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(54) CHIMERIC ANTIGEN RECEPTOR COMPRISING CO-STIMULATORY RECEPTOR AND APPLICATION THEREOF

(57) Provided by the present invention is a chimeric antigen receptor comprising a co-stimulatory receptor, the chimeric antigen receptor having a structure of scFv(X)-(Y)CD3zeta-2A-(Z); X comprises a tumortargeting antibody or a ligand or receptor capable of specifically binding to a tumor; Y is an intracellular region of the co-stimulatory receptor, and Z is a co-stimulatory receptor that is selected from among ICOS, CD28, CD27, HVEM, LIGHT, CD40L, 4-1BB, OX40, DR3, GITR, CD30, TIM1, SLAM, CD2, CD226. Further provided by the present invention are CAR-T cells that are constructed by means of a recombinant expression vector of the described chimeric antigen receptor, a preparation method therefor and an application thereof. The CAR-T cells described in the present invention significantly improve the tumor-killing abilities and amplification abilities thereof.

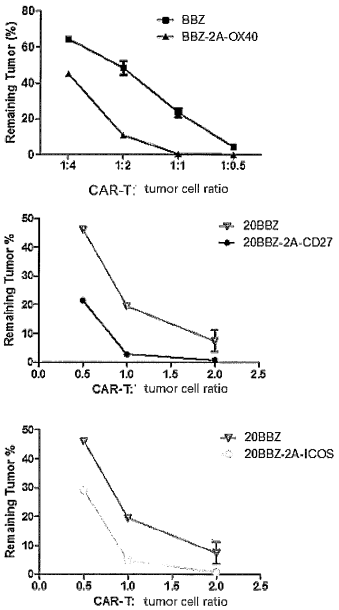

FIG. 13

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Description

Technical Field

[0001] The present invention relates to the field of cellular immunotherapeutic technology, especially relates to a chimeric antigen receptor comprising a co-stimulatory receptor and use thereof.

Background of the Invention

[0002] The use of immunological therapy for overcoming tumors has always been an important direction in the application of immunology in translational medicine. With the development of various omics (genomics, proteomics, etc.), tumor cells have been widely recognized due to their immunogenicity caused by mutations, which lays a theoretical foundation for tumor immunotherapy. At the same time, with the accumulation of tumor immunology research itself, tumor immunotherapy has recently made a great progress, and a series of new immunotherapy methods have gradually entered into the clinic. The current tumor immunology research has established the central position of T cell killing in tumor immunotherapy, and the chimeric antigen receptor T cell (CAR-T cell) is a tumor-killing cell which has combined the targeted recognition of antibody and the tumor-killing function of T cell, and been generated by artificial modification.

[0003] The concept of chimeric antigen receptor T cell was first proposed by Gross, Waks and Eshhar in 1989. They expressed TNP-recognizing antibodies on T cells, achieving antigen-specific, non-MHC-restricted T cell activation and enhanced effect, and proposed the concept of the application of CAR-T technology in tumor treatment. According to this principle, tumor-specific antibodies are embedded into T cells, which will give T cells new tumor-killing capabilities. After that, CAR-T technology was introduced into anti-tumor clinical trials, but the final clinical results of early CAR-T cells are not ideal since their intracellular signal transmission domain contains only the first signal, and the selected tumor type is a solid tumor. In 2008, the Fred Hutchison Cancer Institute and other institutions used CAR-T to treat B cell lymphoma, although the treatment results are not ideal, the key to this clinical trial is to demonstrate that CAR-T treatment with CD20-expressing B cells as the target is relatively safe. Subsequently, in 2010, NCI reported a case of successful treatment of B-cell lymphoma, using CAR-T targeting CD19, the patient's lymphoma was controlled, normal B cells were also eliminated, and serum Ig was significantly reduced, providing a theoretical and practical support for the effectiveness of CAR-T in the treatment of B cell-derived lymphomas. In 2011, a team led by Dr. Carl June of the University of Pennsylvania in the United States used CAR-T that specifically recognizes CD19 for the treatment of chronic lymphocytic leukemia derived from B cells, showing a "cure" effect. After that, clinical trials have also been launched in relapsed and refractory acute lymphoblastic cell leukemia, and good results have also been achieved. Due to this breakthrough progress and the development of other immune regulation methods, Science magazine ranked tumor immunotherapy as the number one scientific and technological breakthrough in 2013. This success has caused widespread influence in countries around the world, and countries have begun to carry out a large number of CAR-T-based scientific research and clinical trials of tumor treatment.

[0004] The structure of CAR consists of an extracellular antigen recognition domain, an extracellular hinge region, a transmembrane domain, and an intracellular signal transduction domain. The extracellular antigen recognition domain generally consists of a single-chain antibody, which specifically recognizes membrane surface molecules of the tumor cell, or can be a ligand or receptor of certain tumor-specific antigens, etc. The extracellular hinge region is a spatial structure that separates the antigen recognition domain from the transmembrane domain, and its purpose is to provide a suitable spatial position, so that the extracellular antigen recognition domain can maintain the correct structure and transmit the intracellular signals before and after recognizing the antigen. The transmembrane domain is a domain for ensuring the positioning of the CAR molecule on the membrane surface. The intracellular signal transduction domain is a key part of mediating the CAR signal transduction, and is usually a combination of one or several first signals (for the recognition of TCR and MHC-I-peptide complex) and second signals (for the recognition of costimulatory receptor and costimulatory ligand). The first-generation CAR contains only the first signal, the second-generation CAR has one first signal and one second signal, and the third-generation CAR has one first signal and two second signal domains. Although CAR-T has achieved a great success in the treatment of leukemia derived from B cell, its relatively high recurrence rate and low effectiveness for solid tumors are important challenges currently. Therefore, there is an urgent clinic need of developing a new generation of high-efficiency CAR-T currently. In addition to the third-generation CAR-T, there are currently other new CAR-T design strategies, that is, new regulatory molecules independent of CAR are introduced on the basis of the second-generation CAR-T to further enhance the function of CAR-T.

[0005] The application of CAR-T targeting the B cell surface targeting molecules CD19 and CD20 prepared from the patient's own blood cells in the treatment of B cell leukemia has been relatively mature, but there are a large number of recurrences, even though the response rate is high. In addition, the treatment efficiency for solid lymphoma is relatively low, which is related to the immunosuppressive microenvironment in solid tumors.

[0006] In solid tumors, there are a variety of immune cells, tumor cells and stromal cells, which together constitute the

tumor microenvironment. The tumor microenvironment is usually immunosuppressive, and can inhibit endogenous anti-tumor T cell responses or adoptive T cells (such as CAR-T) at multiple levels, for example, leading to exhaustion of T cells and loss of tumor killing function, and eventually leading to the clearance of T cells. How to enhance the activation ability of CAR-T in solid tumors so that CAR-T can fight against the immune suppression in the tumor microenvironment is an important idea and direction for expanding CAR-T to solid tumor treatment.

[0007] However, the current CAR-T domains in clinical use still have insufficient tumor killing and expansion abilities, and have poor efficacy in controlling solid tumors/metastasis. Some CAR-T use novel regulatory molecules such as IL-12, 4 - 1BBL, etc. These molecules will also produce non-specific activation effects on other non-CAR-T cells in addition to affecting the CAR-T, which may cause immune side effects.

Summary of the Invention

[0008] An object of the present invention is to address the defects in the prior art, provide a chimeric antigen receptor including a co-stimulatory receptor and use thereof, and provide a CAR-T cell constructed by a recombinant expression vector of the chimeric antigen receptor. For example, OX40 is an important co-stimulatory receptor which is primarily expressed in activated CD4 and CD8 T cells, and displays a variety of functions during the activation of T cells. They can promote the activation of T cells, exhibit more effector molecules, and reduce the expression of gene associated with apoptosis. Integrating the co-stimulatory receptor signal into the CAR-T has a potential effect-enhancing function.

[0009] To address the aforesaid object, the present invention utilizes the following technical solutions:

a first object of the present invention is to provide a chimeric antigen receptor including a co-stimulatory receptor and having a structure of scFv(X)-(Y)CD3zeta-2A-(Z); wherein X is a tumor-targeting antibody or a ligand or receptor capable of specifically binding to a tumor; Y is an intracellular domain of a co-stimulatory receptor, and said co-stimulatory receptor is selected from ICOS, CD28, CD27, HVEM, LIGHT, CD40L, 4-1BB, OX40, DR3, GITR, CD30, TIM1, SLAM, CD2, CD226; Z is a co-stimulatory receptor, and said co-stimulatory receptor is selected from ICOS, CD28, CD27, HVEM, LIGHT, CD40L, 4-1BB, OX40, DR3, GITR, CD30, TIM1, SLAM, CD2, CD226.

[0010] For further optimizing the aforesaid chimeric antigen receptor, the technical means used in the present invention further includes:

Further, the X is selected from anti-CD 19 antibody, anti-CD20 antibody, EGFR antibody, HER2 antibody, EGFRVIII antibody, anti-PSMA antibody, anti-BCMA antibody, anti-CD22 antibody, anti-CD30 antibody. Understandably, X can also be other protein capable of specifically binding to a tumor.

Further, said X is anti-CD20 antibody, said Y is 4-1BB, said Z is one selected from OX40, HVEM, ICOS, CD27, 4-1BB.

Further, said scFv(X)-(Y)CD3zeta is scFv-antihCD20-20BBZ having a sequence of SEQ ID No. 1; said OX40 has a sequence of SEQ ID No.2; said HVEM has a sequence of SEQ ID No.3; said ICOS has a sequence of SEQ ID No.4; said CD27 has a sequence of SEQ ID No.5; said 4-1BB has a sequence of SEQ ID No.6; and said 2A has a sequence of SEQ ID No.7, SEQ ID No.8, SEQ ID No.9 or SEQ ID No. 10.

