

Chimeric Antigen Receptor against Human CD19 Antigen and Its Application

TECHNICAL FIELD

The present invention relates to the genetic engineering field, and more particularly relates to anti-human CD19 antigen chimeric antigen receptor and the application thereof. Furthermore, it relates to a lentiviral vector containing the anti-human CD19 chimeric antigen receptor and the application thereof.

BACKGROUND

Treatment with murine-derived antigen will cause human anti-mouse antibody (HAMA) reaction due to the heterogeneity of murine-derived antigen and will also cause a short half-time period and a quick-erase effect, and ultimately efficacy loss. Therefore, the therapeutic murine-derived antigen needs to be humanized to increase the degree of humanization, and to weaken HAMA. Antibody is one of the most important immune system molecules, and the stability of antigen produced by humoral immunity in vivo is the result of natural evolution and selection. However, murine-derived or humanized antibodies produced by cell engineering and genetic engineering are recombinant proteins, which can be easily degraded in the process of production, transportation, storage and use in vivo. When antibodies are applied in vivo for tumor therapy, the affinity and specificity of antibodies and the tolerance of antibodies under different PH conditions, especially slightly acidic environment can all affect antibody stability and biological activity. Hence, it is important to improve the ability of antibody drugs, and to optimize the function of antibody effect, which plays an important role in clinical application. However, the stability and reliability of the antibody have been plaguing antibody industry for a long time, which is also a serious bottleneck that needs to be broken through in the whole biological field. It is necessary for us to humanize murine-derived antibodies and to modify their stability, specificity and affinity.

Chimeric antigen receptor (CAR) is an artificial receptor that simulates the TCR function, comprising an antigen recognition domain, a hinge region, a transmembrane region, an intracellular signaling domain, etc. When the antigen (receptor) on the surface of cancer cells binds to CAR antibody (ligand), the signal is transmitted into the cells through hinge region and transmembrane region, and then intracellular signaling domain converts the signal into an activation signal to activate effector cells. The effector cells then kill the tumor cells by secreting perforin or producing cytokines while the effector cell itself has undergone amplification, to further expand the immune killing effect.

CD19 is a B cell surface transmembrane protein (Cluster of differentiation 19 protein, CD19), which is closely related to activation, signal transmitting and growth regulating of B cells. In addition, CD19 is a functional receptor molecule on the surface of B lymphocyte cells that can produce the dual antigen binding mode when B-cell antigen receptor (BCR) recognizes an antibody, can also be involved in the transport of Ca^{2+} in B cells, and can regulate the activation and proliferation of B cell. CD19 can be used in the diagnosis and prognosis of leukemia, lymphoma and immune system diseases as an important marker.

Although there are many antibodies for clinical applications, they can far from meet the clinical needs. There are still other technical problems: lack of a comprehensive consideration addressing the extent of humanization, CAR's optimal combination and specificity, stability and better tumor clearance ability. Using non-humanized monoclonal antibodies, for example,

murine-derived monoclonal antibodies, may cause many defects. On one hand, murine-derived antibody usually cannot mediate complement-dependent lysis or dissolve human target cells through antibody-dependent cytotoxicity or Fc receptor-mediated phagocytosis. On the other hand, human body may recognize non-humanized monoclonal antibodies as exogenous proteins to induce harmful allergies immune response: human anti-mouse antibody (HAMA) reaction. Therefore, it is necessary to conduct more researches to further optimize the CD19 antibody.

Currently, CAR technology has been used in many hematological tumors and solid tumors researches, especially in hematological tumors. However, how to select specific and stable scFv sequences to form the optimal combination of CAR still needs to be explored.

SUMMARY

In view of this, the present invention is directed to chimeric antigen receptor (CAR) against human CD19 antigen (hereinafter referred to as anti-CD19 CAR). The said anti-CD19 CAR can be expressed in T lymphocyte stably with high specificity and have better ability to remove tumor cells.

To achieve the above objective, the technical proposal of the present invention is showed below:

Provided is a chimeric antigen receptor (CAR) against human CD19 antigen, consisting of a single-chain antibody (scFv) recognizing human CD19 antigen, a hinge region, a transmembrane region, and an intracellular signaling domain connected in sequence. The single-chain antibody (scFv) recognizing human CD19 antigen has an amino acid sequence as shown in SEQ ID NOs. 1, 2, 3, 4, 35, 36, or 37.

The present invention needs to overcome two technical obstacles, 1. seek more stable and effective humanized monoclonal antibody (scFV) to recognize the CD19 antigen, 2. obtain the best combination of CAR.

The amino acid sequence of the scFV recognizing human CD19 antigen is obtained by humanized modification. Why? Because the currently used anti-CD19 CAR cannot stably express in T lymphocytes, especially in patents' T lymphocytes, and the expression of the anti-CD19 CAR decreased significantly with the prolongation of culture time after transfection. The higher degree of humanized scFV can effectively reduce the immunogenicity of CAR and enhance the sustainability and safety of CAR-T in vivo. The scFv recognizing human CD19 antigen in present invention is modified from mouse anti-human CD19 monoclonal antibody FMC63. Amino acid sequences of CDR region in light and heavy chain variable region of FMC63 monoclonal antibody strain of mouse anti-human CD19 antigen are shown in SEQ ID NOs. 1,2,3,4,5 or 6.

	The amino acid sequence
1	QDISKY
2	HTS
3	QQGNTLPYT
4	GVSLPDYG
5	IWGSETT
6	AKHYYYGGSYAMDY

Humanization methods is: Humanization of the backbone sequence other than the key residues refer to the sequence of human antibody skeleton region, then the humanized antibody is randomly mutated, to preferably obtain a humanized ScFv of anti-human CD19 antigen, and the amino acid sequences are shown in SEQ ID NOs. 1, 2, 3, 4, 35, 36 or 37. The said monoclonal antibody is composed of a light chain variable region, a heavy chain variable region, and an IgG Fc constant region. The nucleotide sequence of the said scFV of anti-human CD19 antigen are shown in SEQ ID NOs. 51, 52 or 53 which are corresponding to SEQ ID NOs. 35, 36 or 37 respectively.

Preferably/In typical embodiment, the amino acid sequences of the heavy chain variable region of the said humanized ScFv of anti-human CD19 antigen are shown in SEQ ID NOs. 38, 39 or 40. And the amino acid sequences of the light chain variable region of the said humanized ScFv of anti-human CD19 antigen are shown in SEQ ID NOs. 41, 42 or 43.

Preferably/In another embodiment, the amino acid sequences of the said heavy chain variable region contained in SEQ ID NOs. 4, 5 or 6 include at least 1, 2 or 3 modifications but not more than 30, 20 or 10 amino acid sequences, and the amino acid sequences of the said heavy chain variable region have 95-99% identity with the amino acid sequence shown in SEQ ID NOs. 38, 39 or 40.

In another embodiment, the amino acid sequences of the said light chain variable region contained in SEQ ID NOs. 41, 42 or 43 include at least 1, 2 or 3 modifications but not more than 30, 20 or 10 amino acid sequences, and the amino acid sequences of the said light chain variable region have 95-99% identity with the amino acid sequence shown in SEQ ID NOs. 41, 42 or 43.

Furthermore, the amino acid sequences of hinge region are shown in SEQ ID NOs 5 or 44.

Furthermore, the transmembrane region is CD8TM or CD28TM or the amino acid sequence shown in SEQ ID NO.59; the CD8TM amino acid sequence is shown in SEQ ID NO: 6, the CD28TM amino acid sequence is shown in SEQ ID NO: 7.

