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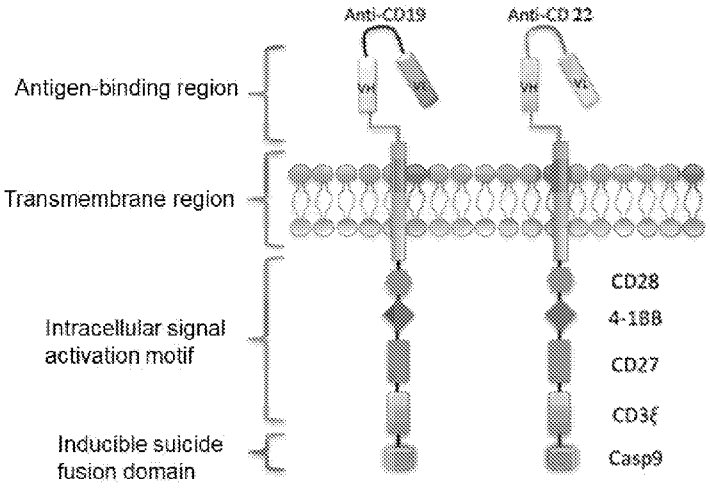

Figure 1

(57) Abstract: Provided is an immune cell mixture comprising an immune cell genetically modified with a chimeric antigen receptor targeting CD19 and an immune cell genetically modified with a chimeric antigen receptor targeting CD22. The chimeric antigen receptor targeting CD19 and the chimeric antigen receptor targeting CD22 each comprises an antigen-binding domain, a transmembrane domain, a costimulatory signaling region, a CD3ζ signaling domain, and an inducible suicide fusion domain in tandem arrangement. The chimeric antigen receptors specifically recognize tumor surface antigens CD19 and CD22. Compared to using other single-targeted chimeric antigen receptor T cells, using CAR-T cells targeting two antigens achieves better therapeutic effects, which makes CD19 escape not easy to occur, and allows the disease to be easily relieved.

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CD19- AND CD22-BASED COMBINED CAR-T IMMUNOTHERAPY

FIELD

The present application relates to the field of cellular immunotherapy for tumors, in particular to an immune cell mixture comprising an immune cell genetically modified with a chimeric antigen receptor targeting CD19 and an immune cell genetically modified with a chimeric antigen receptor targeting CD22, and an application thereof, and specifically to a method for constructing a chimeric antigen receptor T (CAR-T) cell technology based on tumor specific targets CD19 and CD22 and its application in anti-tumor therapy.

BACKGROUND

With the development of immunology theory and clinical technology for tumors, chimeric antigen receptor T cell (CAR-T) immunotherapy has become one of the most promising immunotherapies for cancer treatment. The chimeric antigen receptor (CAR) typically consists of a tumor-associated antigen-binding region, an extracellular hinge region, a transmembrane region, and an intracellular signaling region. The CAR generally comprises a single chain fragment variable (scFv) region of an antibody or a binding domain specific for a tumor-associated antigen (TAA), which is coupled to the cytoplasmic domain of a T cell signaling molecule via hinge and transmembrane regions. The most common lymphocyte activation moieties include a T cell costimulatory domain in tandem with a T-cell effector function triggering (e.g. CD3ζ) moiety. The CAR-mediated adoptive immunotherapy allows CAR-transplanted T cells to directly recognize the TAAs on target tumor cells in a non-HLA-restricted manner.

Most patients with B-cell malignancies including B cell acute lymphocytic leukemia (B-ALL) and chronic lymphocytic leukemia (CLL) will die from their disease. One approach to treat these patients is to genetically modify T cells to target the antigens expressed on tumor cells through the expression of CARs. CAR is an antigen receptor designed to recognize cell surface antigens in a human leukocyte antigen (HLA)-independent manner. Attempts in using genetically modified cells expressing CARs to treat these types of patients have achieved promising success.

