injection

5) Tumor volume and body weight of the mice were measured twice a week. The body weight and tumor volume of the mice were measured for the last time 48 days after inoculation of the tumor cells. After the mice were euthanized, the tumors of each mouse were dissected, the tumor weight was weighed, and the tumors were photographed.

Results and discussion

The tumor growth curve and tumor picture of each group of mice are shown in the figures, the tumors of mice in the NT group increased rapidly with the prolongation of the inoculation time, indicating that the CDX transplantation model was successfully established in this experiment; compared with the NT group, all other 3 engineered T cell treated group showed certain tumor growth inhibition. In contrast to the mild tumor growth inhibition and 2 tumor-free mice in HER2 CAR-T treated group, both HER2-CAB^R-T and HER2-CAB-T showed a much better tumor growth inhibition, and with 5 tumor-free mice and 2 tumor-free mice respectively.

The result trends of tumor growth curve, tumor picture, and tumor weights of each group were consistent (FIG. 24A, C, and D). There was no significant decrease in body weight of the mice in each group throughout the experimental period, indicating that each test sample had

good safety.

Example 13 Dasatinib works as a safety-switch and inhibits cytokine release of CAB-T

As a live cell therapy, CAB-T may lead to cytokine release syndrome (CRS) and on-target, off-tumor toxicities etc. and clinical management of CRS includes the anti-IL-6R agonist Tocilizumab and steroids treatment. Here, we developed the method to manage potential toxicities using Dasatinib.

Dasatinib has been developed as an inhibitor of BCR-ABL fusion protein, and is clinically approved for chronic myelogenous leukemia and acute lymphoblastic leukemia treatment. In addition, dasatinib was also reported can blocks the lymphocyte-specific protein tyrosine kinase (LCK) and thereby inhibits phosphorylation of CD3 ζ and ZAP70, thereby ablating signaling in CAR and TCR signaling. The following example demonstrate that dasatinib is able to inhibit cytokine release of CAB-T dramatically.

1×10^5 effector cells (CAIX CAB-T or HER2 CAB-T) and 1×10^5 target cells (CAIX+MDA-MB231 or SKBR3) were seeded at 200 μ L/well with a gradient concentration of dasatinib (100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 0nM) in 96-well cell culture plate, the plate were centrifuged at 300 g for 5 min after co-cultured overnight, and then 150 μ L of the supernatant/well was pipetted into a new 96-well cell culture plate using a multichannel pipette, and cytokine detections were performed as showed in Example 5.

Result and discussion

As shown in FIG 25A, 25B and FIG 26A, 26B, dasatinib inhibited IFN γ and IL-2 secretion effectively in both activated CAIX CAB-T and HER2 CAB-T at a low dose around 25 nM. The result imply that dasatinib could be used as a safety-switch to control the potential CRS syndrome and on-target, off-tumor toxicity of CAB-T in clinical treatment.

Example 14 Dasatinib works as a safety-switch and inhibits killing activity of CAB-T

As described in Example 13, T cell therapy has on-target, off-tumor toxicity potential issues clinically. To manage the potential toxicity of CAB-T, inhibition of the killing activity of CAB-T was evaluated using dasatinib.

We used LDH cytotoxicity assay method to detect the killing inhibition capability of dasatinib. The detailed protocol was described in Example 9. Briefly, 5×10^4 effector cells (CAIX CAB-T or HER2 CAB-T) and 1×10^4 target cells (CAIX+MDA-MB231 or SKBR3) were co-cultured with a gradient concentration of dasatinib (100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 0nM), Cytotoxicity of CAB-T to its target cells were determined in 2-hour intervals over a period of 12 hours [effector-to-target cell (E:T) ratio, 5:1].

Result and discussion

As shown in FIG 25C and FIG 26C, dasatinib inhibited cytotoxicity of CAIX CAB-T and HER2 CAB-T effectively to their target cells at a low dose around 50 nM. The result imply that dasatinib could be used as a safety-with to control the potential on-target, off-tumor toxicity of CAB-T in clinical treatment.

Summary and discussion:

The CAB-T technology utilizes BiTA secreted by itself to simultaneously recognize chimeric CD3 or tumor antigen and endogenous CD3 in T cells, and thereafter induces tumor antigen-dependent endogenous TCR activation and chimeric CD3 activation. Both activations of endogenous TCR complexes and chimeric CD3 are dependent on the level of expression and secretion of BiTA by CAB-T cells. Thus, BiTA can induce CAB-T cells by the way of autocrine and induce non-engineered T cell activation in the tumor microenvironment by the way of paracrine; meanwhile, CAB-T cells can release higher levels of BiTA in tumor tissues after activation in tumor tissues, thereby mobilizing the activation and anti-tumor effects of more non-engineered T cells in tumor tissues.