Furthermore, the intracellular signaling domain is CD28 and / or CD137 and / or CD3; the CD28 amino acid sequence is shown in SEQ ID NO: 8, the CD137 amino acid sequence is shown in SEQ ID NO: 9, and the CD3 amino acids sequence is shown in SEQ ID NO: 10.

As a preference/Preferably, the intracellular signaling domain is CD28 or CD3.

As a preferred/ Preferably, the intracellular signaling domain is CD137 or CD3.

Furthermore, the amino acid sequences of the CAR are shown in SEQ ID NOs.11, 12, 13, 14, 15, 16, 17, 18, 45, 46 or 47.

The present invention is also directed to a preparation method for a lentiviral vector of the CAR, including the following steps:

1) Synthesis of CAR gene sequence of anti-human CD19 antigens: including leader peptide, different single-chain antibodies of anti-human CD19 antigen, the hinge region, the transmembrane region and the intracellular signaling domain. The nucleotide sequence of the leader peptide is shown in SEQ ID NO.31.

2) The lentiviral vector expressing CAR is obtained by connecting the gene sequences of the DNA fragments with restriction endonuclease or Gibson cloning method.

Furthermore, step 1), the nucleotide sequences of the CAR are shown in SEQ ID NOs. 23, 24, 25, 26, 27, 28, 29, 30, 48, 49 or 50.

Furthermore, after step 2), pack and purify the lentiviral vector.

The present invention is also directed to a lentiviral vector obtained by the method above.

The said lentiviral vector infected with cells has high positive expression rate, and the infection rate of primary immune cells (T lymphocytes) is high for the patients who are difficult to be infected, and the CAR transfected with the said lentiviral vector can express CAR cells stably in the culture process, and the positive rate of CAR do not decrease over time. Thus, T cells infected with the said lentiviral vector have the function to kill target cells.

The present invention is also directed to an application of the above-mentioned CAR or lentiviral vector in the preparation of CAR immune cells expressing human CD19 antigen.

Furthermore, the immune cells are T lymphocytes, NK cells, macrophages or DC cells.

The method of importing the lentiviral vector into immune cells includes gene gun, electroporation or viral transduction. Preferably/In typical embodiment, the method is viral transduction.

The present invention is also directed to a T cells infected with the lentiviral vector.

The present invention is also directed to an application of the above-mentioned CAR or lentiviral vector or T cells in the preparation of B-cell malignancy drugs.

Furthermore, the cells or tissues of B-cell malignancy can express CD19.

CD19 mutations are associated with severe immunodeficiency syndrome and B-cell lymphoma. Specific B-cell lymphoma tumors expressing CD19 antigen include but are not limited to B-cell acute lymphoid leukemia (B-ALL), B-cell presenile lymphoid leukemia, maternal plasma-like dendritic cell tumors, diffuse large B-cell lymphoma, follicular lymphoma, small or large cell follicular lymphoma, malignancy lymphoproliferative disorder, MALT lymphoma, mantle cell lymphoma, marginal zone lymphoma, non-Hodgkin lymphoma, plasma-blastic lymphoma, plasma-like dendritic cell tumors and leukemia.

Furthermore, the B-cell lymphoma tumors include acute lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia, B- (B-CLL), B cell lymphoma, Hodgkin's (B-HL) and non-Hodgkin's lymphoma (B-NHL).

The present invention is also directed to an application of the amino acid sequence of the scFV recognizing human CD19 antigen in the preparation of B-cell malignancy drugs.

Furthermore, the cells of B-cell malignancy can express CD19.

The present invention is also directed to a method for the treatment of diseases associated with CD19 expression in cells or tissues, including the application of an effective dose of cells expressing the above-mentioned CAR to mammals.

Furthermore, CD19-related diseases expressed in the cells or tissues are B-cell-related diseases and B-cell malignant tumors.

Furthermore, combination of cells expressing CAR molecule with active agents for enhancing the efficacy of cells expressing CAR molecule or alleviating side effects.

The side effects are associated with the cells expressing CAR.

Furthermore, cells expressing CAR molecule are combining with drugs targeting or acting on CD19 molecule. The cells expressing CAR molecule can be applied in combination with other known active agent and therapeutic drugs. "Combination" in this article is meant herein, two or more different treatments are delivered to an individual in the course of illness, for example, after an individual is diagnosed with a disease and before the disease is cured or eliminated or treatment is discontinued for some reasons, two or more treatments are delivered to an individual. In some embodiments, the first treatment continues at the beginning of the second one. This is sometimes referred to as a "simultaneous" or "parallel delivery" due to overlap in application. In other embodiments, after one treatment delivery is completed, other treatment delivery is initiated. In some embodiments of either case, treatment is more effective due to combination. For example, the second treatment is more effective than the second treatment without the first one. For example, the same effect is observed in case of fewer second treatments, or the second treatment reduces symptoms to a greater extent, or similar conditions are observed in the first treatment. In some embodiments, the reduction in other parameters associated with delivery is greater than that observed when one treatment is delivered without another. The effects of the two treatments can be partially, completely, or more than additive. Delivery can be made so that the effect of the first treatment delivered can be detected when the second treatment is delivered.

The cells expressing CAR molecule and at least one other therapeutic agent can be concurrently administered in the same composition or in separate compositions, or administered sequentially.

In some embodiments, the expressed CAR molecule can be co-expressed with other target CAR molecules in the same cell for the treatment of malignant tumors, such as the co-expression of the expressed CAR molecule and the gene modified cells targeting CD22 molecule for the treatment of malignant tumors, or the cells expressing CAR molecule may be co-expressed with CAR expression cells targeting other targets, such as CAR-T cells, use in combination to treat the malignant tumor.

In some embodiments, the expressed CAR molecule can be modified. Specifically, the "switch" molecule can be designed at the 3' or 5' ends of the expressed CAR molecule to regulate the expression of CAR. Specifically, the tag gene expression tag protein can be designed at the 3' or 5' ends of the expressed CAR molecule to track the expression of CAR cells or regulate the expression of CAR.

In certain circumstances, the expressed CAR molecule can be used in combination with the following treatments: surgery, chemotherapy, radiation, immunosuppressive agents, such as cyclosporine, azathioprine, methotrexate, mycophenolate and FK506, antibodies or other

immunoablative agents, such as CAMPATH, anti-CD3 antibodies or other antibodies, cytoxan, fludarabine, cyclosporine, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines and irradiation. The expressed CAR molecule can be used in combination with active agents targeting extracellular matrix proteins like tenascin, such as anti-tenascin antibody and ²¹¹At-labeled anti-tenascin antibody. Some embodiments may be used in combination with immunomodulators, such as interferon- α , interferon- β , TGF- β 2 peptide inhibitors, or poly-ICLC.

The present invention is also directed to a drug for the mammalian diseases related to cells or tissues expressing CD19. The drug comprises cells expressing the above CAR molecule and pharmaceutically acceptable vectors or auxiliaries.

The "vector" mentioned above is a cloning vector or an expression vector, including but not limited to, one or more plasmids (expression plasmids, cloning vectors, small loops, microcarriers, double minute chromosomes), retroviruses and lentivirus vector.

The present invention is also directed to an application of the amino acid sequences of the scFV recognizing human CD19 antigen in the preparation of the vectors capable of capturing B-cell malignant tumors expressing CD19. The amino acid sequences mentioned above are shown in SEQ ID NOs. 1, 2, 3 or 4. The modified polypeptide fragments are more stable and specific than those before modification.

The present invention is also directed to an application of the amino acid sequences in the preparation of CAR backbone, the said amino acid sequences are shown in SEQ ID NOs. 1, 2, 3, 4, 35, 36 or 37.