CD19 molecule is a potential target for the treatment of B lymphocyte tumors, and is also a

focus in CAR research. The expression of CD19 is restricted to normal and malignant B cells and thus is a widely accepted CAR target for safety tests. Although T cells genetically modified with a chimeric antigen receptor targeting the CD19 molecule (CD19 CAR-T) have achieved great success in the treatment of multiple, refractory acute B lymphocytic leukemia, they have significant poor therapeutic effects in the treatment of refractory, recurrent chronic B lymphocytic leukemia and B lymphocyte lymphoma.

CN 104788573 A discloses a chimeric antigen receptor hCD19scFv-CD8α-CD28-CD3ζ and use thereof. This second-generation chimeric antigen receptor is composed of variable regions of light and heavy chains of anti-human CD19 monoclonal antibody HI19a (hCD19scFv), a human CD8α hinge region, human CD28 transmembrane and intracellular regions, and a human CD3ζ intracellular region in tandem arrangement. In this patent, the expression level of CD19 is decreased after a single infusion of CAR-T cells, causing the tumor cells to easily escape immune mechanisms. In addition, this second-generation CART causes a strong immune factor storm which has safety concerns.

In addition, according to the actual statistics on the treatment of lymphoma with a fourth generation CD19-targeted chimeric antigen receptor accomplished by our center, among 9 patients, 4 patients showed CD19 strong positive in tumor tissue immunohistochemical staining and achieved complete remission after receiving CD19-targeted chimeric antigen receptor therapy, while among another 5 patients that showed weak CD19 expression, only 1 achieved remission after the therapy, 2 only achieved partial remission, 1 maintained stable disease and 1 had progressive disease. This result indicates that the treatment with only CD19-targeted chimeric antigen receptors is difficult.

Therefore, for CD19-negative relapse, as well as CD19 low-expression B-cell tumors, it is particularly important to combine another potential chimeric antigen receptor to address the problem of easy mutation and low expression of CD19. In addition to CD19, CD22 is also a potential target for the treatment of malignant B-cell tumors. Like CD19, CD22 is expressed only on the cell surface of B cell lines. According to previous studies on clinical trials using anti-CD22 antibodies, CD22 has been evaluated and confirmed to be a good target for the treatment of B cell malignancies.

Therefore, it is particularly important to find a chimeric antigen receptor that is highly specific

and highly targeted and can effectively improve the therapeutic effects of CARTs.

SUMMARY

In view of the fact that the current single-targeted CAR-T therapies for treating tumors don't have a desired long-term effect, and of the influence of tumor microenvironment on the therapeutic effects of the CAR-T technique, the present application provides an immune cell mixture comprising an immune cell genetically modified with a chimeric antigen receptor targeting CD19 and an immune cell genetically modified with a chimeric antigen receptor targeting CD22, and an application thereof. The present application initiates combining the two tumor targets, CD19 and CD22, possesses advantages including strong specificity and high targeting ability, and it can effectively improve and prolong the therapeutic effects of CARTs, shows a better therapeutic effect on surface antigens CD19 and CD22-positive leukemia or B-cell lymphoma and can effectively avoid the off-target escape as found in single-targeted therapy.

To achieve this purpose, the present application uses the following technical solutions:

In one aspect, the present application provides an immune cell mixture comprising an immune cell genetically modified with a chimeric antigen receptor targeting CD19 and an immune cell genetically modified with a chimeric antigen receptor targeting CD22.

In the present application, T cells are modified with lentiviral vectors encoding antigen binding domains that bind to tumor surface antigens CD19 and CD22, thus allowing the tumor surface antigens CD19 and CD22 to specifically bind to the chimeric antigen receptors of the present application. Thereby, CAR-T cells eliminate both tumor cells expressing CD19 and those expressing CD22, effectively avoiding the escape of tumor cells resulting from a low antigen expression, and enhancing the long-term immune effects of CAR-T cells.