Therefore, compared with TRuC-T and TAC-T, CAB-T does not only have the advantage of activating endogenous TCR signals, but also mobilizes the anti-tumor activity of infiltrating T cells in tumor tissues, and theoretically has better therapeutic potential for solid tumors. In addition, unlike BiTE drugs, BiTA drugs that are continuously secreted by CAB-T cells solve the clinical application problem of short half-life of the single drug of BiTE. In addition, since BiTA targeting the target antigen will exert the maximum effect in the tumor microenvironment where the CAB-T cells reach, and will not be enriched at a high concentration in a non-tumor tissue site, it has better safety and greater clinical application potential, compared with the systemic administration of the single drug of BiTE.

The mechanism of action and clinical application potential of CAB-T are stated as follows.

- 1) In tumor tissues, BiTA, which is underexpressed in CAB-T cells, can bind to its own endogenous TCR complex or CD3e-BBζ in an autocrine fashion (FIG. 27B), thereby stimulating CAB-T to release more BiTA to achieve a local activation loop (FIG. 27C). This allows CAB-T to achieve the maximum activation levels at the local tumor site, exerting its greatest anti-tumor effect, and achieving safety and effectiveness similar to the local administration of drugs at the tumor site. It is anticipated that CD3e-BBζ would form heterodimers with endogenous CDδ or CD3γ chains on T cell membranes, and hence further enhancing CD3-based signaling.
- 2) Binding and activation of chimeric CD3e in CAB-T by autocrine BiTA (FIG. 27A) confers sensitivity-enhanced proliferation and activation capacity on CAB-T compared to non-engineered T cells, further enhancing antitumor activity of CAB-T.
- 3) Activation of CAB-T (FIG. 27 A, B, and C) and non-engineered T cells (FIG. 27D) by autocrine and paracrine of BiTA, respectively, solves the problem where CAR-T cells cannot activate endogenous TCR signals, conferring a potential for treating solid tumors with CAB-T cells.
- 4) CAB-T can be used as a drug synthesis plant for BiTA, which solves the problem of the short in vivo half-life of BiTA; the activation of CAB-T depends on the release level of BiTA, and the two rely on each other and cooperate with each other to collectively determine the safety and effectiveness of CAB-T for clinical application.

The schematic diagram of the mechanism of action of CAB-T is shown in FIG. 27.

All documents mentioned in the present disclosure are cited as references herein, as if each document is cited separately as a reference. In addition, it should be understood that various modifications or alterations of the present disclosure can be made by those skilled in the art after having read the above teachings of the present disclosure, and these equivalent forms also fall within the scope defined by the claims appended hereto.

CLAIMS

1. A nucleic acid molecule encoding a chimeric CD3 fusion protein and a bispecific T cell activating element.
2. The nucleic acid molecule according to claim 1, wherein the chimeric CD3 fusion protein comprises of one or more polypeptide(s) recognizable by an anti-CD3 antibody, and optionally one or more of the following domains: hinge or linker regions, transmembrane domains (TM), co-stimulatory domains and CD3 signal activation domains.
3. The nucleic acid molecule according to claim 1, the bispecific T cell activating element is a fusion protein which comprises of one or more tumor antigen recognition region(s) and one or more CD3 antigen-binding antibody fragment(s).
4. The nucleic acid molecule according to claim 1, wherein the fusion protein has a structure shown in the following formula I:



wherein in the formula,

L is absent or a signal peptide sequence;

EC is a polypeptide binding domain recognizable by an anti-CD3 antibody and binds to the anti-CD3 antibody;

H is absent or a linker or a hinge region;

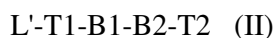
TM is a transmembrane domain;

C is absent or a costimulatory signaling molecule;

CD3 ζ is absent or a cytoplasmic signaling sequence derived from CD3 ζ ;

each "-" is independently a linker peptide or a peptide bond.

5. The nucleic acid molecule according to claim 1, wherein the bispecific T cell activating element has a structure shown in the following formula II: :



wherein:

L' is absent or a signal peptide sequence;

T1 is absent or a tag element;

B1 is a tumor antigen recognition region or a CD3 antigen-binding antibody fragment;
B2 is a CD3 antigen-binding antibody fragment or a tumor antigen recognition region; T2 is absent or a tag element;

each "-" is independently a linker peptide or peptide bond.

6. The nucleic acid molecule according to claim 4, wherein EC is a polypeptide from or derived from CD3e.

7. The nucleic acid molecule according to claim 6, wherein EC comprises position 1 to 104 of a CD3e protein, having an amino acid sequence as set forth by SEQ ID NO: 4 and/or a nucleotide sequence as set forth by SEQ ID NO.: 3.

8. The nucleic acid molecule according to claim 4, wherein C when present is a costimulatory signal molecule of a protein selected from the following group: OX40, CD2, CD7, CD27, CD28, CD30, CD40, CD70, CD134, 4-1BB (CD137), PD1, Dap10, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), NKG2D, GITR, TLR2, or a combination thereof.