In general, the scFV-CAR in the present invention is expressed in immune cells, not only to maintain the CAR positive rate during patients' cells culturing, but also to enhance the proliferation and tumor killing ability of CAR-T, and has no toxic side effects on antigen-negative cells and can also be used for targeted therapy of tumors.

The term "human CD19" refers to a human cluster of differentiation 19 protein (CD19), an antigen that can be detected and expressed in B-cells tumors. The amino acid sequence of human CD19 is found in UniProt / Swiss-Prot with ID No. P15391, while nucleotide sequences encoding human CD19 can be found in ID No. NM_001178098. Most human CD19 can be expressed in B cell tumors, e.g. acute lymphoblastic leukemia, chronic lymphocytic leukemia and non-Hodgkin's lymphoma.

The term "humanized monoclonal antibody" refers to an artificially modified mouse monoclonal antibody, and murine-derived monoclonal antibody is modified and re-expressed by gene clone and DNA recombination technology. Most amino acid sequences are replaced by humanized sequences, retain the basic affinity and specificity of parental murine-derived monoclonal antibody, but reduce its heterogeneity, in favor of the antibody used in the human body.

The term "Single-chain antibody variable region fragment, ScFv" refers to the antibody fragment with antigen binding ability, which is connected by the amino acid sequences of VL region and that of VH region through the joint polypeptide.

The term "chimeric antigen receptor (CAR)" is an artificially modified receptor that can anchor the specific molecules (e.g. antibody) of tumor antigens on immune cells (e.g. T cells),

so that immune cells recognize the tumor antigens or virus antigens and kill tumor cells or virus-infected cells.

The term "co-stimulation signal molecule" refers to some adhesion molecules on the surface of the immune cells, such as CD28, CD134 / OX40, CD137 / 4-1BB, and CD40. By binding with ligands, the second signal of immune cells is activated, the proliferation ability of immune cells and the secretion function of cytokines are enhanced, and the survival time of activated immune cells is prolonged.

The term "Immunoreceptor tyrosine-base activation motifs, ITAM" refers to the immune cells activate associated receptor (e.g., BCR / 1α / 1β , TCR / CD3, Fc α R and Fc γ R). The common amino acid sequence in the cytoplasmic domain is based on tyrosine residues (Y). Two tyrosine residues are separated by about other 13 amino acid residues (YXX [L/V] X7-11YXX [L/V]). Tyrosine is the phosphorylation site of protein kinase and can bind to signal molecules downstream of signal transduction pathway after phosphorylation, and lead to cell activation.

Advantages of the present invention:

1) The CAR provided by the present invention can recognize anti human CD19 antigen, and can be more stably expressed in immune cells (T cells, NK cells, etc.) and can effectively kill CD19 antigen positive tumor cells, and has a better tumor cells cleaning ability. It can not only maintain the stable expression of CAR molecules in primary immune cells (e.g. T lymphocytes) of patients not easily infected, but also can enhance the proliferation and tumor killing ability of CAR-T, has no toxic side effects on antigen-negative cells, and can be used in targeted therapy of cancer.

2) The CAR provided by the present invention is modified with high humanization degree, which can effectively reduce the immunogenicity of CAR, and enhance the sustainability and safety of CAR-T in vivo.

3) The CAR provided by the present invention can stably express in T lymphocytes, especially T lymphocytes derived from patients, and can be used in preparation of drugs for the treatment of hematological malignancies aimed at adoptive cells of tumors.

BRIEF DESCRIPTION

FIG 1 is Structural simulation of variable region of humanized monoclonal antibody against human CD19 antigen;

FIG 2 is Structural schematic diagram of CAR targeting CD19 and design and identification of lentivirus expression vector;

FIG 3 is Cell phenotype detected result after CAR-T expressing targeted CD19 was cultured for 12 days;

FIG 4 is Cytotoxicity to CD19-positive and CD19-negative tumor cells by CR-T expressing targeted CD19;

FIG 5 is Proliferation of CAR-T targeting CD19 after stimulation by CD19 positive cells;

FIG 6 is Treatment of CD19-positive transplanted tumors by targeting CAR-T in mice model of blood transplantation tumors;

FIG 7 is Purification of humanized monoclonal antibodies against human CD19 antigen;

FIG 8 is Affinity Map of Humanized Monoclonal Antibody;

FIG 9 is Half-life map of humanized monoclonal antibodies;

FIG 10 is Structural sketch of CAR targeting CD19;

FIG 11 is Cell phenotype detected result after CAR-T expressing targeted CD19 was cultured for 12 days;

FIG 12 is Cytotoxicity to CD19-positive and CD19-negative tumor cells by CR-T expressing targeted CD19;

FIG 13 is Cytokine release from CAR-T targeting CD19 stimulated by CD19 positive cells;

FIG 14 is Treatment of CD19-positive transplanted tumors by targeting CRR-T in mice model of blood transplantation tumors;

FIG 15 is Therapeutic effects of CAR-T targeting CD19 on human Raji-Luc tumor cells in mice under different stimulus signals.

EMBODIMENTS

Hereinafter, preferred embodiments of the present invention will be described in detail with reference to the accompanying drawings. Wherever there are no specific experimental conditions specified, the experimental methods in general follow the conventional conditions, such as those described in the Molecular Cloning Experiment Guide (third edition, J. Sambrooke et al.), or those recommended by the manufacturers. The described embodiments are intended to better illustrate the contents of the invention, but the contents of the invention are not limited to the embodiments. Therefore, any non-essential improvement and adjustment implemented by the person skilled in the art to the above-mentioned inventions shall still fall within the scope of protection of the present invention.

Embodiment Design of humanized monoclonal antibodies against human CD19 antigen

(1) The amino acid sequences of CDR region in the heavy and light chain variable regions of FMC63 monoclonal antibody strain of mouse anti-human CD19 antigen are shown in SEQ ID NO.38, SEQ ID No.39, SEQ ID No.40, and SEQ ID No.41, SEQ ID No.42, SEQ ID No.43. FIG 1 is a Structural simulation of the variable region of humanized monoclonal antibody against human CD19 antigen. After sequence analysis and alignment in IMGT/BLAST database, the highest homologous human antibody sequence was selected as the template for modification.

(2) Two methods have been adopted to modify the template sequence

a. After simulating the molecular docking, the amino acid residues most closely related to antigen-antibody docking are shown in Table 1. Humanized modification of backbone sequence other than key residues with reference to human antibody backbone region, then random mutation of humanized antibodies was performed, and then affinity evaluation and prediction were carried out by means of antigen-antibody affinity screening and molecular docking simulation. Preferred amino acid sequence of humanized single chain antibody (ScFv) against human CD19 antigen is obtained, which is shown in SEQ ID NOs.35, 36 or 37.

Table 1 Key Amino Acid Residues

Key amino acid residues	
VL	DIQMTQTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHS GVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEIK
VH	EVKLQESGPGLVAPSQLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSE TTYNSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDY WGQGTSTVTVSS

b. 15 ScFV sequences were obtained by random mutation after replacing the human antibody skeleton. The optimal sequence was screened by antibody purification and stability characteristics.

Table 2 shows the result of random mutations of the monoclonal antibody.

The subsequent embodiments screen the ScFV sequences obtained by the two methods respectively, so the subsequent embodiments are expressed separately.