[0011] Wherein the aforesaid sequences are as follows:

SEQ ID No.1:

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATS
NLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEI
KGGGGSGGGGSGGGGSQVQLQQPGAELVKPGASVKMSCKASGYTFTSYNM
HWVKQTPGRGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSL
TSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVSAAAATTTPAPRPPTPAPT
IASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYC
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAP
AYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL
QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR ;

SEQ ID No.2:

MCVGARRLGRGPCAALLLLGLGLSTVTGLHCVGDTYPSNDRCCHECRP
GNGMVSRCSRSQNTVCRPCGPGFYNDVVSSKPCKPCTWCNLRSGSERKQLCT
ATQDTVCRCRAGTQPLDSYKPGVDCAPCPPGHFSPGDNQACKPWTNCTLAG
KHTLQPASNSSDAICEDRDPPATQPQETQGPPARPITVQPTEAWPRTSQGPSTRP
VEVPGGRAVAAILGLGLVLGLLGPLAILLALYLLRRDQRLPPDAHKPPGGGSFR
TPIQEEQADAHSTLAKI;

SEQ ID No.3:

MEPPGDWGPPPWRSTPKTDVLRLVLYLTFLGAPCYAPALPSCKEDEYPVG
SECCPKCSPGYRVKEACGELTGTVCEPCPPGTYIAHLNGLSKCLQCQMCDPA
MGLRASRNCSRTENAVCGCSPGHFCIVQDGDHCAACRAYATSSPGQRVQKGG
TESQDTLCQNCPPGTFSPNGTLEECQHQTKCSWLVTKAGAGTSSSHWVWWF
LSGSLVIVIVCSTVGLIICVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQ
APPDVTTVAVEETIPSFTGRSPNH;

SEQ ID No.4:

MKSGLWYFFLFCLRIKVLTGEINGSANYEMFIFHNGGVQILCKYPDIVQQ
FKMQLLKGGQILCDLTKTKGSGNTVSIKSLKFCHSQLSNNSVSFFLYNLDHSH
ANYYFCNLSIFDPPPFKVTLTGGYLHIYESQLCCQLKFWLPIGCAAFVVVCILG
CILICWLTKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTL;

SEQ ID No.5:

MARPHPWWLCVLGTLVGLSATPAPKSCPERHYWAQGKLCCQMCEPGTF
LVKDCDQHRKAAQCDPCIPGVSFSPDHHTRPHCESCRHCNSGLLVRNCTITAN
AECACRNGWQCRDKECTECDPLPNPSLTARSSQALSPHPQPTHLPYVSEMLEA
RTAGHMQTLADFRQLPARTLSTHWPPQRSLCSSDFIRILVIFSGMFLVFTLAGA
LFLHQRRKYRSNKGESPVEPAEPCRYSCPREEEGSTIPIQEDYRKPEPACSP;

SEQ ID No.6:

MGNSCYNIVATLLLVLNFERTRSLQDPCSNCPAGTFCDNNRNQICSPCPPN
SFSSAGGQRTCDICRQCKGVFRTRKECSSTSNAECDCTPGFHCLGAGCSMCEQ
DCKQGQELTKKGCKDCCFGTFNDQKRGICRPWTNCSLDGKSVLVNGTKERD
VVCGPSPADLSPGASSVTPPAPAREPGHSPQIISFFLALTSTALLFLLFFLTLRFSV
VKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL;

SEQ ID No.7: GSGATNFSLLKQAGDVEENPGP;
SEQ ID No.8: GSGEGRGSLLTCGDVEENPGP;
SEQ ID No.9: GSGQCTNYALLKLAGDVESNPGP;
SEQ ID No.10: GSGVKQTLNFDLLKLAGDVESNPGP.

[0012] Further, the extracellular hinge region of said chimeric antigen receptor is a region selected from CD8a or IgG; and the transmembrane domain of said chimeric antigen receptor is one selected from CD8a, CD28, CD137 or CD3.

[0013] A second object of the present invention is to provide a recombinant expression vector of any one of the aforesaid chimeric antigen receptors.

[0014] A third object of the present invention is to provide a CAR-T cell constructed by a recombinant expression vector of any one of the aforesaid chimeric antigen receptors.

[0015] A fourth object of the present invention is to provide a method of preparing the aforesaid CAR-T cell which includes the following steps:

step 1: construction of lentiviral vector and production of virus;
incorporating 2A between scFv(X)-(Y)CD3zeta and Z to form a fusion protein, adding a lentiviral vector to both ends of the fusion protein, and co-transfecting with lentiviral packaging plasmid to obtain an scFv(X)-(Y)CD3zeta-2A-(Z) virus;
step 2, preparation of scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cell;
culturing purified human PBMC and infecting said PBMC with the scFv(X)-(Y)CD3zeta-2A-(Z) virus obtained in Step 1, and subjecting them to cell expansion under suitable conditions to prepare scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cell.

[0016] For further optimizing the method of preparing the aforesaid CAR-T cell, the technical means used in the present invention further includes:
Further, said construction of lentiviral vector and production of virus include: incorporating 2A between scFv(X)-(Y)CD3zeta and Z by overlap PCR to form a fusion protein, and adding restriction sites to both ends of the fusion protein to clone a lentiviral vector; subjecting the clones sequenced correctly to a large scale endotoxin-free extraction, and co-transfecting with lentiviral packaging plasmid, after a predetermined period of time, collecting a supernatant, filtering, centrifuging to concentrate the virus to obtain an scFv(X)-(Y)CD3zeta-2A-(Z) virus.

[0017] Still further, the specific steps of the construction of lentiviral vector and production of virus are as follows: incorporating 2A sequence between scFv(X)-(Y)CD3zeta and OX40 by overlap PCR, adding EcoRI and SalI restriction sites to both ends of the fusion protein to clone the pCDH-MSCVEF vector, subjecting the clones sequenced correctly to a large scale endotoxin-free extraction, and co-transfecting with lentiviral packaging plasmid into 293X; after 48 and 72 hours, collecting the supernatant, filtering it by a 0.45uM filter and performing centrifugation at 25000RPM for 2 hours to concentrate the viruses to obtain the scFv(X)-(Y)CD3zeta-2A-(Z) virus.

[0018] Further, the specific steps of the preparation of scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cell include: isolating human PBMC for purification, inoculating into a culture plate under suitable stimulation conditions, culturing for a predetermined period of time, infecting said PBMC with the scFv(X)-(Y)CD3zeta-2A-(Z) virus obtained in Step 1, and subjecting it to cell expansion under suitable stimulation conditions, after 2 rounds of expansion under stimulation, the obtained cells are the scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cells.

[0019] Further, the stimulation conditions for culturing the isolated and purified human PBMC are anti-hCD3 and anti-hCD28; and the stimulation conditions for cell expansion are stimulation by use of artificial antigen presenting cell or anti-hCD3/28 every 6 days.

[0020] Still further, the specific steps of preparing the scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cell are as follows: purifying human PBMC by a Stemcell T cell isolation kit, inoculating into a 96-well culture plate coated by anti-hCD3 and anti-

hCD28. After 2 days, infecting the cells with the scFv(X)-(Y)CD3zeta-2A-(Z) virus at MOI=10-20. After 1 day, continuing to culture the cells with the medium changed, and stimulating them by artificial antigen presenting cell or anti-hCD3/28 every 6 days. After 2 rounds of stimulation, the obtained cells are scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cells.

[0021] Further, said X is selected anti-CD 19 antibody, anti-CD20 antibody, EGFR antibody, HER2 antibody, EGFRVIII antibody.

[0022] Further, said X is anti-CD20 antibody, said Y is 4-1BB, said Z is one selected from OX40, HVEM, ICOS, CD27, 4-1BB.

[0023] Further, said scFv(X)-(Y)CD3zeta is scFv-antihCD20-20BBZ having a sequence of SEQ ID No. 1; said OX40 has a sequence of SEQ ID No.2; said HVEM has a sequence of SEQ ID No.3; said ICOS has a sequence of SEQ ID No.4; said CD27 has a sequence of SEQ ID No.5; said 4-1BB has a sequence of SEQ ID No.6; and said 2A has a sequence of SEQ ID No.7.

[0024] Further, the lentiviral packaging plasmid in Step 1 includes VSV-g, pMD Gag/Pol, RSV-REV, and the centrifugation is performed with Beckman ultracentrifuge and SW28 head.

[0025] A fifth object of the present invention is to provide a formulation including the aforesaid CAR-T cell or the CAR-T cell prepared by the aforesaid preparation method. Further, the formulation also includes a pharmaceutically diluents or excipient.

[0026] A sixth object of the present invention is to provide a use of the aforesaid chimeric antigen receptor, the aforesaid CAR-T cell or the CAR-T cell prepared by the aforesaid preparation method in preparation of a medicament for treating or preventing tumor.

[0027] Further, said tumors are solid tumors. Examples of said solid tumors include, but are not limited to, lymphomas, renal tumors, neuroblastoma, germ cell tumor, osteosarcoma, chondrosarcoma, soft tissue sarcoma, liver tumor, thymoma, pulmonary blastoma, pancreatoblastoma, hemangioma, etc.

[0028] As compared with the prior art, the present invention has the following beneficial effects:
the CAR-T cell of the present invention significantly increases the tumor killing ability and expansion ability, and exhibits a greatly increased solid/metastasis tumor killing ability. The CAR-T cell of the present invention includes a co-stimulatory receptor (ICOS, CD28, CD27, HVEM, LIGHT, CD40L, 4-1BB, OX40, DR3, GITR, CD30, TIM1, SLAM, CD2, CD226, etc.), instead of a conventionally used ligand or excreted factor, and works only on the CAR-T cell, thereby reducing the risk of causing an immune side effect.

[0029] The present invention first utilizes the co-stimulatory receptor in the construction of CAR-T. As compared with the current CAR-T technology in clinic use, the present invention significantly increases the activation ability and survival ability of CAR-T cell in tumors, and controls the ability of solid/metastatic tumors, thereby improving the therapeutic effect of the CAR-T cell to get a more superior anti-tumor therapeutic effect.

Brief Description of the Drawings

[0030]

FIG. 1 is an illustrative schematic view showing the molecular structure of chimeric antigen receptor (CAR) including the third signal receptor in embodiments of the present invention;

FIG. 2 is a schematic view showing the virus titer measured after 293 cells were infected with BBZ-2A-OX40 virus in an embodiment of the present invention;

FIG. 3 is a schematic view showing the virus titer measured after 293 cells were infected with BBZ-2A-HVEM virus in an embodiment of the present invention;

FIG. 4 is a schematic view showing the virus titer measured after 293 cells were infected with BBZ-2A-ICOS virus in an embodiment of the present invention;

FIG. 5 is a schematic view showing the virus titer measured after 293 cells were infected with BBZ-2A-CD27 virus in an embodiment of the present invention;

FIG. 6 is a schematic view showing the virus titer measured after 293 cells were infected with BBZ-2A-4-1BB virus in an embodiment of the present invention;

FIG. 7 is a schematic view showing the results of phenotypic analysis of BBZ CAR-T cell and BBZ-2A-OX40 CAR-T cell in an embodiment of the present invention;

FIG. 8 is a schematic view showing the results of phenotypic analysis of BBZ CAR-T cell and BBZ-2A-HVEM CAR-T cell in an embodiment of the present invention;

FIG. 9 is a schematic view showing the results of phenotypic analysis of BBZ CAR-T cell and BBZ-2A-ICOS CAR-T cell in an embodiment of the present invention;

FIG. 10 is a schematic view showing the results of phenotypic analysis of BBZ CAR-T cell and BBZ-2A-CD27 CAR-T cell in an embodiment of the present invention;

FIG. 11 is a schematic view showing the results of phenotypic analysis of BBZ CAR-T cell and BBZ-2A-4-1BB CAR-

T cell in an embodiment of the present invention;
FIG. 12 is a schematic view showing the expansion ability of BBZ CAR-T cell and BBZ-2A-OX40 CAR-T cell in an embodiment of the present invention;
FIG. 13 is a schematic view showing the tumor killing ability of BBZ CAR-T cell and BBZ-2A-OX40 CAR-T cell in an embodiment of the present invention;
FIG. 14 is a schematic view showing the anti-tumor ability of BBZ CAR-T cell and BBZ-2A-OX40 CAR-T cell in an embodiment of the present invention;
FIG. 15 is a schematic view showing the *in vivo* survival ability of BBZ CAR-T cell and BBZ-2A-OX40 CAR-T cell in an embodiment of the present invention.