In the present application, the two chimeric antigen receptors may be a separate chimeric antigen receptor targeting CD19 and a separate chimeric antigen receptor targeting CD22, respectively. Alternatively the chimeric antigen receptor targeting CD19 may be combined with the chimeric antigen receptor targeting CD22 to express as a dual chimeric antigen receptor, *i.e.*, the antigen binding domain thereof binds to tumor surface antigens CD19 and CD22. Both cases can achieve a combination therapy of the two chimeric antigen receptors.

According to the present application, the chimeric antigen receptor targeting CD19 and the chimeric antigen receptor targeting CD22 each comprises an antigen-binding domain, a transmembrane domain, a costimulatory signaling region, a CD3ζ signaling domain, and an inducible suicide fusion domain in tandem arrangement.

Preferably, in the case of a chimeric antigen receptor targeting CD19, the antigen-binding domain is a single chain antibody against tumor surface antigen CD19, and in the case of a chimeric antigen receptor targeting CD22, the antigen-binding domain is a single chain antibody against tumor surface antigen CD22.

According to the present application, the chimeric antigen receptor (CAR) of the genetically modified T cell and the single chain antibodies (scFv) of the antigen binding domains for CD19 and CD22 of the genetically modified T cells are exemplified below.

In a specific embodiment, the single chain antibody against tumor surface antigen CD19 has an amino acid sequence selected from any one of the group consisting of

(I) the amino acid sequence as shown in SEQ ID NO.1;

(II) an amino acid sequence that shares ≥90%, preferably ≥95%, more preferably ≥98%, most preferably ≥99% homology with the amino acid sequence as shown in SEQ ID NO. 1;

(III) an amino acid sequence that is obtained by modifying, substituting, deleting or adding one or several amino acids to the amino acid sequence as shown in SEQ ID NO. 1; and

the amino acid sequence has the activity of a single chain antibody against tumor surface antigen CD19.

The amino acid sequence (SEQ ID No. 1) of the single-chain antibody against the tumor surface antigen CD19 is listed as follows:

DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVP
SRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGSTSGSGKPGSGEGS
TKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTY
YNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTV
SS.

According to the present application, the amino acid sequence that shares more than 90% homology or the amino acid sequence that is obtained by modifying, substituting, deleting or

adding one or several amino acids can be replaced by other single chain antibodies or humanized CD19 single chain antibodies. The amino acid mutant still functions as a CD19 single-chain antibody.

In a specific embodiment, the single chain antibody against the tumor surface antigen CD22 has an amino acid sequence selected from any one of the group consisting of

(I) the amino acid sequence as shown in SEQ ID NO. 2;

(II) an amino acid sequence that shares ≥90%, preferably ≥95%, more preferably ≥98%, most preferably ≥99% homology with the amino acid sequence as shown in SEQ ID NO. 2;

(III) an amino acid sequence that is obtained by modifying, substituting, deleting or adding one or several amino acids to the amino acid sequence as shown in SEQ ID NO. 2; and

the amino acid sequence has the activity of a single chain antibody against tumor surface antigen CD22.

The amino acid sequence (SEQ ID No. 2) of the single-chain antibody against tumor surface antigen CD22 is listed as follows:

DIQMTQTTSSLSASLGDRVTISCRASQDISNYLNWYQQKPDGTVKLLIYYTSILHSGVP SRFSGSGSGTDYSLTISNLEQEDFATYFCQQGNTLPWTFGGGTKLEIKGSTSGSGKPGSSEGS TKGEVQLVESGGGLVKPGGSLKLSCAASGFAFSIYDMSWVRQTPEKRLEWVAYISSGGGTT YYPDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCARHSGYGTHWGVLFAYWGQGT LVTVSA.

According to the present application, the amino acid sequence that shares more than 90% homology or the amino acid sequence that is obtained by modifying, substituting, deleting or adding one or several amino acids can be replaced by other single chain antibodies or humanized CD22 single chain antibodies. The amino acid mutant still functions as a CD22 single-chain antibody.

According to the present application, T cells are genetically modified with the chimeric antigen receptor by lentiviral vectors. The CD19- and CD22-based CAR-T cells bind to tumor surface antigens CD19 and CD22, exhibiting a stronger tumor-killing effect.