Table 2

humanized1	DIQMTQSPSSLSASVGDRTVSGCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVTLPDYGVSWIRQPPGKALEWLGVIWGGSETTYNSALKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized2	DIQMTQSPSSLSASVGDRTVIGCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVTLPDYGVSWIRQPPGKALEWLGVIWGGIETTYNSALKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized3	DIQMTQSPSSLSASVGDRTVITCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVTLPDYGVSWIRQPPGKALEWLGVIWGGIETTYNSALKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized4	DIQMTQSPSSLSASVGDRTITCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPGKALEWLGVIWGGSETTYNSALKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized5	DIQMTQSPSSLSASVGDRTITCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPGKALEWLGVIWGGSETTYNTSLKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized6	DIQMTQSPSSLSASVGDRTVIGCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPKALEWLGVIWGGSETTYTTSLKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized7	DIQMTQSPSSLSASVGDRTVIGCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPKALEWLGVIWGGSETTYTTSLKTRLTISKDNSKNQVLTMTNMDGVDTATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized8	QVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPGKALEWLGVIWGGSETTYNTSLKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSSGGGGGGGGGGGGGGGGGGGGDIQMTQSPSSLSASVGDRTVITCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIK
humanized9	DIQMTQSPSSLSASVGDRTVITCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPGKALEWLGVIWGGSETTYSTSLKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized10	DIQMTQSPSSLSASVGDRTVITCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPKALEWLGVIWGGSETTYQTSLKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized11	DIQMTQSPSSLSASVGDRTVITCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYFCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPGKALEWLGVIWGGIETTYNSLSLKTRLTISKDNSKNQVLTMTNLDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized12	DIQMTQSPSSLSASVGDRTVIGCRASQDISKYLWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVTLPDYGVSWIRQPPGKALEWLGVIWGGIETTYNSLSLKTRLTISKDNSKNQVLTMTNLDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized13	QVQLQESGPGLVKPSQTLSTCTVSGVTLPDYGVSWIRQPPGKALEWLGVIWGGIETTYNSLSLKTRLTISKDNSKNQVLTMTNLDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSSGGSTSGSGKPGSGEGSTKGDIQMTQSPSSLSASVGDRTVIGCRASQDISKYLWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIK
humanized14	DIQMTQSPSSLSASVGDRTVITCRASQDISKYLNWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIKGSTSGSGKPGSGEGSTKGQVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPGKALEWLNVIWGGSETTYSTSLKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSS
humanized15	QVQLQESGPGLVKPSQTLSTCTVSGVSLPDYGVSWIRQPPGKALEWLNVIWGGSETSYTSLKTRLTISKDNSKNQVLTMTNMDPVDATYYCAKHHYYGGSYAMDYWGQGSSVTVSSGGGGGGGGGGGGGGGGGGGGDIQMTQSPSSLSASVGDRTVITCRASQDISKYLWYQQKPGKAPRLIYHTSRLHSGVPSRFSGSGSGTDYTLTISSSLQPEDFATYYCQQGNTLPYTFGGGTRLEIK

Part 1 Reform version of scheme a

Embodiment 1 Preparation of lentiviruses targeting CAR expressing human CD19 antigen (CD19)

(1) Preparation of CAR gene sequence targeting human CD19 antigen

ScFv, hFc hinge region, transmembrane region and intracellular signal domain were synthesized, and their structures were shown in Fig. 2. The nucleotide sequence of the leading peptide is shown in SEQ ID NO.5 and the amino acid sequence is shown in SEQ ID NO.6. The amino acid sequences of hCD19-1, hCD19-2, and hCD19-3, the humanized ScFV of anti-human CD19, are shown in SEQ ID NOs. 35, 36 or 37, the nucleotide sequences of which are shown in SEQ ID NOs. 51, 52 or 53. The nucleotide sequence of the hFc hinge region is shown in SEQ ID NO. 54, and the amino acid sequence is shown in SEQ ID NO.44. The nucleotide sequence of transmembrane region is shown in SEQ ID NO.55, and the amino acid sequence is shown in SEQ ID NO.59. The nucleotide sequences of intracellular signal domain are shown in SEQ ID NOs.56 or 57.

The nucleotide sequences of finally synthesized humanized anti-CD19 CAR are shown in SEQ ID NOs.48, 49 or 50, and unhumanized mouse anti-control antibody was named mCD19. It serves as the CAR nucleotide sequence of ScFv as shown in SEQ ID NO.58.

Then the CAR sequence was used as template for PCR amplification. The reaction system was added according to the instructions of KOD FX NEO DNA polymerase (purchased from TOYOBO Company). The reaction conditions were pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 s, annealing at 55°C for 15 s, and extension at 68°C for 30 cycles. After the amplification products were identified, the DNA fragments were recovered by the Recovery Kit (Promega Company). The specific method was described in the instructions, and CAR was recovered, and the DNA recovery fragments were sent to Genscript (Nanjing) for sequencing.

(2) Construction of lentiviral vector pl-CAG-CD19-8h-28TM-28Z expressing CAR

The gene sequence encoding CAR was cloned by restriction endonuclease, and enzyme digestion followed the instructions. The recombinant products were recovered and linked to the synthesized CAR sequence fragments. The expression vectors of pl-CAG-CD19-8h-28TM-28Z loaded with different ScFv were obtained. The expression vectors of CAR were named mCD19, hCD19-1, hCD19-2 and hCD19-3 lentivirus vectors according to the different loadings of ScFv.

The structure is shown in Figure 2. The lentiviral vectors were transformed into *E.coli* TOP10. The monoclones were cultured for 12 hours. The plasmids were extracted by Plasmid Extraction Kit (Invitrogen Company). The specific method is shown in the instruction. The extracted plasmids were identified by restriction endonuclease HindIII digestion electrophoresis. The results are shown in Fig. 2.

Embodiment 2. Preparation of CAR of CD19 antigen modified T cells

(1) lentivirus packaging

In this embodiment, calcium phosphate method is used for packaging lentivirus. The specific steps are described in the molecular cloning experiment guide (Third Edition, J. Sam Brook et al).

(2) lentivirus purification

The virus supernatant was collected and filtered by centrifugation and transferred to a new centrifugal tube. According to the amount of virus supernatant, 50% PEG6000 (w/v) and 4M NaCl were added respectively, and then the final concentration of PEG6000 was 8.5% and the final concentration of NaCl was 0.3M. The sample was centrifuged at 4°C and at a rate of 5000r/min for 30 minutes, and the supernatant was discarded, and the virus was re-suspended in DMEM medium containing 10% FBS at 200 mu, 1.5mL EP. Pipe packing, stored at - 80°C for reserve.

(3) Determination of titer lentivirus

293T cells were infected with the virus and centrifuged for 5 minutes at 1000r/min after 72 hours to collect the cells. QIAamp DNA Blood Mini Kit was purchased from Qiagen Company (No. 511004) for genome extraction kit. According to the kit instruction, the viral titer was determined by qRT-PCR and analyzed by analytical software. The viral titer was calculated according to the standard curve. The results were expressed by TU/mL.

(4) lentivirus infected T cells

1) Isolation of Human Peripheral Blood Monocytes

Peripheral blood samples were collected into the blood collection tube with anticoagulant and poured into 50mL centrifugal tubes, with 30 mL in each tube. They were diluted with 7.5mL hydroxyethyl starch and naturally settled at room temperature (18-25°C), and superficial plasma was collected, centrifuged for 15 minutes. Then, normal saline was used for re-suspension and precipitation, adding at 1:1 volume ratio to lymphocyte separation solution, and gradient centrifugation was used to extract the blood samples. The second layer of white lymphocyte was washed twice with normal saline and the cells were suspended in normal saline to obtain human peripheral blood mononuclear cells.

2) lentiviral vector infected T lymphocytes

PBMC was cultured in RPMI 1640 complete medium containing 10% FBS. On the first day, anti-CD3 monoclonal antibody was added to activate PBMC. Lentiviral infection was carried out on the first three days. Lentiviral vectors including mCD19, hCD19-1, hCD19-2 and hCD19-3 were added, and uninfected peripheral blood lymphocyte (PBMC) was used as blank control. Medium was replaced by RPMI 1640 complete medium containing 500 IU/mL recombinant human IL-2 and continued for 10-20 days.