Detailed Description of the Invention

[0031] The present invention provides a chimeric antigen receptor including a co-stimulatory receptor having a structure of scFv(X)-(Y)CD3zeta-2A-(Z); wherein X is a tumor-targeting antibody or other protein; Y is an intracellular domain of a co-stimulatory receptor, and said co-stimulatory receptor is selected from ICOS, CD28, CD27, HVEM, LIGHT, CD40L, 4-1BB, OX40, DR3, GITR, CD30, TIM1, SLAM, CD2, CD226; Z is a co-stimulatory receptor, and said co-stimulatory receptor is selected from ICOS, CD28, CD27, HVEM, LIGHT, CD40L, 4-1BB, OX40, DR3, GITR, CD30, TIM1, SLAM, CD2, CD226. The present invention also relates to a CAR-T cell constructed by a recombinant expression vector of any one of the aforesaid chimeric antigen receptor and a preparation method therefor, a formulation including the CAR-T cell, and a use of the CAR-T cell.
[0032] Hereinafter the embodiments of the present invention are further described with reference to the accompanying drawings and examples. The following examples are only for more clearly illustrating the technical solutions of the present invention, but not for limiting the protective scope of the present invention.
[0033] The chimeric antigen receptor (CAR) molecules including a co-stimulatory receptor used in the following examples of the present invention are BBZ-2A-OX40, BBZ-2A-HVEM, BBZ-2A-ICOS, BBZ-2A-CD27, BBZ-2A-4-1BB, respectively, and their structures are shown in FIG. 1.

EXAMPLE 1 - Preparation of 20BBZ-2A-OX40 CAR-T cell

[0034] The preparation of the 20BBZ-2A-OX40 CAR-T cell in this example includes the following steps:

1. Construction of lentiviral vector pCDH-MSCVEF-20BBZ-2A-OX40 and production of virus
incorporating 2A (SEQ ID No. 7) sequence between scFv-antihCD20-20BBZ (SEQID No.1) and OX40 (SEQ ID No.2) by overlap PCR, and adding EcoRI and SalI restriction sites to both ends to clone the pCDH-MSCVEF vector. Subjecting the clones sequenced correctly to a large scale endotoxin-free extraction, and co-transfecting with lentiviral packaging plasmid (VSV-g, pMD Gag/Pol, RSV-REV) into 293X. After 48 and 72 hours, collecting the supernatant, filtering it by a 0.45uM filter, and performing centrifugation with Beckman ultracentrifuge and SW28 head at 25000 RPM for 2 hours to concentrate the virus, which is pCDH-MSCVEF-20BBZ-2A-OX40 virus (briefly, 20BBZ-2A-OX40 virus) for the subsequent production of CAR-T cell. Meanwhile, producing the control pCDH-MSCVEF-20BBZ virus (briefly, 20BBZ virus), and infecting 293 cells with the obtained virus to measure the virus titer, as shown in FIG. 2.
2. Preparation of 20BBZ-2A-OX40 CAR-T cell and 20BBZ CAR-T cell
purifying human PBMC by a Stemcell T cell isolation kit, and inoculating into a 96-well culture plate coated with anti-hCD3 and anti-hCD28. After 2 days, infecting the cells with 20BBZ virus and 20BBZ-2A-OX40 virus at MOI=10-20. After 1 day, continuing to culture the cells with the medium changed, and stimulating them by artificial antigen presenting cell or anti-hCD3/28 every 6 days. After 2 rounds of stimulation, the obtained cells are 20BBZCAR-T cell and 20BBZ-2A-OX40 CAR-T cell for subsequent experiments and phenotypic analysis. The results are shown in FIG. 7. It can be seen that the obtained cells are CAR-POSITIVE.

EXAMPLE 2- Preparation of 20BBZ-2A-HVEM CAR-T cell

[0035] The preparation of the 20BBZ-2A-HVEM CAR-T cell in in this example includes the following steps:

1. Construction of lentiviral vector pCDH-MSCVEF-20BBZ-2A-HVEM and production of virus
incorporating 2A (SEQ ID No. 8) sequence between scFv-antihCD20-20BBZ (SEQID No. 1) and HVEM (SEQID No.3) by overlap PCR, and adding EcoRI and SalI restriction sites to both ends to clone pCDH-MSCVEF vector. Subjecting the clones sequenced correctly to a large scale endotoxin-free extraction, and co-transfecting with lentiviral packaging plasmid (VSV-g, pMD Gag/Pol, RSV-REV) into 293X. After 48 and 72 hours, collecting the super-

natant, filtering it by a 0.45uM filter, and performing centrifugation with Beckman ultracentrifuge and SW28 head at 25000 RPM for 2 hours to concentrate the virus, which is pCDH-MSCVEF-20BBZ-2A-HVEM virus (briefly, 20BBZ-2A-HVEM virus) for the subsequent production of CAR-T cell. Meanwhile, producing the control pCDH-MSCVEF-20BBZ virus (briefly, 20BBZ virus). Infecting 293 cells with the obtained virus to measure the virus titer, as shown in FIG. 3.

2. Preparation of 20BBZ-2A-HVEM CAR-T cell and 20BBZ CAR-T cell

purifying human PBMC by a Stemcell T cell isolation kit, and inoculating into a 96-well culture plate coated with anti-hCD3 and anti-hCD28. After 2 days, infecting the cells were infecte with 20BBZ virus and 20BBZ-2A-HVEM virus at MOI=10-20. After 1 day, continuing to culture the cells with the medium changed, and stimulating them by artificial antigen presenting cell or anti-hCD3/28 every 6 days. After 2 rounds of stimulation, the obtained cells are 20BBZCAR-T cell and 20BBZ-2A-HVEM CAR-T cell for subsequent experiments and phenotypic analysis. The results are shown in FIG. 8. It can be seen from the figure that the obtained cells are CAR-POSITIVE.

EXAMPLE 3 - Preparation of 20BBZ-2A-ICOS CAR-T cell

[0036] The preparation of the 20BBZ-2A-ICOS CAR-T cell in this example includes the following steps:

1. Construction of lentiviral vector pCDH-MSCVEF-20BBZ-2A-ICOS and production of virus

incorporating 2A (SEQ ID No. 9) sequence between scFv-antihCD20-20BBZ (SEQID No. 1) and ICOS (SEQID No.4) by overlap PCR, and adding EcoRI and SalI restriction sites to both ends to clone pCDH-MSCVEF vector. Subjecting the clones sequenced correctly to a large scale endotoxin-free extraction, and co-transfecting with lentiviral packaging plasmid (VSV-g, pMD Gag/Pol, RSV-REV) into 293X. After 48 and 72 hours, collecting the supernatant, filtering it by a 0.45uM filter, and performing centrifugation with Beckman ultracentrifuge and SW28 head at 25000 RPM for 2 hours to concentrate the virus, which is pCDH-MSCVEF-20BBZ-2A-ICOS virus (briefly, 20BBZ-2A-ICOS virus) for the subsequent production of CAR-T cell. Meanwhile, producing the control pCDH-MSCVEF-20BBZ virus (briefly, 20BBZ virus), and infecting 293 cells with the obtained virus to measure the virus titer, as shown in FIG. 4.

2. Preparation of 20BBZ-2A-ICOS CAR-T cell and 20BBZ CAR-T cell purifying human PBMCs by a Stemcell T cell isolation kit, and inoculating into a 96-well culture plate coated with anti-hCD3 and anti-hCD28. After 2 days, infecting the cells with 20BBZ virus and 20BBZ-2A-ICOS virus at MOI=10-20. After 1 day, continuing to culture the cells with the medium changed, and stimulating them by artificial antigen presenting cell or anti-hCD3/28 every 6 days. After 2 rounds of stimulation, the obtained cells are 20BBZCAR-T cell and 20BBZ-2A-ICOS CAR-T cell for subsequent experiments and phenotypic analysis. The results are shown in FIG. 9. It can be seen from the figure that the obtained cells are CAR-POSITIVE.

EXAMPLE 4- Preparation of 20BBZ-2A-CD27 CAR-T cell

[0037] The preparation of 20BBZ-2A-CD27 CAR-T cell in this example includes the following steps:

1. Construction of lentiviral vector pCDH-MSCVEF-20BBZ-2A-CD27 and production of virus

incorporating 2A (SEQ ID No. 10) sequence between scFv-antihCD20-20BBZ (SEQID No. 1) and CD27 (SEQID No.5) by overlap PCR, and adding EcoRI and SalI restriction sites to both ends to clone pCDH-MSCVEF vector. Subjecting the clones sequenced correctly to a large scale endotoxin-free extraction, and co-transfecting with lentiviral packaging plasmid (VSV-g, pMD Gag/Pol, RSV-REV) into 293X. After 48 and 72 hours, collecting the supernatant, filtering it by a 0.45uM filter, and performing centrifugation with Beckman ultracentrifuge and SW28 head at 25000 RPM for 2 hours to concentrate the virus, which is pCDH-MSCVEF-20BBZ-2A-CD27 virus (briefly, 20BBZ-2A-CD27 virus) for the subsequent production of CAR-T cell. Meanwhile, producing the control pCDH-MSCVEF-20BBZ virus (briefly, 20BBZ virus), and infecting 293 cells with the obtained virus to measure the virus titer, as shown in FIG. 5.