According to the present application, the transmembrane domain is a CD28 transmembrane domain and/or a CD8α transmembrane domain. In some particular embodiments, the

transmembrane domain can be selected or modified by amino acid substitution.

According to the present application, the costimulatory signaling region is any one selected from the group consisting of a CD28 signaling domain, a CD27 signaling domain or a CD137 signaling domain, or a combination of at least two thereof. A person skilled in the art can adjust the arrangement of the CD28 signaling domain, CD27 signaling domain and CD137 signaling domain according to requirements. Different arrangements of the CD28 signaling domain, CD27 signaling domain and CD137 signaling domain will not affect the chimeric antigen receptor. The present application employs the order of CD28-CD27.

According to the present application, the fourth-generation CAR comprises an inducible suicide fusion domain which contains a Caspase 9 domain having the amino acid sequence as shown in SEQ ID NO. 3, which is as follows:

GSGATNFSLLKQAGDVEENPGPMGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGK
KVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHA
TLVFDVELLKLEGGGGSGGGGSGAMVGALESLRGNADLAYILSMEPCGHCLIINNVNFCRE
SGLRTRTGSNIDCEKLRRRFSSLHFMVEVKGDLTAKKMVLALLELARQDHGALDCCVVVIL
SHGCQASHLQFPGAVYGTDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFE
VASTSPEDESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGS
WYVETLDDIFEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTSAS.

According to the present application, the inducible suicide fusion domain is connected in tandem with the CD3ζ signaling domain via a 2A sequence. The 2A sequence will cause the protein expressed by the inducible suicide fusion domain to cleave off from the chimeric antigen receptor protein, thereby allowing the chimeric antigen receptor to exert its function. While the suicide fusion domain can be activated by injecting an activator, thereby causing the T cells expressing the chimeric antigen receptor to die to lose their functions.

According to the present application, the chimeric antigen receptor further comprises a signal peptide which is capable of directing transmembrane transfer of the chimeric antigen receptor. A person skilled in the art can select a signal peptide conventional in the art according to requirements. The signal peptide is a Secretory signal peptide, which is the signal peptide for gene GMCSFR and may have the amino acid sequence as shown in SEQ ID NO. 8, which is as follows: MLLLVTSLLLCELPHPAFLLIP.

Preferably, the Secretory signal peptide is a signal peptide for CD8a gene, and the Secretory signal peptide has the amino acid sequence as shown in SEQ ID NO. 9, which is as follows: MALPVTALLLPLALLLHAARP.

The chimeric antigen receptor of the present application may further comprise a hinge region. The hinge region may be selected by those skilled in the art according to actual situation, and is not particularly limited herein. The presence of a hinge region will not affect the performance of the chimeric antigen receptor of the present application.

According to the present application, the chimeric antigen receptor targeting CD19 and the chimeric antigen receptor targeting CD22 each comprises a signal peptide, an antigen-binding domain, a transmembrane domain, a costimulatory signaling region, a CD3ζ signaling domain, a 2A sequence and an inducible suicide fusion domain in tandem arrangement.

As a preferable technical solution, the chimeric antigen receptor is obtained by connecting a Secretory signal peptide, a CD19 antigen-binding domain and/or a CD22 antigen-binding domain, CD8α and/or CD28 transmembrane domain(s), a CD28 extracellular signaling domain, a CD28 intracellular signaling domain, a CD27 intracellular signaling domain, a CD3ζ intracellular signaling domain, a 2A sequence and a FBKP.Casp9 domain in tandem. Specifically, the arrangement is as follows:

Secretory-CD19 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9;

Secretory-CD22 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9.