Embodiment 3. Detection of CAR expression targeting human CD19 antigen

Flow cytometry was used to detect the expression of CAR in T cells. T cells infected with the virus were collected and cultured for 14 days. The cells were suspended in PBS solution containing 1% FBS (v/v) and the cell density was adjusted to 1×10^6 /mL. The collected cells were put into six tubes, with 100μL in each tube. 20μL FITC-labeled mouse anti-human CD3 monoclonal antibody (Biolegend Company), PerCP-labeled mouse anti-human CD8

monoclonal antibody (Biolegend Company), and Alexa Fluor 647 labeled Protein L (Thermo Fisher Company) were added to each tube. They were incubated at 4°C for 30 minutes, washed with PBS solution twice, and detected by flow cytometry. The results are as showed in Figure 3: FL1 channel was CD3 positive, and FL4 channel was Protein L positive, indicating that the positive rate of CAR expression in T cells was over 70%. Above all, FL3 channel is the proportion of CD3 positive cells and CD8 positive cells in Protein L positive cells. These results showed that mCD19, hCD19-1, hCD19-2 and hCD19-3 lentiviral vectors were successfully transfected into T cells. The positive rate of CAR expression was over 70% and 40%, which could be used for further identification experiments.

Embodiment 4. Validation of anti-tumor effect of T lymphocyte expressing CAR targeting CD19

The target cells were CD19 positive Raji cells (Raji-luc) and K562 cells (K562-hCD19-luc) which stably express luciferase and human CD19 antigen, and the CD19-negative K562 cells (K562-luc) stably expressing luciferase were used as contrast. The effector cells were mCD19, hCD19-1, hCD19-2 and hCD19-3 lentiviral vectors infected T cells. The PBMC cells without infection by virus were used as contrast. On the basis of the ratio of 10: 1, 5: 1 and 1: 1 of effector cells, each group sets 3 duplicate wells. According to the standard method provided by the Steady-Glo®Luciferase Assay System (Promega Cat. #E2520) kit, the killing rate was calculated by the following formula.

$$\text{Cytotoxicity} = (1 - \text{Fluorescence intensity of target cells co-cultured with effector cells} / \text{Fluorescence intensity of target cell}) \times 100\%$$

The killing effect of CAR-T cells with different target-to-target ratios is shown in Fig. 4A/B/C, and the release of IFN- γ factor in CAR-T cells after 24 hours is shown in Fig. 4D. The results showed that T lymphocyte of the CAR expressing targeting CD19 antigen has significant killing effect on CD19 positive tumor cells, but weaker killing effect on CD19 negative cells and no significant difference from PBMC group. ELISA assay showed that CAR-T cells release IFN- γ , and stimulation signals of CAR-T cells work properly. Meanwhile, by comparing the killing effect of different CAR-T cells, it was found that the killing effect of CAR-T on CD19 positive cells in hCD19-3 lentivirus transfection group was not significantly different from that of mCD19 lentivirus transfection group before modification, but the killing effect on CD19 negative cells was significantly reduced, suggesting that the humanized CAR on ScFv improved the specific killing performance.

Embodiment 5. Detection of Long-term Proliferation and Cytokine Secretion Ability of CAR-T Expressing CD19 after Stimulation

(1) Detection of Long-term Proliferation of CAR-T Expressing CD19 after Stimulation

CAR-T cells infected with viral vectors were stimulated and activated in vitro and cultured for a long time. Flow cytometry was used regularly to detect the proportion of CAR-T cells with CD3 and double positive protein-L. Then the number of CAR-T cells was calculated by multiplying the total number of cells with the proportion of CAR-T cells, and the proliferation curve was drawn to test whether CAR-T cells possessed the long-term proliferation ability after stimulation and evaluate their clinical therapeutic potential. CAR-T cells were co-cultured with CD19-positive Raji cells and K562 cells transfected with CD19 (K562-hCD19) to stimulate the activation of CAR-T cells. Compared with CD19-negative K562 cells, CAR-T cells were co-cultured with target cells at a ratio of 5:1. The release of cytokines was

detected by ELISA. On the 3rd and 7th day after stimulation, the number of CAR-T cells was counted, and the CD4 and CD8 positive ratios of CD3 and protein-L double positive CAR-T cells, as well as CD4 and CD8 positive cells, CD45RA+/RO-/CD62L+, CD45RA-/RO+/CD62L+ and CD45RA+/-/CD62L-cell ratios were detected by flow cytometry. If the number of cells increased on the 7th day, the second stimulation was carried out using the target cells. When the number of cells did not increase after stimulating N times, the proliferation curve of CAR-T cells during target cell stimulation was analyzed statistically. The results were shown in Fig. 5A. Experiments showed that the proliferation of CAR-T cells stimulated by hCD19-2 and hCD19-3 was not significantly different from that of mCD19, suggesting that the two CAR-T cells with humanized ScFv maintained the same post-stimulation proliferation as that of mCD19.

(2) Detection of Cytokine Release Ability of CAR-T Expressing CD19 after Stimulation

Target cells were plated in 24-well plate with 5×10^4 cells / well, and CAR-T cells were co-cultured with target cells at a target-to-target ratio of 5:1 for 24 hours and supernatants were collected by centrifugation. Fresh medium was added at a ratio of 1:1 after 48 hours of stimulation. If the number of cells constantly increased on the 7th day, the target cells were added for the second stimulation and supernatants were collected after 24 hours of stimulation activation. When the number of cells did not increase after stimulating N times, the cell culture supernatant was collected for the last time. Test results found that the number of CAR-T cells did not proliferate after three stimulation activations. Supernatants were collected 24 hours after each stimulation activation. The release of IFN- γ , IL-2 and TNF- α factors were detected by ELISA after centrifugation. The results were shown in Fig. 5B/C/D. Experiments showed that the hCD19-3 lentivirus infection group had better IFN- γ , IL-2 and TNF- α release ability after three stimulations, which indicated that the long-term stimulation of hCD19-3 lentivirus had better factor release performance than mCD19, and had better clinical application prospects than mCD19 CAR-T cells.

Embodiment 6. Anti-tumor effect of CAR-T targeting CD19 in animal models

A mouse transplanted tumor model of human CD19 positive tumor cell line was established to verify the anti-tumor effect of CAR-T targeting CD19 in animal models.

The use of mice as NOD.Cg-PrkdcscidII2rgtm1Sug/JicCrl (abbreviated as NOG) in vivo validation was developed by Mamoru Ito from the Laboratory Animal Research Institute of Japan (CIEA). It is a mouse strain with severe immune dysfunction established by hybridizing NOD/SCID mice and γ -chain IL-2 receptor knockout (IL2ryKO) mice and is the most common strain of carcinogenic test in vivo in the world. The mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd, and were fed and studied in accordance with national laboratory animal regulations under the SPF level. Tumor-targeting cells used for in vivo validation are CD19 positive cell line Raji (Raji-luc) stably expressing firefly luciferase, which was previously validated in vitro. The effector cells were CAR-T cells infected with lentiviral vectors mCD19, hCD19-1, hCD19-2 and hCD19-3. The control group was PBMC cells infected with no virus.

Perkin Elmer's IVS in vivo imaging system was used to photograph and image the growth of tumors. The imaging results and survival curve of mice were shown in Fig. 6. The results showed that the transfection of CAR-T cells with hCD19-3 lentivirus showed better anti-tumor effect than other groups. All the 8 experimental mice in this group survived on the 30th day.