2. Preparation of 20BBZ-2A-CD27 CAR-T cell and 20BBZ CAR-T cell purifying human PBMC by a Stemcell T cell isolation kit, and inoculating into a 96-well culture plate coated with anti-hCD3 and anti-hCD28. After 2 days, infecting the cells with 20BBZ virus and 20BBZ-2A-CD27 virus at MOI=10-20. After 1 day, continuing to culture the cells with the medium changed, and stimulating them by artificial antigen presenting cell or anti-hCD3/28 every 6 days. After 2 rounds of stimulation, the obtained cells are 20BBZCAR-T cell and 20BBZ-2A-CD27 CAR-T cell for subsequent experiments and phenotypic analysis. The results are shown in FIG. 10. It can be seen from the figure that the obtained cells are CAR-POSITIVE.

EXAMPLE 5- Preparation of 20BBZ-2A-4-1BB CAR-T cell

[0038] The preparation of the 20BBZ-2A-4-1BB CAR-T cell in this example includes the following steps:

1. Construction of lentiviral vector pCDH-MSCVEF-20BBZ-2A-4-1BB and production of virus incorporating 2A (SEQ ID No. 7) sequence between scFv-antihCD20-20BBZ (SEQID No. 1) and 4-1BB (SEQID No.6) by overlap PCR, and adding EcoRI and SalI restriction sites to both ends to clone pCDH-MSCVEF vector. Subjecting the clones sequenced correctly to a large scale endotoxin-free extraction, and co-transfecting with lentiviral packaging plasmid (VSV-g, pMD Gag/Pol, RSV-REV) into 293X. After 48 and 72 hours, collecting the supernatant, filtering it by a 0.45uM filter, and performing centrifugation with Beckman ultracentrifuge and SW28 head at 25000 RPM for 2 hours to concentrate the virus, which is pCDH-MSCVEF-20BBZ-2A-4-1BB virus (briefly, 20BBZ-2A-4-1BB virus) for the subsequent production of CAR-T cell. Meanwhile, producing the control pCDH-MSCVEF-20BBZ virus (briefly, 20BBZ virus), infecting 293 cells with the obtained virus to measure the virus titer, as shown in FIG. 6.
2. Preparation of 20BBZ-2A-4-1BB CAR-T cell and 20BBZ CAR-T cell purifying human PBMC by a Stemcell T cell isolation kit, and inoculating into a 96-well culture plate coated with anti-hCD3 and anti-hCD28. After 2 days, infecting the cells with 20BBZ virus and 20BBZ-2A-4-1BB virus at MOI=10-20. After 1 day, continuing to culture the cells with the medium changed, and stimulating them by artificial antigen presenting cell or anti-hCD3/28 every 6 days. After 2 rounds of stimulation, the obtained cells are 20BBZCAR-T cell and 20BBZ-2A-4-1BB CAR-T cell for subsequent experiments and phenotypic analysis. The results are shown in FIG. 11. It can be seen from the figure that the obtained cells are CAR-POSITIVE.

EXAMPLE 6 - Comparison of expansion abilities of 20BBZ CAR-T cell and 20BBZ-2A-OX40 CAR-T cell

[0039] 20BBZ CAR-T cell and 20BBZ-2A-OX40 CAR-T cell prepared in Step 2 of Example 1 were continuously cultured for 14 days, and stimulated with artificial antigen presenting cell once every 6 days. The cells were counted, and the results are shown in FIG. 12. It can be seen from the figure that 20BBZ-2A-OX40 CAR-T cell has enhanced proliferation ability as compared with 20BBZCAR-T cell.

EXAMPLE 7 - Comparison of tumor-killing abilities of 20BBZ CAR-T cell and 20BBZ-2A-OX40 CAR-T cell

[0040] 20BBZ CAR-T cell and 20BBZ-2A-OX40 CAR-T cell obtained in Step 2 of Example 1, 20BBZ-2A-ICOS CAR-T cell obtained in Step 2 of Example 3, and 20BBZ-2A-CD27 CAR-T cell obtained in Step 2 of Example 4 were inoculated into a 96-well plate, and Raji tumor cells were added at a CAR-T:tumor cell ratio of 1:1, 1:2, 1:4. After 24 and 48 hours, the survival rates of tumor cells were compared, and the results are shown in FIG. 13. It can be seen from the figure that 20BBZ-2A-OX40/ICOS/CD27 CAR-T cell has similar tumor killing ability as compared with 20BBZ CAR-T cell, and some CAR-T including the co-stimulatory receptor has a stronger tumor killing ability.

EXAMPLE 8 - Comparison of anti-tumor ability and in vivo survival ability of 20BBZ CAR-T cell and 20BBZ-2A-OX40 CAR-T cell

[0041] 10^6 Nalm-6 tumor cells were intravenously inoculated into B-NDG mice, which were treated with 10^7 20BBZ CAR-T cells and 20BBZ-2A-OX40 CAR-T cells after 6 days. The mice were observed for their survival rates, and some mice were detected for the level of tumor cells and CAR-T cells in their marrow on Day 7. The results are shown in FIG. 14 and FIG. 15, respectively. It can be seen from the figure that 20BBZ-2A-OX40 CAR-T cell, as compared with 20BBZ CAR-T cell, significantly prolongs the survival of mice, and expanded more in vivo.

[0042] It can be seen from the aforesaid examples that the present invention constructs a novel CAR-T cell including a co-stimulatory receptor, which significantly increases the activation ability, survival ability, expansion ability of the CAR-T cells in tumors, as compared with the current CAR-T technology in clinic use, and has a more superior anti-tumor therapeutic effect.

[0043] Hereinbefore the specific embodiments of the present invention are described in details. However, they are only used as examples, and the present invention is not limited to the specific embodiments as described above. For those skilled in the art, any equivalent modifications and substitutions made to the present invention are encompassed in the scope of the present invention. Therefore, all the equal transformations and modifications without departing from the spirit and scope of the present invention should be covered in the scope of the present invention.

SEQUENCE LISTING

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        35                  40                  45
Ala Thr Ser Asn Leu Ala Ser Gly Val Pro Val Arg Phe Ser Gly Ser
    50                  55                  60
Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Val Glu Ala Glu
65                  70                  75                  80
Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Thr Ser Asn Pro Pro Thr
                85                  90                  95
Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Gly Gly Gly Gly Ser Gly
            100                 105                 110
Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln Val Gln Leu Gln Gln Pro
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Thr Pro Gly Arg Gly Leu Glu Trp Ile Gly Ala Ile Tyr Pro Gly Asn
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Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr
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Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr
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Gly Asp Trp Tyr Phe Asn Val Trp Gly Ala Gly Thr Thr Val Thr Val
225                 230                 235                 240
Ser Ala Ala Ala Ala Thr Thr Thr Pro Ala Pro Arg Pro Pro Thr Pro
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Arg Pro Ala Ala Gly Gly Ala Val His Thr Arg Gly Leu Asp Phe Ala
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        275                 280                 285
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Leu Leu Tyr Ile Phe Lys Gln Pro Phe Met Arg Pro Val Gln Thr Thr
                325                 330                 335
Gln Glu Glu Asp Gly Cys Ser Cys Arg Phe Pro Glu Glu Glu Glu Gly
            340                 345                 350
Gly Cys Glu Leu Arg Val Lys Phe Ser Arg Ser Ala Asp Ala Pro Ala
        355                 360                 365
Tyr Gln Gln Gly Gln Asn Gln Leu Tyr Asn Glu Leu Asn Leu Gly Arg
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385                 390                 395                 400
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                405                 410                 415
Glu Leu Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser Glu Ile Gly Met
            420                 425                 430
Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly Leu Tyr Gln Gly
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Gly Asp Thr Tyr Pro Ser Asn Asp Arg Cys Cys His Glu Cys Arg Pro
        35                  40                  45
Gly Asn Gly Met Val Ser Arg Cys Ser Arg Ser Gln Asn Thr Val Cys
    50                  55                  60
Arg Pro Cys Gly Pro Gly Phe Tyr Asn Asp Val Val Ser Ser Lys Pro
65                  70                  75                  80
Cys Lys Pro Cys Thr Trp Cys Asn Leu Arg Ser Gly Ser Glu Arg Lys
                85                  90                  95
Gln Leu Cys Thr Ala Thr Gln Asp Thr Val Cys Arg Cys Arg Ala Gly
            100                 105                 110
Thr Gln Pro Leu Asp Ser Tyr Lys Pro Gly Val Asp Cys Ala Pro Cys
        115                 120                 125
Pro Pro Gly His Phe Ser Pro Gly Asp Asn Gln Ala Cys Lys Pro Trp
    130                 135                 140
Thr Asn Cys Thr Leu Ala Gly Lys His Thr Leu Gln Pro Ala Ser Asn
145                 150                 155                 160
Ser Ser Asp Ala Ile Cys Glu Asp Arg Asp Pro Pro Ala Thr Gln Pro
                165                 170                 175
Gln Glu Thr Gln Gly Pro Pro Ala Arg Pro Ile Thr Val Gln Pro Thr
            180                 185                 190
Glu Ala Trp Pro Arg Thr Ser Gln Gly Pro Ser Thr Arg Pro Val Glu
        195                 200                 205
Val Pro Gly Gly Arg Ala Val Ala Ala Ile Leu Gly Leu Gly Leu Val
    210                 215                 220
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Leu Gly Leu Leu Gly Pro Leu Ala Ile Leu Leu Ala Leu Tyr Leu Leu
225 230 235 240
Arg Arg Asp Gln Arg Leu Pro Pro Asp Ala His Lys Pro Pro Gly Gly
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35 40 45
Val Gly Ser Glu Cys Cys Pro Lys Cys Ser Pro Gly Tyr Arg Val Lys
50 55 60
Glu Ala Cys Gly Glu Leu Thr Gly Thr Val Cys Glu Pro Cys Pro Pro
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Gly Thr Tyr Ile Ala His Leu Asn Gly Leu Ser Lys Cys Leu Gln Cys
85 90 95
Gln Met Cys Asp Pro Ala Met Gly Leu Arg Ala Ser Arg Asn Cys Ser
100 105 110
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Glu Glu Cys Gln His Gln Thr Lys Cys Ser Trp Leu Val Thr Lys Ala
180 185 190
Gly Ala Gly Thr Ser Ser Ser His Trp Val Trp Trp Phe Leu Ser Gly
195 200 205
Ser Leu Val Ile Val Ile Val Cys Ser Thr Val Gly Leu Ile Ile Cys
210 215 220
Val Lys Arg Arg Lys Pro Arg Gly Asp Val Val Lys Val Ile Val Ser
225 230 235 240
Val Gln Arg Lys Arg Gln Glu Ala Glu Gly Glu Ala Thr Val Ile Glu
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Gln Gln Phe Lys Met Gln Leu Leu Lys Gly Gly Gln Ile Leu Cys Asp
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Lys Phe Cys His Ser Gln Leu Ser Asn Asn Ser Val Ser Phe Phe Leu
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Tyr Asn Leu Asp His Ser His Ala Asn Tyr Tyr Phe Cys Asn Leu Ser
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Ile Cys Trp Leu Thr Lys Lys Lys Tyr Ser Ser Ser Val His Asp Pro
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35 40 45
Leu Val Lys Asp Cys Asp Gln His Arg Lys Ala Ala Gln Cys Asp Pro
50 55 60
Cys Ile Pro Gly Val Ser Phe Ser Pro Asp His His Thr Arg Pro His
65 70 75 80
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Cys Arg Gln Cys Lys Gly Val Phe Arg Thr Arg Lys Glu Cys Ser Ser
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Thr Lys Lys Gly Cys Lys Asp Cys Cys Phe Gly Thr Phe Asn Asp Gln
        115                 120                 125
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    130                 135                 140
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Ser Pro Ala Asp Leu Ser Pro Gly Ala Ser Ser Val Thr Pro Pro Ala
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Pro Ala Arg Glu Pro Gly His Ser Pro Gln Ile Ile Ser Phe Phe Leu
            180                 185                 190
Ala Leu Thr Ser Thr Ala Leu Leu Phe Leu Leu Phe Phe Leu Thr Leu
        195                 200                 205
Arg Phe Ser Val Val Lys Arg Gly Arg Lys Lys Leu Leu Tyr Ile Phe
    210                 215                 220
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Claims