In a specific embodiment, the chimeric antigen receptor Secretory-CD19 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9 has the amino acid sequence as shown in SEQ ID NO. 4 or an amino acid sequence that shares more than 90% homology therewith. The amino acid sequence as shown in SEQ ID NO. 4 is as follows:

MLLLVTSLLLCELPHPAFLLIPQVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGMN
WVRQAPGQGLEWMGWINTYTGEPTYADAFKGRVTMTTDTSTSTAYMELRSLRSDDTAVY
YCARDYGDYGMDYWGQGTTVTVSSGSTSGSGKPGSSEGSTKGDIVMTQSPDSLAVSLGER
ATINCRASKSVSTSGYSFMHWYQQKPGQPPKLLIYLASNLESGVPDRFSGSGSGTDFTLTISS
LQAEDVAVYYCQHSREVPWTFGQGTKVEIKAAAIEVMYPPPYLDNEKSNGTIIHVKGKHLC
PSPLFPGPSKPFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTR

KHYQPYAPPRDFAAYRSASGGGGSGGGGSQRRKYRSNKGESPVEPAEPCHYSCPREEEGSTI
PIQEDYRKPEPACSPGGGGSGGGGSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLD
KRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST
ATKDTYDALHMQALPPRTSGSGATNFSLLKQAGDVEENPGPMGVQVETISPGDGRTFPKRG
QTCVVHYTGMLEDGKKVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISP
DYAYGATGHPGIIPPHATLVFDVELLKLEGGGGSGGGGSGAMVGALESLRGNADLAYILSM
EPCGHCLIINNVNFCRESGLRTRTGSNIDCEKLRRRFSSLHFMVEVKGDLTAKKMVLALLEL
ARQDHGALDCCVVVILSHGCQASHLQFPGAVYGTDGCPVSVEKIVNIFNGTSCPSLGGKPK
LFFIQACGGEQKDHGFEVASTSPEDESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFVS
YSTFPGFVSWRDPKSGSWYVETLDDIFEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFN
FLRKKLFFKTSAS.

In a specific embodiment, the chimeric antigen receptor Secretory-CD19 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9 has the nucleotide sequence as shown in SEQ ID NO. 5 or an nucleotide sequence that shares more than 95% homology therewith. The nucleic acid sequence as shown in SEQ ID NO. 5 is as follows:

ATGCTGCTGCTGGTCACAAGCCTGCTGCTGTGCGAGCTGCCCCACCCCGCCTTTCT
GCTGATCCCCGACATCCAGATGACCCAGACCACCAGCAGCCTGAGCGCCAGCCTGGGC
GACAGAGTGACCATCAGCTGCCGGGCCAGCCAGGACATCAGCAAGTACCTGAACTGGT
ATCAGCAGAAACCCGACGGCACCGTGAAGCTGCTGATCTACCACACCAGCCGGCTGCA
CAGCGGCGTGCCCAGCAGATTTTCTGGCAGCGGATCTGGCACCGACTACAGCCTGACCA
TCTCCAACCTGGAACAGGAAGATATCGCTACCTACTTCTGTCAGCAGGGCAACACCCTG
CCCTACACCTTCGGCGGAGGCACCAAGCTGGAAATCACCGGCAGCACCAGCGGCTCCG
GCAAGCCTGGATCTGGCGAGGGCAGCACCAAGGGCGAAGTGAAGCTGCAGGAAAGCG
GCCCTGGCCTGGTCGCCCCTAGCCAGAGCCTGTCCGTGACCTGTACCGTGTCCGGCGTG
TCCCTGCCCGACTACGGCGTGTCCTGGATCAGACAGCCCCCCAGAAAGGGCCTGGAATG
GCTGGGCGTGATCTGGGGCAGCGAGACAACCTACTACAACAGCGCCCTGAAGTCCCGG
CTGACCATCATCAAGGACAACAGCAAGAGCCAGGTGTTCCTGAAGATGAACAGCCTGC
AGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACTACGGCGGCAGCTAC
GCCATGGACTACTGGGGCCAGGGCACCAGCGTGACAGTCTCTTCTGCGGCCGCAATTGA
AGTTATGTATCCTCCTCCTTACCTAGACAATGAGAAGAGCAATGGAACCATTATCCATGT