Based on the above results, compared with the non-humanized mouse-anti-human CAR-T, the anti-human CD19 antigen CAR-T obtained by the present invention has better killing specificity in the in vitro cell experiment, and has better therapeutic effect in animal experiments in vivo. It has application value for clinical treatment of CD19 positive hematological tumors such as relapsed B cell acute lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia (CLL) and B cell non-Hodgkin lymphoma (B-NHL) in children and adults.

Part 2 Reform version of scheme b

Embodiment 1. Purification of humanized monoclonal antibodies targeting human CD19 antigen

Stably transfected cell line that stably expresses a humanized antibody was constructed, the cell supernatant harvested liquid was filtered through 0.45μm membrane, and the filtrate was collected. The PH value was measured by diluting it with a balanced buffer of equal volume. The specific steps of purification with AKTAPrime are in GE product manual as follows: ultrafiltration concentration, then sterilization and filtration with 0.22μm needle filter, aliquoting of samples, marking and storage in -80°C refrigerator. Sampling and testing. Use SDS and Western blot for detection. The specific steps can be found in the GE antibody purification manual. The test results are shown in Figure 7. Four monoclonal antibodies were selected to obtain high purity antibodies, namely, ScFv-humanized4 amino acid sequence shown in SEQ ID NO.1, ScFv-humanized5 amino acid sequence shown in SEQ ID NO.2, ScFv-humanized9 amino acid sequence shown in SEQ ID NO.3, and ScFv-humanized11 amino acid sequence shown in SEQ ID NO.4.

Embodiment 2. Specific detection of humanized monoclonal antibodies targeting human CD19 antigen

The monoclonal antibody scFV labeled with His label was cloned into plasmid vector and transfected into HEK293 cells. The supernatant of HEK293 cells was harvested through a 0.45μm filter and filtrate was extracted. The PH value was measured by diluting it with a balanced buffer of equal volume. Ultrafiltration concentration, sterilization and filtration with 0.22μm needle filter, aliquoting, marking and storage in -80°C refrigerator. Different humanized antibodies were incubated with Raji of CD positive cells and K562 of CD19 negative cells for 30 minutes and then the redundant antibodies were washed off. After staining with FITC-His antibody for 30 minutes, the unlabeled antibodies were washed out for flow cytometry. The results showed that four monoclonal antibodies could recognize Raji of positive cells but could not recognize K562 of negative cells as shown in Table 3. The amino acid sequence of ScFv-humanized4 is shown in SEQ ID NO. 1, the positive cell detection rate was 90.82%, and the negative cell detection rate was 0.7%. The amino acid sequence of ScFv-humanized5 is shown in SEQ ID NO.2, the positive cell detection rate was 84.04%, and the negative cell detection rate was 0.5%. The amino acid sequence of ScFv-humanized9 is shown in SEQ ID NO. 3, the positive cell detection rate was 87.75%, and the negative cell detection rate was 0.4%. The amino acid sequence of ScFv-humanized11 is shown in SEQ ID NO. 4, the positive cell detection rate was 80.60%, and the negative cell detection rate was 0.19%. Compare with commercialized antibodies, the positive cell detection rate was 91.47%, and the negative cell detection rate was 0.88%.

Table 3

Group	Antibody Specificity Detection %				
	Raji	K562	Group	Raji	K562
Humanized 1	10.00	0.90	Humanized 8	6.14	0.20
Humanized 2	0.87	0.60	Humanized 9	87.75	0.40
Humanized 3	3.71	6.80	Humanized 10	19.81	8.2
Humanized 4	90.82	0.70	Humanized 11	80.60	0.19
Humanized 5	84.04	0.50	Humanized 12	9.65	0.80
Humanized 6	4.50	0.90	Humanized 13	1.00	0.40
Humanized 7	9.87	1.23	Humanized 14	0.98	0.33
Humanized 15	0.87	0.12	Commercialized	91.47	0.88

Embodiment 3. Stability determination of humanized monoclonal antibody targeting CD19

1) avidity test at room temperature

The optimized monoclonal antibody scFV labeled with His was cloned into plasmid vector and transfected into HEK293 cells. 6 gradients of purified scFV-His, scFV-His and negative control were diluted with PBS to incubate CD19-positive Raji cells. The positive rate of Raji CD19 was detected by flow cytometry. The affinity of different scFV to CD19 was analyzed by flow cytometry and MFI statistics. 3 independent repetitions were carried out. The results are shown in Fig. 8 and Table 4. The larger the K_d (nM), the smaller the affinity.

Table 4

	humanized4	humanized5	humanized9	humanized11
Affinity	1.993ug/mL	6.216ug/mL	4.757ug/mL	18.496ug/mL
WM (KDa)	27.29	27.32	27.31	27.30
K _d (nM)	73.0304	227.5256	174.1853	677.2794

2) Detection of half-life of antibody

4 kinds of scFV were obtained by adding 4 groups of CD19-expressing cancer cells. Each group was incubated with 5 gradient concentrations for 1 hour at 4°C to remove the unconjugated antibody. The antibody was dissociated at 37°C. The activity of the antibody was detected 15, 30, 45 and 60 minutes after dissociation. The results are shown in Fig. 9 and Table 5, in which the dissociation rate responds to the half-life of the antibody, and the smaller the absolute value of the dissociation rate, the longer the half-life of the antibody.

Table 5

	humanized4	humanized5	humanized9	humanized11
Dissociation rate (MFI)	-4.138 ± 0.2746	-2.744 ± 0.2476	-0.593 ± 0.0809	-0.976 ± 0.2076

Embodiment 4. Preparation of lentiviruses expressing targeting human CD19 antigen

(1) The CAR sequences of ScFv, hIgG-Fc hinge region, transmembrane region and intracellular signal domain containing leading peptide (signal peptide) and anti-CD19 antigen were synthesized. The structure of the sequences is shown in Fig. 10. The nucleotide sequence of leading peptide is shown in SEQ ID NO. 31, the nucleotide sequences of ScFv of anti-CD 19 antigen are shown in SEQ ID NOs. 19, 20, 21 or 22, the amino acid sequence of hIgG-Fc hinge region is shown in SEQ ID NO.5, the amino acid sequences of transmembrane region (CD8TM or CD28TM) are shown in SEQ ID NOs. 6 or 7, and the amino acid sequences of intracellular signal domain (CD28, CD137, CD3) are shown in SEQ ID NOs. 8, 9 or 10. The nucleotide sequences of finally synthesized humanized anti-CD19 CAR are shown in SEQ ID NOs. 23, 24, 25, 26, 27, 28, 29 or 30, the amino acid sequences of which are shown in SEQ ID NOs.11, 12, 13, 14, 15, 16, 17 or 18. The un-humanized mouse antibody as contrast was named mCD19.

The amplification and reaction system were operated according to the instructions of KOD FX NEO DNA polymerase (purchased from TOYOBO Company). The DNA fragment was then recovered by using the Recovery Kit (Promega Company). The specific method was described in the instructions, recovered for CAR, and the DNA recovery fragment was sent to Genscript (Nanjing) for sequencing.

(2) Construction of lentiviral vector expressing CAR

According to the different loads of ScFv, CAR expression vectors were abbreviated as PCAR M19, PCAR M20, PCAR 011, PCAR 012, PCAR 013, PCAR 014, PCAR 015, PCAR 016, PCAR 017, PCAR 018, PCAR 019, PCAR 020, PCAR 021, PCAR 022, PCAR 023, PCAR 024, PCAR 025, and PCAR 026 lentivirus vectors. PCAR M19 and PCAR M20 were mouse-derived controls.