1. A chimeric antigen receptor comprising a co-stimulatory receptor, wherein said chimeric antigen receptor has a structure of scFv(X)-(Y)CD3zeta-2A-(Z);
wherein X comprises a tumor-targeting antibody or a ligand or receptor capable of specifically binding to a tumor;
Y is an intracellular domain of a co-stimulatory receptor, and said co-stimulatory receptor is selected from ICOS, CD28, CD27, HVEM, LIGHT, CD40L, 4-1BB, OX40, DR3, GITR, CD30, TIM1, SLAM, CD2, CD226; and
Z is a co-stimulating receptor, and said co-stimulatory receptor is selected from ICOS, CD28, CD27, HVEM, LIGHT, CD40L, 4-1BB, OX40, DR3, GITR, CD30, TIM1, SLAM, CD2, CD226.

2. The chimeric antigen receptor comprising a co-stimulatory receptor according to claim 1, wherein said X is selected from anti-CD 19 antibody, anti-CD20 antibody, EGFR antibody, HER2 antibody, EGFRVIII antibody, anti-PSMA antibody, anti-BCMA antibody, anti-CD22 antibody, anti-CD30 antibody.

3. The chimeric antigen receptor comprising a co-stimulatory receptor according to claim 1, wherein said X is anti-

CD20 antibody, said Y is 4-1BB, said Z is one selected from OX40, HVEM, ICOS, CD27, 4-1BB.

4. The chimeric antigen receptor comprising a co-stimulatory receptor according to claim 3, wherein said scFv(X)-(Y)CD3zeta is scFv-antihCD20-20BBZ with a sequence of SEQ ID No. 1; said OX40 has a sequence of SEQ ID No.2; said HVEM has a sequence of SEQ ID No.3; said ICOS has a sequence of SEQ ID No.4; said CD27 has a sequence of SEQ ID No.5; said 4-1BB has a sequence of SEQ ID No.6; and said 2A has a sequence of SEQ ID No.7, SEQ ID No.8, SEQ ID No.9 or SEQ ID No.10.

5. A CAR-T cell constructed by a recombinant expression vector of said chimeric antigen receptor according to any one of claims 1-4.

6. A method of preparing said CAR-T cell according to claim 5, comprising the following steps:

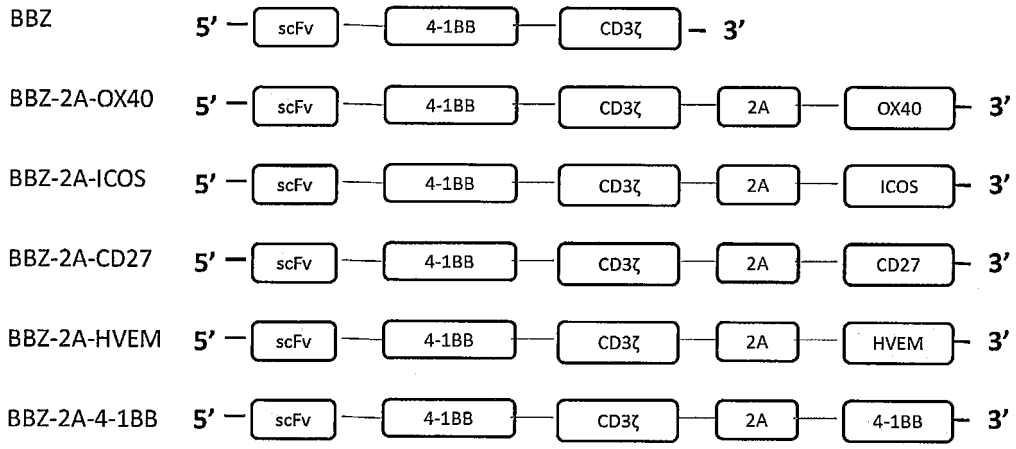
step 1, construction of lentiviral vector and production of virus;
incorporating 2A between scFv(X)-(Y)CD3zeta and Z to form a fusion protein, adding a lentiviral vector to both ends of the fusion protein, and co-transfecting with a lentiviral packaging plasmid to obtain an scFv(X)-(Y)CD3zeta-2A-(Z) virus; and
step 2, preparation of scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cell;
culturing purified human PBMC , and infecting said PBMC with the scFv(X)-(Y)CD3zeta-2A-(Z) virus obtained in Step 1, subjecting them to cell expansion under suitable conditions to prepare the scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cell.

7. The method of preparing said CAR-T cell according to claim 6, wherein said construction of lentiviral vector and production of virus comprises:
incorporating 2A between scFv(X)-(Y)CD3zeta and Z by overlap PCR to form a fusion protein, and adding restriction sites to both ends of the fusion protein to clone a lentiviral vector; subjecting the clones sequenced correctly to a large scale endotoxin-free extraction, and co-transfecting with a lentiviral packaging plasmid; after a predetermined time period, collecting a supernatant, filtering, centrifuging to concentrate the virus to obtain an scFv(X)-(Y)CD3zeta-2A-(Z) virus.

8. The method of preparing said CAR-T cell according to claim 6, wherein said preparation of said scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cell comprises: isolating human PBMC for purification, inoculating into a culture plate under suitable stimulation conditions, culturing them for a predetermined period of time, infecting said PBMC with the scFv(X)-(Y)CD3zeta-2A-(Z) virus produced in Step 1, and subjecting them to cell expansion under suitable stimulation conditions, after 2 rounds of expansion under stimulation, the obtained cells are the scFv(X)-(Y)CD3zeta-2A-(Z) CAR-T cells.

9. A formulation, comprising said CAR-T cell according to claim 5.

10. Use of said chimeric antigen receptor according to any one of claims 1-4 or said CAR-T cell according to claim 5 in the preparation of a medicament for treating or preventing tumors.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5'
scFv
4-1BB
CD3ζ
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BBZ-2A-OX40
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scFv
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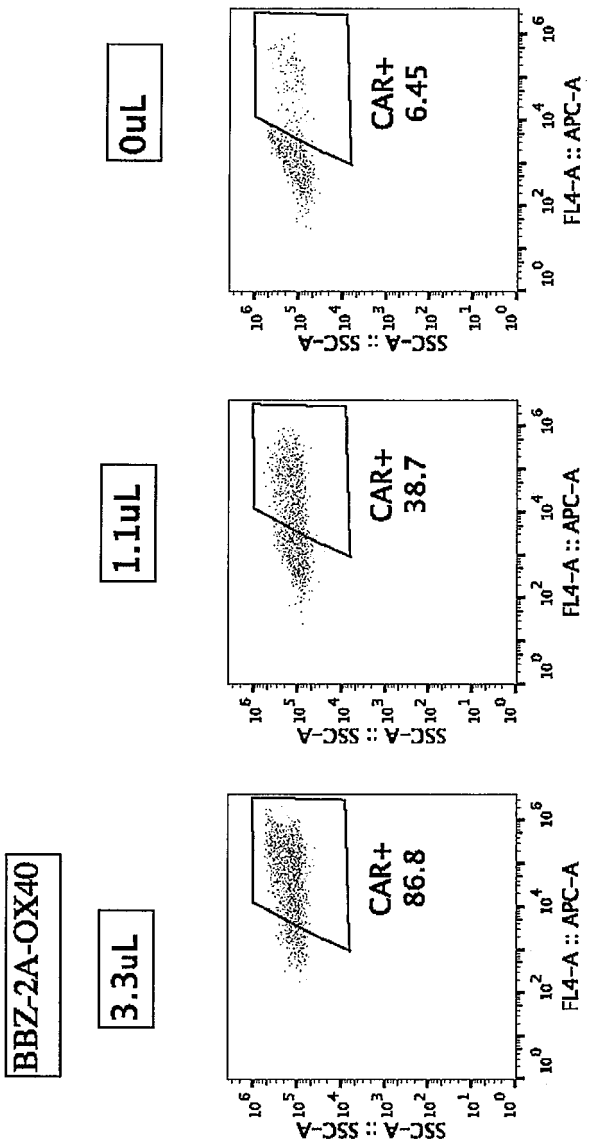
FIG. 1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3.3uL
1.1uL
0uL
CAR+
86.8
CAR+
38.7
CAR+
6.45
SSC-A :: SSC-A
FL4-A :: APC-A