9. The nucleic acid molecule according to claim 4, wherein C when present comprises a 4-1BB-derived costimulatory signal molecule having an amino acid sequence as set forth by SEQ ID NO: 10 and/or a nucleotide sequence as set forth by SEQ ID NO.: 9, and/or a CD28-derived costimulatory signal molecule having an amino acid sequence as set forth by SEQ ID NO: 62 and/or a nucleotide sequence as set forth by SEQ ID NO.: 61.

10. The nucleic acid molecule according to claim 4, wherein CD3 ζ when present is a cytoplasmic signaling sequence having an amino acid sequence as set forth by SEQ ID NO: 12 and/or a nucleotide sequence as set forth by SEQ ID NO.: 11.

11. The nucleic acid molecule according to claim 1, wherein the construct is co-expressed with polypeptides from or derived from Caspase 9 (iCasp9), CD19, CD20, EGFR, HER2, CD30, CD19, c-Met, Claudin 18.2, or a combination thereof.

12. The nucleic acid molecule according to claim 5, wherein B1 is a tumor antigen recognition region and B2 is a CD3 antigen recognition region.

13. The nucleic acid molecule according to claim 5, wherein the tumor antigen recognition region comprises of receptor or ligand binding domain, an antibody fragment, and/or a T-cell receptor (TCR) sequence.

14. The nucleic acid molecule according to claim 5, wherein the tumor antigen recognition

region targets tumor antigen CAIX and/or HER2.

15. The nucleic acid molecule according to claim 5, wherein the tumor antigen recognition region is an antibody fragment, and having an amino acid sequence as set forth by SEQ ID NO: 18 and/or a nucleotide sequence as set forth by SEQ ID NO.: 17.

16. The nucleic acid molecule according to claim 5, wherein the tumor antigen recognition region is an antibody fragment, targeting HER2 and having an amino acid sequence as set forth by SEQ ID NO: 66 and/or a nucleotide sequence as set forth by SEQ ID NO.: 65.

17. The nucleic acid molecule according to claim 5, wherein the CD3 antigen- binding antibody fragment(s) comprises of a single domain antibody sequence (VHH), a single-chain antibody variable region sequence (scFv), and/or an antigen-binding fragment (Fab) that targets CD3.

18. The nucleic acid molecule according to claim 5, wherein the CD3 antigen-binding antibody fragment is derived from anti-CD3 Ab clones of L2K, UCHT, OKT3, F6A, SP34 etc.

19. The nucleic acid molecule according to any one of the preceding claims, comprising the sequence as set forth by SEQ ID NO.: 73.

20. The nucleic acid molecule according to any one of the preceding claims, comprising the sequence as set forth by SEQ ID. NO.: 67.

21. The nucleic acid molecule according to claim 1, wherein the nucleic acid molecule encoding the chimeric CD3 fusion protein and the nucleic acid molecule encoding the bispecific T cell activating element are encoded by separate nucleic acid molecules.

22. The nucleic acid molecule according to claim 21, wherein the nucleic acid molecule encoding the chimeric CD3 fusion protein and the nucleic acid molecule encoding the bispecific T cell activating element are co-expressed in the same immune cell.

23. A vector, wherein the vector comprises the nucleic acid molecule according to any one of the preceding claims.

24. A genetically engineered immune cell, wherein the immune cell expresses the nucleic acid molecule according to any one of the preceding claims.

25. The genetically engineered immune cell according to claim 24, wherein the immune cell is T cell.

26. The genetically engineered immune cell according to claim 25, wherein the immune

cell is engineered to express the chimeric CD3 fusion protein and the bispecific T cell activating element, whereby the nucleic acid molecule encoding chimeric CD3 fusion protein and the nucleic acid molecule encoding the bispecific T cell activating element are not provided on the same DNA construct.

27. A non-naturally occurring T cell population, comprising the genetically engineered immune cell according to claim 25, wherein the T cells are present in the T cell population at a ratio C1 of 10% or more, based on the total number of T cells in the T cell population.

28. A composition, comprising: (a) the genetically engineered immune cell according to claim 25 and/or the T cell population according to claim 27, and (b) a pharmaceutically acceptable carrier, diluent and/or excipient.

29. Use of the genetically engineered immune cell according to claim 25 and/or the T cell population according to claim 27, in the preparation of a medicament for the prevention and/or treatment of cancer or tumor.

30. The use according to claim 29, wherein the tumor is selected from the following group: a hematological tumor, a solid tumor, or a combination thereof.

31. The use according to claim 29, wherein the medicament is used in combination with dasatinib.

32. Use of dasatinib in the preparation of a medicament for reducing the toxicity of an immune cell engineered with a chimeric CD3 fusion protein and a bispecific T cell activating element.