GAAAGGGAAACACCTTTGTCCAAGTCCCCTATTTCCCGGACCTTCTAAGCCCTTTTGGG
TGCTGGTGGTGGTTGGGGGAGTCCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTTA
TTATTTTCTGGGTGAGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATG
ACTCCCCGCCGCCCTGGGCCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGA
CTTCGCAGCCTATCGCTCCGCTAGCGGAGGTGGAGGTTCTGGAGGTGGTGGAAGTCAA
AGAAGGAAGTACCGCAGCAACAAAGGAGAATCTCCCGTCGAGCCAGCCGAGCCCTGTC
ATTATTCATGCCCAAGGGAGGAGGAGGGAAGTACAATCCCAATTCAAGAAGACTACAGG
AAGCCCGAACCTGCATGCAGTCCAGGTGGAGGCGGTTCTGGAGGCGGTGGCTCCCGGG
TGAAATTCTCACGGTCTGCAGACGCACCCGCTTACCAGCAAGGCCAGAACCAACTCTAT
AACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCC
GGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACA
ATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGA
GCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAG
GACACCTACGACGCCCTTCACATGCAGGCCCTGCCCCCTCGCACTAGTGGCTCCGGAGC
CACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATG
GGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTCCCCAAGCGCGGCC
AGACCTGCGTGGTGCACTACACCGGGATGCTTGAAGATGGAAAGAAAGTGGACTCCTC
CCGGGACAGAAACAAGCCCTTTAAGTTTATGCTAGGCAAGCAGGAGGTGATCCGAGGC
TGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGAGAGCCAAACTGACTATATCTC
CAGATTATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCACTCTCG
TCTTCGATGTGGAGCTTCTAAAACTGGAAGGTGGAGGCGGTTCAGGCGGCGGCGGCAG
CGGCGCCATGGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCC
TGAGCATGGAGCCCTGTGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGT
CCGGGCTCCGCACCCGCACTGGCTCCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTC
TCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCCAAGAAAATGGTGCT
GGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTC
ATTCTCTCTCACGGCTGTCAGGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCAC
AGATGGATGCCCTGTGTCGGTCGAGAAGATTGTGAACATCTTCAATGGGACCAGCTGCC
CCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGGGGAGCAGAA
AGACCATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACC

CCGAGCCAGATGCCACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGC
CATATCTAGTTTGCCCACACCCAGTGACATCTTTGTGTCCTACTCTACTTTCCCAGGTTTT
GTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGACATCTT
TGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTG
TTTCGGTGAAAGGGATTTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAAC
TTTTCTTTAAAACATCAGCTAGTTAA.

In a specific embodiment, the chimeric antigen receptor Secretory-CD22 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9 has the amino acid sequence as shown in SEQ ID NO. 6 or an amino acid sequence that shares more than 90% homology therewith. The amino acid sequence as shown in SEQ ID NO. 6 is as follows:

MLLLVTSLLLCELPHPAFLLIPDIQMTQTTSSLSASLGDRVTISCRASQDISNYLNWYQQ
KPDGTVKLLIYYTSILHSGVPSRFSGSGSGTDYSLTISNLEQEDFATYFCQQGNTLPWTFGGG
TKLEIKGSTSGSGKPGSSEGSTKGEVQLVESGGGLVKPGGSLKLSCAASGFAFSIYDMSWVR
QTPEKRLEWVAYISSGGGTTYYPDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCARH
SGYGTHWGVLFAYWGQGTLVTVSAAAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFP
GPSKPFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQP
YAPPRDFAAYRSASGGGGSGGGGSQRRKYRSNKGESPVEPAEPCHYSCPREEEGSTIPIQED
YRKPEPACSPGGGGSGGGGSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRG
RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD
TYDALHMQALPPRTSGSGATNFSLLKQAGDVEENPGPMGVQVETISPGDGRTFPKRGQTCV
VHYTGMLEDGKKVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAY
GATGHPGIIPPHATLVFDVELLKLEGGGGSGGGGSGAMVGALESLRGNADLAYILSMEPCG
HCLIINNVNFCRESGLRTRTGSNIDCEKLRRRFSSLHFMVEVKGDLTAKKMVLALLELARQD
HGALDCCVVVILSHGCQASHLQFPGAVYGTDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQ
ACGGEQKDHGFEVASTSPEDESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYSTFP
GFVSWRDPKSGSWYVETLDDIFEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRK
KLFFKTSAS.