FIG. 2

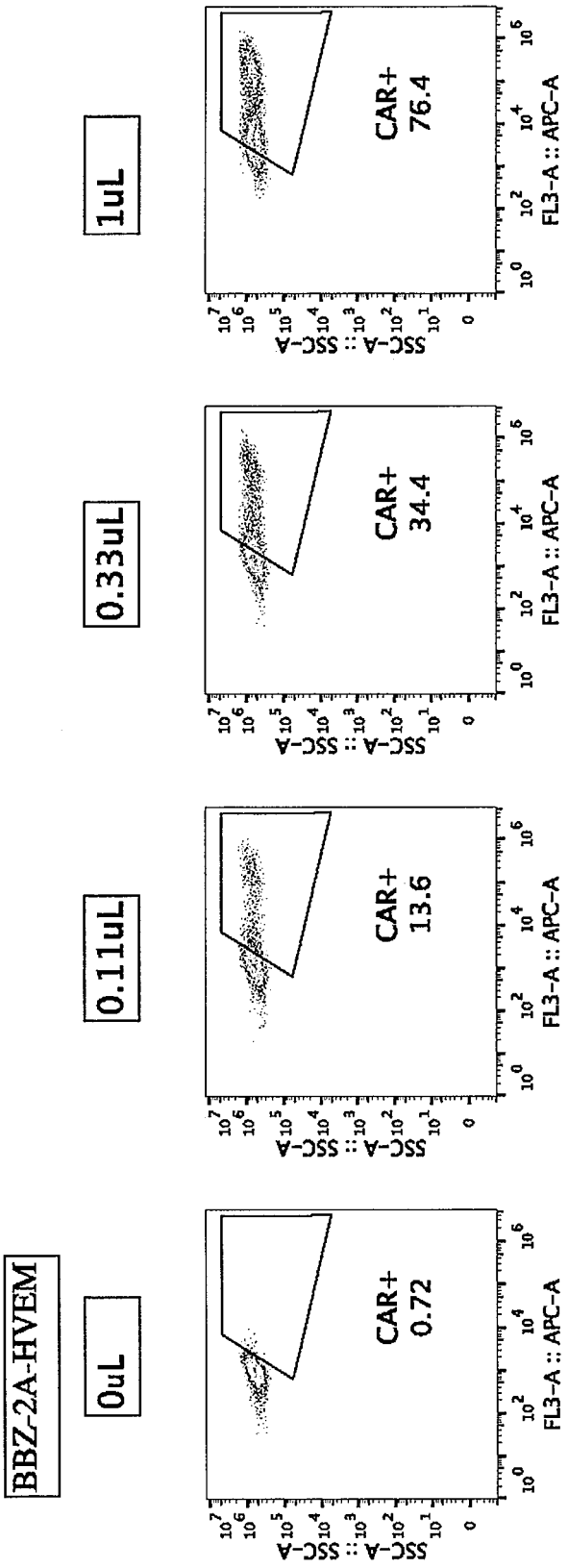
BBZ-2A-HVEM
0uL
0.11uL
0.33uL
1uL
CAR+
0.72
CAR+
13.6
CAR+
34.4
CAR+
76.4
SSC-A :: SSC-A
FL3-A :: APC-A

FIG. 3

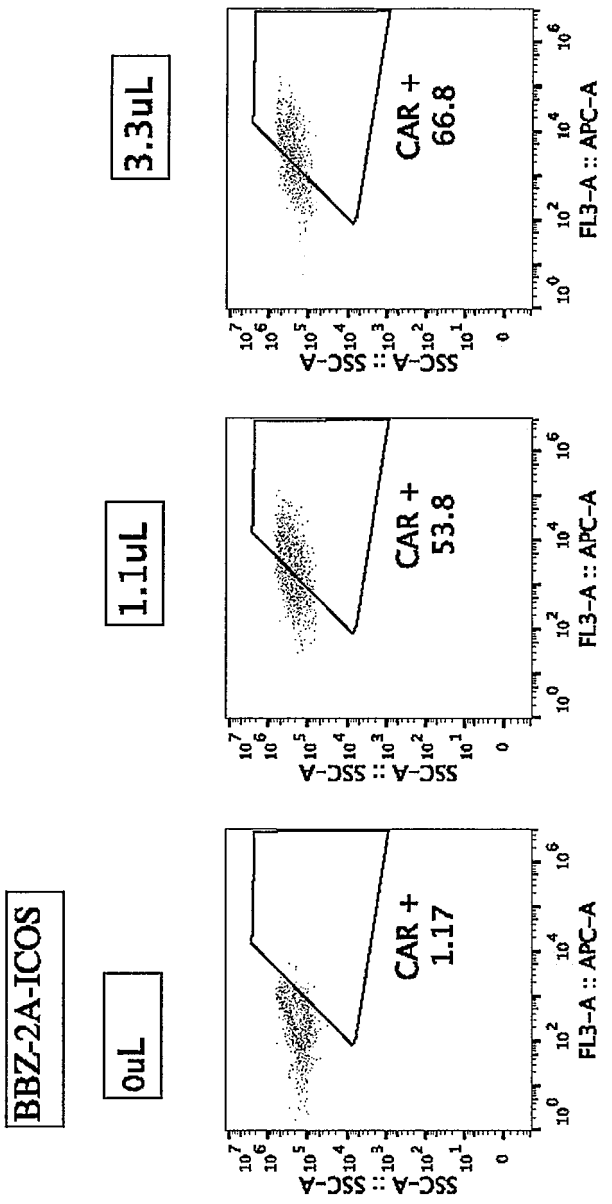
BBZ-2A-ICOS
0uL
1.1uL
3.3uL
CAR + 1.17
CAR + 53.8
CAR + 66.8
SSC-A :: SSC-A
FL3-A :: APC-A

FIG. 4

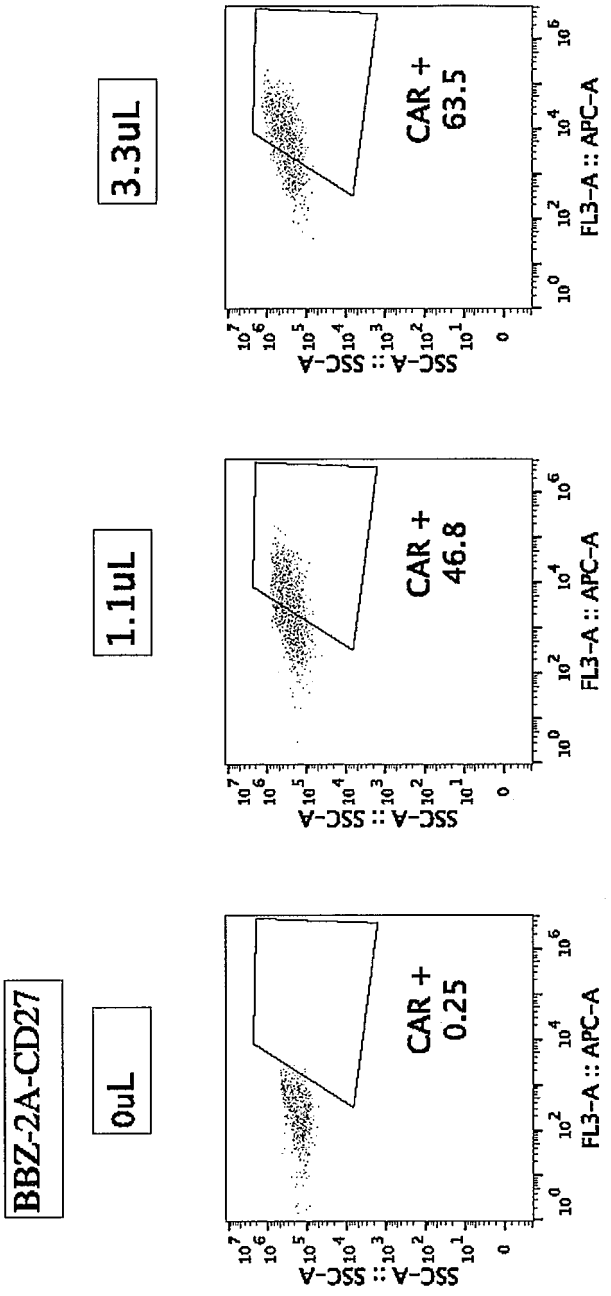
BBZ-2A-CD27
0uL
1.1uL
3.3uL
CAR + 0.25
CAR + 46.8
CAR + 63.5
SSC-A :: SSC-A
FL3-A :: APC-A

FIG. 5

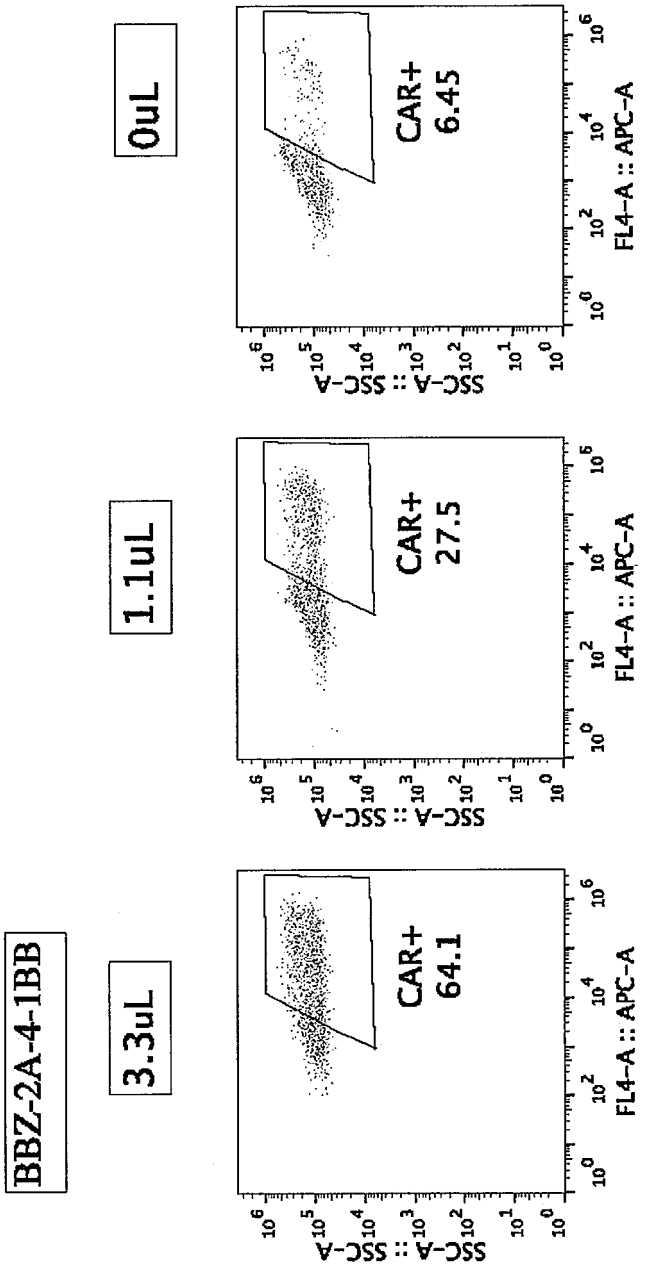
BBZ-2A-4-1BB
3.3uL
1.1uL
0uL
CAR+
64.1
CAR+
27.5
CAR+
6.45
SSC-A :: SSC-A
FL4-A :: APC-A