Enzyme digestion was carried out according to the instructions. DNA fragments were recovered by agarose gel electrophoresis, and then the target fragments and vector fragments were linked by T4 ligase (purchased from Promega Company). Lentiviral vectors expressing CAR were obtained. The structures of four CAR structures and four humanized antibody combinations shown in FIG. 10 were 16 combinations. Plasmid extraction kit (Invitrogen Company) is used to extract plasmids. The specific method is described in the instructions.

Embodiment 5. Preparation of CAR-T of CD19 antigen

(1) Lentiviral packaging: the same as Embodiment 2 in Part 1

(2) Purification of lentivirus: the same as Embodiment 2 in Part 1

(3) Measurement of titer lentivirus: the same as Embodiment 2 in Part 1

(4) T cells infected with lentivirus

1) Isolation of human peripheral blood monocytes: the same as Embodiment 2 in Part 1

2) Lentivirus vector infection of T lymphocyte

PBMC was cultured in RPMI 1640 complete medium containing 10% FBS. The anti-CD3 monoclonal antibody was activated and then infected with lentivirus. Lentivirus vectors were added to PBMC as blank control. After 24 hours, the medium was replaced with RPMI 1640

complete medium containing 500 IU/mL recombinant human IL-2 and continued to be cultured for 10-20 days. The CAR-T cells obtained were named PCAR M19, PCAR M20, PCAR 011, PCAR 012, PCAR 013, PCAR 014, PCAR 015, PCAR 016, PCAR 017, PCAR 018, PCAR 019, PCAR 020, PCAR 021, PCAR 022, PCAR 023, PCAR 024, PCAR 025, PCAR 026, and PCAR 003.

3) Detection of CAR expression targeting human CD19 antigen

T cells infected with the virus were collected and cultured for 10 days. The cell density was adjusted to $1 \times 10^6/\text{ml}$. The positive rate of Protein-L was detected by flow cytometry. The results showed that the positive rate of T lymphocyte was expressed by different CD19-CAR combinations on the 4th and 10th day of culture. Results are shown in Table 3: the positive rates of CAR were higher in PCAR 019, PCAR 020, PCAR 021, PCAR 022, PCAR 023, PCAR 024, PCAR 025 and PCAR 026 infected T cells. PCAR M19 and PCAR M20 were used as contrast to detect the positive rates of T cells infected with PCAR 019, PCAR 020, PCAR 021, PCAR 022, PCAR 023, PCAR 024, PCAR 025 and PCAR 026 virus cultured for 4 and 10 days. As shown in Fig. 11 and Table 6, the infection efficiency of T cells PCAR021, PCAR022 and PCAR025 was good, and the positive rate of CAR was stable and self-activation probability was low with culture time.

Table 6

CAR-T Positive Rate Statistics (%)					
Groups	4 DAYS	10 DAYS	Group	4 DAYS	10 DAYS
Control T	0.00	0.00	Control T	0.00	0.00
PCAR M19	24.87	34.60	PCAR M20	54.75	78.4
PCAR 019	53.71	66.80	PCAR 023	39.81	48.2
PCAR 020	65.82	79.70	PCAR 024	20.60	25.90
PCAR 021	64.04	66.50	PCAR 025	69.65	60.80
PCAR 022	54.50	59.90	PCAR 026	61.00	75.40

Embodiment 6. Validation of Anti-Tumor Effect of T Lymphocyte Expressing CAR Targeting CD19

Detailed procedures are the same as Embodiment 4 in Part 1, and the killing efficiency target ratio was 1:1.

The killing results are shown in Fig. 12A/B and Table 7. The results show that the T lymphocyte of the optimized combination of humanized monoclonal antibody scFV has significant killing effect on CD19 positive tumor cells.

Table 7

CAR Killing Rate (%)					
Groups	Raji-Luc	K562-Luc	Group	Raji-Luc	K562-Luc
Control T	10.26	21.53	Control T	6.68	3.96
PCAR 003	29.61	73.39	PCAR M20	50.76	79.24
PCAR 019	35.44	71.46	PCAR 023	49.42	78.94
PCAR 020	38.13	73.68	PCAR 024	46.13	81.67
PCAR 021	31.33	70.33	PCAR 025	44.25	79.64
PCAR 022	35.08	72.46	PCAR 026	50.27	81.82

Embodiment 7. Detection of Cytokine Secretion Ability of CAR-T Expressing CD19

Cytokine IFN- γ was detected by Elisa with BD Biosciences kit. Detection kit NO: 555142, batch 6266958; specific steps are shown in kit instructions.

DETERMINATION: The absorbance (OD) of each hole is measured sequentially at 450 nm wavelength with zero-setting of blank hole. The determination should be carried out within 15 minutes after adding termination solution.

Results are shown in Fig. 13 and Table 8. IFN- γ was secreted 24 hours after killing target cells by 1:1 CAR-T cells. The optimized combination of humanized monoclonal antibody scFV can work normally and secrete IFN- γ .

By comparing the killing effects of different CAR-T cells, it was found that the killing effects of the selected combination of monoclonal antibodies ScFv on the CAR-T cells PCAR 019, PCAR 020, PCAR 021, PCAR 022, PCAR 023, PCAR 024, PCAR 025 and PCAR 026 were stronger than those of the in-situ combination of 8 kinds of CAR-T cells. The CD19 positive cell killing effect is remarkable.

Table 8

	Factor Detection (pg/ml)				
Group	Raji-Luc	K562-Luc	Group	Raji-Luc	K562-Luc
Control T	164.8	84.06	Control T	307.6	283.6
PCAR 003	18490	27500	PCAR M20	7015	8458
PCAR 019	20490	28120	PCAR 023	11580	10620
PCAR 020	20030	29470	PCAR 024	10730	11930
PCAR 021	16940	27930	PCAR 025	10660	12910
PCAR 022	15200	28010	PCAR 026	13190	15490

Embodiment 8. Anti-tumor effect of CAR-T targeting CD19 in animal models

Construction of transplanted tumor model in mice is the same as that in Part 1 of Embodiment 6. The effector cells for injection are lentiviral vectors PCAR 019, PCAR 020, PCAR 021, PCAR 022, PCAR 023, PCAR 024, PCAR 025, and PCAR 026 infected CAR-T cells. The control group is saline group, non-infected PBMC cells and unreformed anti-FMC63 scFV combination of CAR-T cells PCAR M19 and PCAR M20. The obtained monoclonal antibody modified CAR was named PCAR 003 as in vivo killing effect contrast.

CAR-T cells 5E05 cells/mice injected into tail vein 3 days after tumorigenesis. CAR-T cells were injected and then photographed every seven days by Perkin Elmer's IVS in vivo imaging system to show the growth of the tumors. The survival of mice was observed and recorded daily. The results showed that PCAR 021, PCAR 022, PCAR 023, PCAR 025 and PCAR 026 in Table 9 and Table 10 showed better therapeutic effect than those in the control group PCAR M19, PCAR M20 and the combination of pre-modification antibodies CARPCAR003. The smaller the fluorescence value, the better the therapeutic effect of survival mice.