FIG. 6

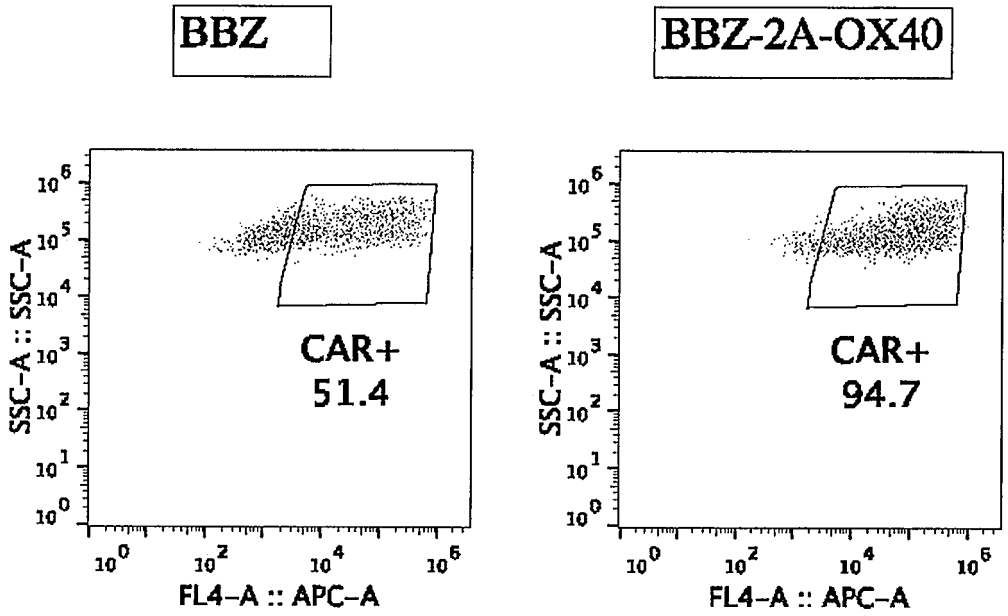
BBZ
BBZ-2A-OX40
SSC-A :: SSC-A
FL4-A :: APC-A
CAR+
51.4
CAR+
94.7

FIG. 7

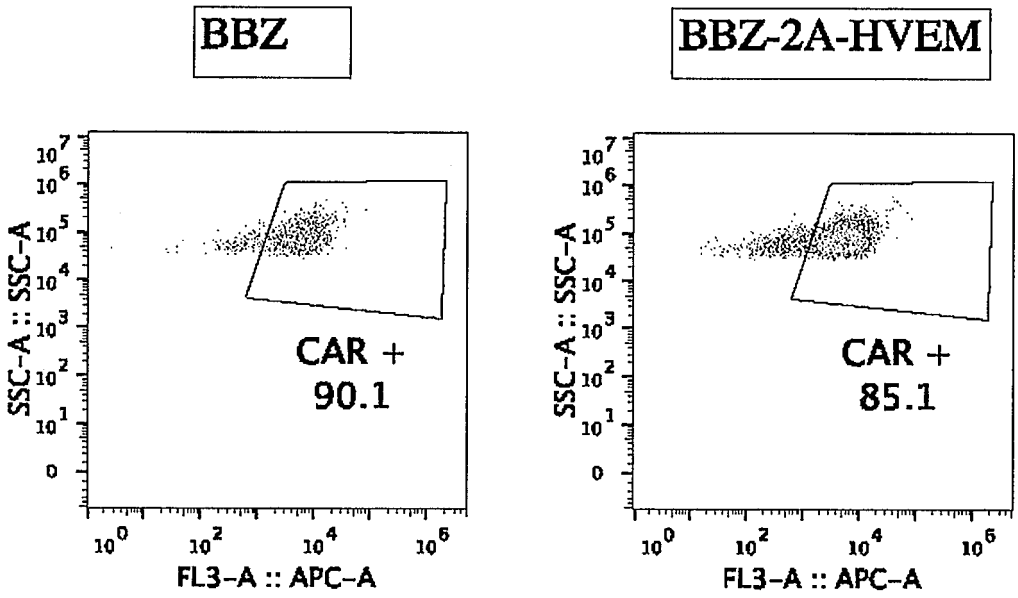
BBZ
BBZ-2A-HVEM
SSC-A :: SSC-A
FL3-A :: APC-A
CAR +
90.1
CAR +
85.1

FIG. 8

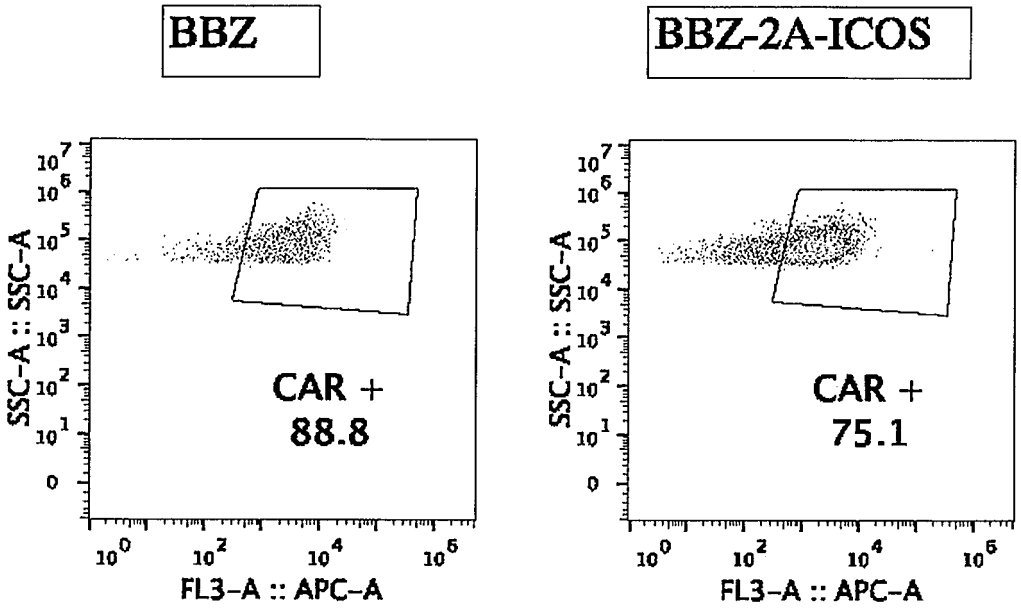
BBZ
BBZ-2A-ICOS
SSC-A :: SSC-A
FL3-A :: APC-A
CAR +
88.8
CAR +
75.1

FIG. 9

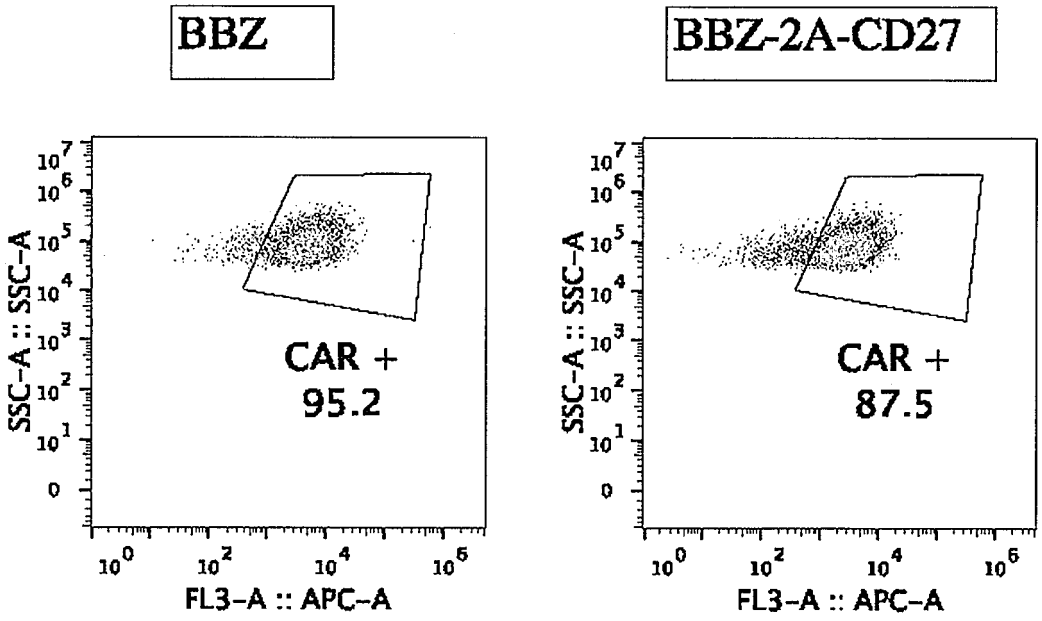
BBZ
BBZ-2A-CD27
SSC-A :: SSC-A
FL3-A :: APC-A
CAR +
95.2
CAR +
87.5

FIG. 10

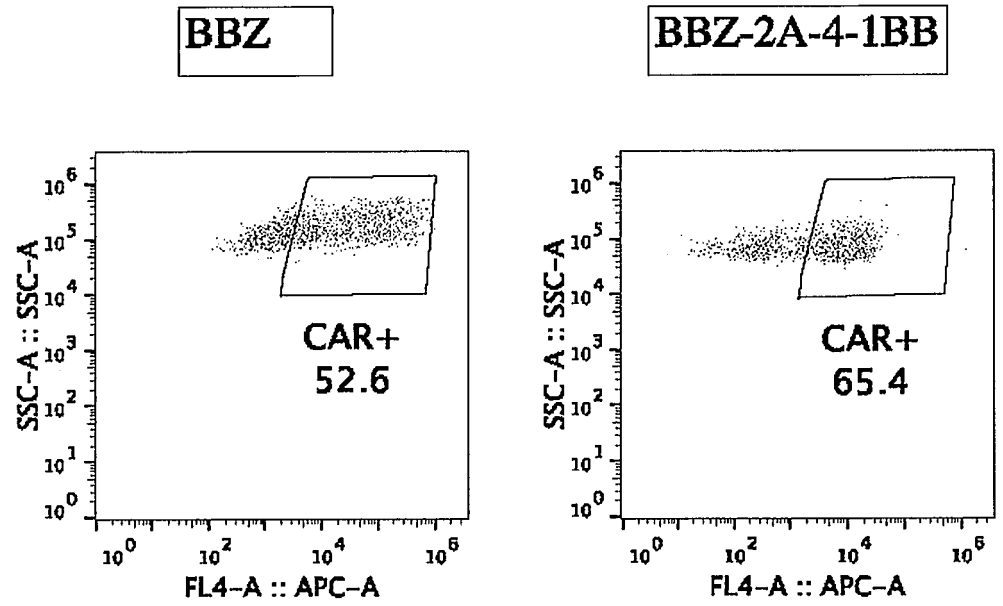
BBZ
BBZ-2A-4-1BB
SSC-A :: SSC-A
FL4-A :: APC-A
CAR+
52.6
CAR+
65.4

FIG. 11

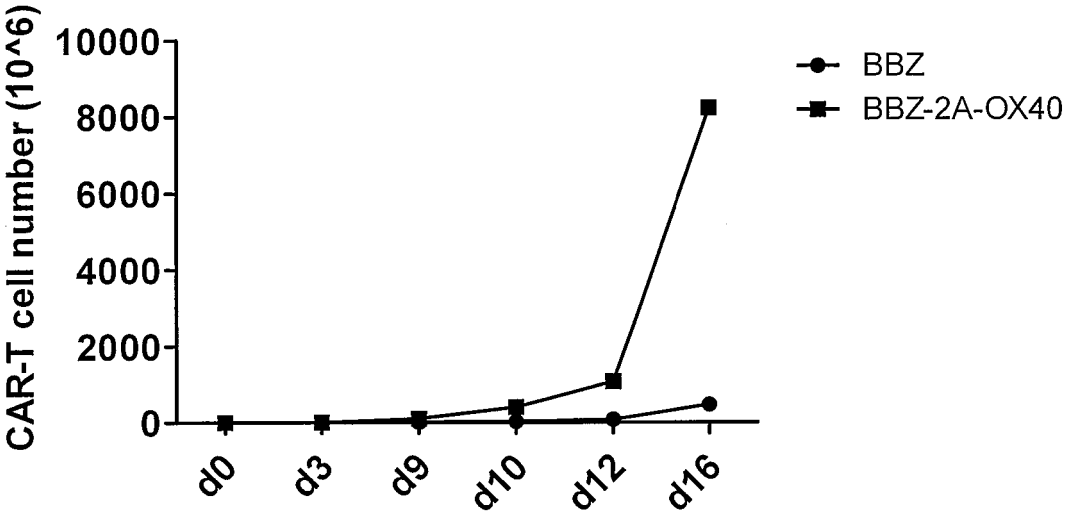
CAR-T cell number (10^6)
10000
8000
6000
4000
2000
0
d0
d3
d9
d10
d12
d16
BBZ
BBZ-2A-OX40

FIG. 12

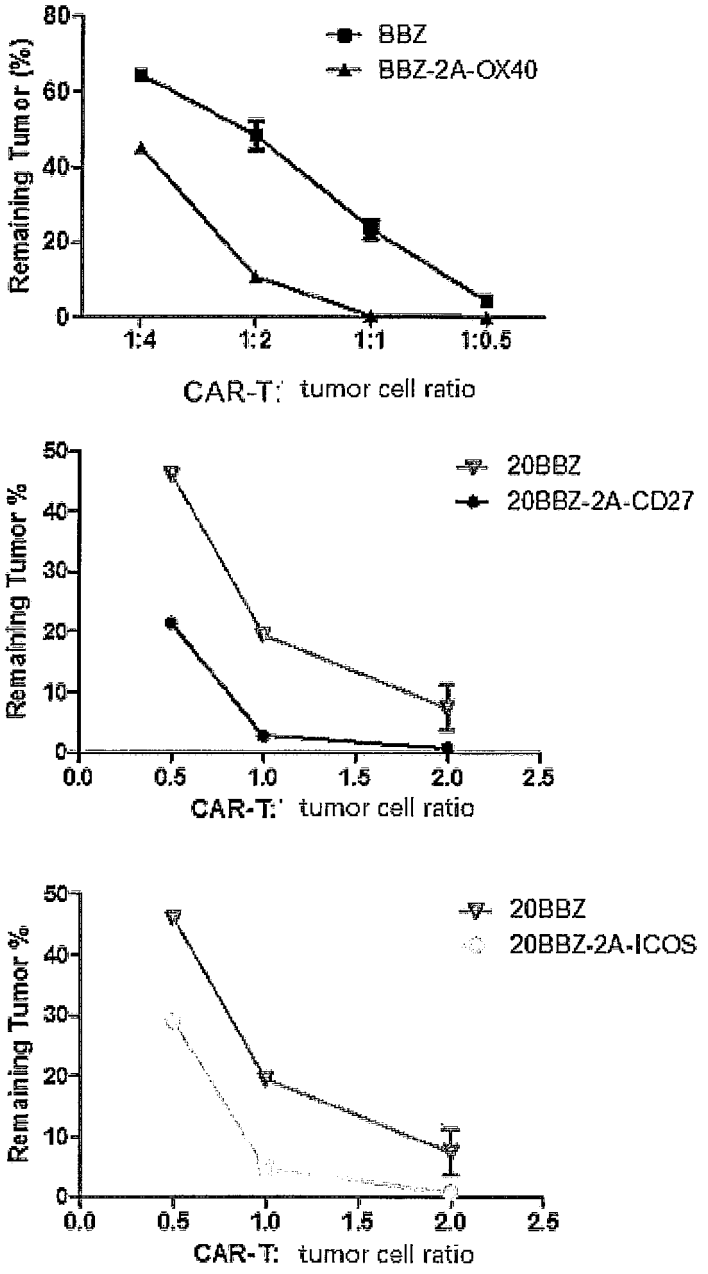
Remaining Tumor (%)
80
60
40
20
0
1:4
1:2
1:1
1:0.5
BBZ
BBZ-2A-OX40
CAR-T: tumor cell ratio
Remaining Tumor %
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
20BBZ
20BBZ-2A-CD27
CAR-T: tumor cell ratio
Remaining Tumor %
20BBZ
20BBZ-2A-ICOS
CAR-T: tumor cell ratio

FIG. 13

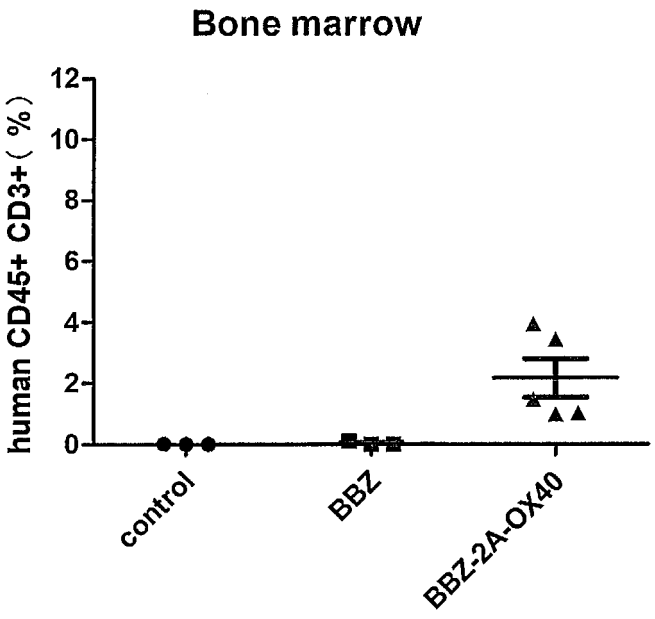
Bone marrow
human CD45+ CD3+ (%)
12
10
8
6
4
2
0
control
BBZ
BBZ-2A-OX40

FIG. 14

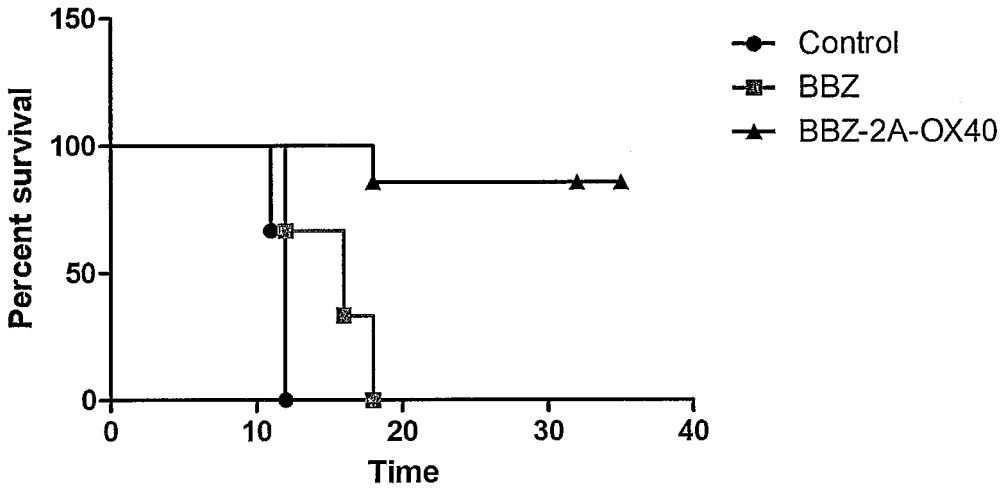
Percent survival
150
100
50
0
0
10
20
30
40
Time
Control
BBZ
BBZ-2A-OX40

FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2019/077922

A. CLASSIFICATION OF SUBJECT MATTER

C07K 19/00(2006.01)i; A61K 48/00(2006.01)i; A61P 35/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K, A61K, A61P

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

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

CNKI, CNABS, CNTXT, DWPI, CPEA, SIPOABS, EPTXT, WOTXT, USTXT, JPTXT, ELSEVIER, EMBASE, and Key Terms: CAR, CAR-T, 嵌合抗原受体, 共表达, co-express+, CD19, CD20, HVEM, OX40, ICOS, CD27, 4-1BB等; GENBANK, EMBL, 中国专利生物序列检索系统和检索的序列, China Patents Biological Sequence Search System and seared sequences: SEQ ID NO: 1-10.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOICE, M. et al. "Loss of the HVEM Tumor Suppressor in Lymphoma and Restoration by Modified CAR-T Cells" *Cell*, Vol. 167, 06 October 2016 (2016-10-06), p. 412, right column, last paragraph, and figure 7	1, 2
Y	BOICE, M. et al. "Loss of the HVEM Tumor Suppressor in Lymphoma and Restoration by Modified CAR-T Cells" *Cell*, Vol. 167, 06 October 2016 (2016-10-06), p. 412, left column, last paragarph to right column, paragarph 2, and p. 412, tight column, paragarph 1, and figure 7	3-10
Y	CN 107384963 A (BELLASTEM BIOTECHNOLOGY LIMITED) 24 November 2017 (2017-11-24) claims 1-7, and description, paragraph [0003]	3-10

☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 2019	**23 May 2019**

Name and mailing address of the ISA/CN	Authorized officer
State Intellectual Property Office of the P. R. China (ISA/CN) **No. 6, Xitucheng Road, Jimenqiao Haidian District, Beijing 100088** **China**	
Facsimile No. **(86-10)62019451**	Telephone No.

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2019/077922

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

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

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2019/077922

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
CN 107384963 A	24 November 2017	None	

Form PCT/ISA/210 (patent family annex) (January 2015)