Table 9

Average value		2d	9d	16d	23d	30d	37d	44d
Aliquot	Survival	2	9	16	23	30	37	44
Salin	0/6	3.27E+06	1.23E + 09	1.32E + 10				
ControlT	0/7	2.78E+06	9.51E + 08	8.15E + 09				
PCAR M19	0/7	7.74E+05	1.84E + 08	5.04E + 09	5.03E + 09			
PCAR003	1/7	3.14E+06	1.59E + 07	2.72E + 08	3.36E + 09	4.77E + 09	4.56E + 08	
PCAR019	1/7	2.87E+06	1.14E + 07	1.95E + 08	1.03E + 09	5.97E + 09	3.94E + 09	
PCAR020	1/7	2.84E+06	3.19E + 06	1.86E + 07	3.05E + 08	6.67E + 08	2.04E + 09	
PCAR021	4/7	3.12E+06	2.48E + 06	8.32E + 06	2.01E + 07	1.56E + 09	6.64E + 08	2.05E + 09
PCAR022	2/5	3.40E+06	4.88E + 06	3.67E + 07	2.91E + 07	2.12E + 09	2.20E + 09	3.40E + 09

Table 10

Average value		7d	21d	35d	49d	63d	77d
Aliquot	Survival	7	21	35	49	63	77
Salin	0/7	1.30E + 06	1.49E + 08	4.98E + 09	2.08E + 08		
PCARM20	1/7	1.29E + 06	8.15E + 08	4.28E + 09	1.51E + 10	5.13E + 08	1.42E + 09
PCAR023	5/7	9.19E + 05	6.66E + 06	5.35E + 07	2.24E + 08	9.13E + 08	3.98E + 09
PCAR024	1/7	1.44E + 06	1.42E + 08	3.77E + 09	7.85E + 09	1.37E + 10	4.70E + 07
PCAR025	6/7	7.97E + 05	2.70E + 06	7.21E + 07	3.39E + 09	1.28E + 10	2.94E + 09
PCAR026	3/6	1.35E + 06	1.52E + 08	2.07E + 09	6.01E + 08	3.35E + 09	6.47E + 09

Imaging result and mice survival curves are as shown in FIG. 14 and FIG. 15. The results show that PCAR 021, PCAR 022, PCAR 023 and PCAR 025 showed better anti-tumor effects than other groups. All the mice survived on the 30th days.

Based on the above results, compared with the non-humanized mouse-anti- human CAR-T, the anti-human CD19 antigen CAR-T obtained by the present invention has better killing specificity in the in vitro cell experiment, and has better therapeutic effect in animal experiments in vivo; especially after the second optimization the obtained humanized antibody ScFv-humanized4 amino acid sequence shown in SEQ ID NO.1 and ScFv-humanized9 amino acid sequence shown in SEQ ID NO.3 and ScFv-humanized11 amino acid sequence shown in SEQ ID NO.4 combination PCAR 021, PCAR 022, PCAR 023, PCAR 025 and PCAR 026 are respectively shown to be good for the treatment of animals as shown in SEQ ID NO. 13, SEQ ID NO. 14, SEQ ID NO. 15, SEQ ID NO. 17 and SEQ ID NO. 18. Our PCAR series CAR has application value for clinical treatment of CD19 positive hematological tumors such as relapsed B cell acute lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia (CLL) and B cell non-Hodgkin lymphoma (B-NHL) in children and adults.

Finally, the above embodiments are only used to illustrate the technical scheme of the present invention, not to limit it. Although the present invention is described in detail with reference to better embodiments, it should be understood by person skilled in the art that the technical scheme of the present invention can be modified or replaced equally, without departing from the purpose and scope of the technical scheme of the present invention, and that they should all be covered by the scope of claims in the present invention.

CLAIMS

1. Chimeric anti-human antigen receptor CD19 antigen (CAR), characterized in that, comprising single-chain antibody (scFv) recognizing human CD19 antigen, hinge region, transmembrane region and intracellular signal domain connected in sequence; the amino acid sequence of the single-chain antibody (scFv) recognizing human CD19 antigen is as shown in SEQ ID NO.1 or SEQ ID NO.2 or SEQ ID NO.3 or SEQ ID NO.4 or SEQ ID NO.35 or SEQ ID NO.36 or SEQ ID NO.37.
2. The CAR according to claim 1, characterized in that, the amino acid sequence of the hinge region is as shown in SEQ ID NO: 5.
3. The CAR according to claim 1, characterized in that, the transmembrane region is the amino acid sequence shown in CD8TM or CD28TM; the amino acid sequence of the CD8TM is as shown in SEQ ID NO: 6, and the amino acid sequence of the CD28TM is as shown in SEQ ID NO: 7.
4. The CAR according to claim 1, characterized in that, the intracellular signaling domain is CD28 and /or CD137 and /or CD3; the amino acid sequence of CD28 is as shown in SEQ ID NO: 8, the amino acid sequence of CD137 is as shown in SEQ ID NO: 9, and the amino acid sequence of CD3 is as shown in SEQ ID NO: 10.
5. The CAR according to any one of claims 1-4, characterized in that, the amino acid sequence of the CAR is as shown in SEQ ID NO.11 or SEQ ID NO.12 or SEQ ID NO.13 or SEQ ID NO.14 or SEQ ID NO.15 or SEQ ID NO.16 or SEQ ID NO.17 or SEQ ID NO.18, or SEQ ID NO.45 or SEQ ID No.46 or SEQ ID NO.47.
6. A method of preparing lentiviral vector expressing the CAR according to any one of claims 1 to 4, characterized in that, comprising the following steps of:
Step 1) Synthesis of CAR gene sequence of anti-human CD19 antigens: different single chain antibodies (ScFv) containing leader peptide and anti-human CD19 antigen, a hinge region, a transmembrane region and an intracellular signal domain; the nucleotide sequence of the leader peptide is as shown in SEQ ID NO.31;
Step 2) The gene sequence of the DNA fragment was connected through restriction enzyme to obtain the lentiviral vector expressing the CAR.
7. The method according to claim 6, characterized in that, the nucleotide sequence of the CAR mentioned in step 1) is as shown in SEQ ID NO.23 or SEQ ID NO.24 or SEQ ID NO.25 or SEQ ID NO.26 or SEQ ID NO.27 or SEQ ID NO.28 or SEQ ID NO.29 or SEQ ID NO.30 or SEQ ID NO.48 or SEQ ID NO.49 or SEQ ID NO.50.
8. The method according to claim 6, characterized in that, after the step 2), the lentiviral vector is packaged and purified.
9. The lentiviral vector obtained by the method according to claim 8.
10. Use of the CAR according to claim 1-5 or the lentivirus vector according to claim 8, for preparing CAR immune cells that express target human CD19 antigen.
11. The use according to claim 10, characterized in that, the CAR immune cells are T lymphocytes.

12. T cells infected by the lentivirus vector of claim 9.
13. Use of the CAR according to claim 1-5 or the lentiviral vector according to claim 8, or the T cells according to claim 12, for the production of a medicament for treating B cell malignancy.
14. The use according to claim 13, characterized in that, cells or tissues of the said B cell malignancy are capable of expressing CD19.
15. The use according to claim 13, characterized in that, the said B-cell malignancy includes B-cell acute lymphoblastic leukemia (B-ALL), B-cell chronic lymphocytic leukemia (B-CLL), B-cell Hodgkin's lymphoma (B-HL) and B-cell non-Hodgkin's lymphoma (B-NHL).
16. Use of the amino acid sequence of scFV recognizing human CD19 antigen according to claim 1, for the production of a medicament for treating B cell malignancy.
17. The use according to claim 16, characterized in that, the said B cell malignancy is capable of expressing CD19.
18. A medicament for treating a mammalian disease associated with CD19-expression cell or tissue,
characterized in that, the medicament comprises cells that express the CAR according to anyone of claim 1-5 and pharmaceutically acceptable carrier or auxiliary.
19. Use of the amino acid sequence of scFv recognizing human CD19 antigen according to claim 1, for the production of vector that is able to accurately capture B cell malignancy that expresses CD19.
20. Use of the amino acid sequences as shown in SEQ ID NO. 1 or SEQ ID NO.2 or SEQ ID NO. 3 or SEQ ID NO. 4 or SEQ ID NO. 35 or SEQ ID NO. 36 or SEQ ID NO. 37, for the production of antigen recognition domain in CAR structure.