In a specific embodiment, the chimeric antigen receptor Secretory-CD22 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9 has the nucleotide sequence as shown in SEQ ID NO. 7 or an nucleotide sequence that shares more than 95% homology therewith. The nucleic acid

sequence as shown in SEQ ID NO. 7 is as follows:

ATGCTGCTCCTCGTTACAAGTCTGCTCCTGTGTGAACTGCCTCACCCTGCATTTCTG
CTGATTCCAGATATACAGATGACTCAAACCACATCCTCTCTGAGTGCTTCCCTGGGCGAC
CGCGTCACCATATCTTGTAGAGCCAGTCAGGACATCAGCAATTACCTGAATTGGTATCAG
CAGAAACCAGACGGAACTGTGAAGCTGCTCATCTACTATACCAGCATTCTGCATAGCGG
CGTTCCATCCCGCTTTAGCGGCAGTGGCAGCGGAACCGATTATTCACTGACTATCAGCAA
CCTGGAACAGGAAGACTTTGCTACCTACTTCTGCCAGCAAGGCAATACCCTGCCCTGGA
CCTTCGGAGGCGGCACCAAGCTGGAAATCAAGGGTTCCACCTCTGGATCTGGGAAGCC
TGGGAGCAGCGAGGGATCTACCAAAGGCGAGGTGCAGCTGGTGGAATCAGGAGGCGG
ACTCGTCAAGCCCGGAGGATCTCTGAAGCTGAGCTGCGCCGCCTCAGGGTTCGCATTCT
CTATATATGACATGTCTTGGGTGAGGCAGACTCCCGAGAAGAGGCTGGAGTGGGTTGCA
TACATCAGTTCTGGCGGCGGTACTACCTATTATCCCGATACTGTCAAGGGTCGGTTTACAA
TTTCTCGGGATAACGCTAAGAACACCCTGTATCTCCAGATGTCATCTCTGAAGAGTGAAG
ATACTGCTATGTATTATTGCGCTAGACACTCCGGGTACGGAACACACTGGGGCGTGCTGT
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AGCCTATCGCTCCGCTAGCGGAGGTGGAGGTTCTGGAGGTGGTGGAAGTCAAAGAAGG
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ATGCCCAAGGGAGGAGGAGGGAAGTACAATCCCAATTCAAGAAGACTACAGGAAGCCC
GAACCTGCATGCAGTCCAGGTGGAGGCGGTTCTGGAGGCGGTGGCTCCCGGGTGAAAT
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CTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACC
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CTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCCAAGAAAATGGTGCTGGCTTT
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GGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCC
AGATGCCACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTA
GTTTGCCCACACCCAGTGACATCTTTGTGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTG
GAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGACATCTTTGAGCAG
TGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTG
AAAGGGATTTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTT
AAAACATCAGCTAGTTAA.

In the present application, the chimeric antigen receptor further comprises a promoter, which is any one of the group consisting of EF1a, CMV-TAR and CMV, or a combination of at least two thereof.

According to the present application, the chimeric antigen receptor is expressed by transducing the nucleic acid encoding the same into T cell.

According to the present application, the transduction is performed by transduction into T cells via any one of the group consisting of a viral vector, an eukaryotic expression plasmid and an mRNA sequence, or a combination of at least two thereof, preferably by transduction into T cells

via a viral vector.

Preferably, the viral vector is any one of the group consisting of a lentiviral vector and a retroviral vector, or a combination of at least two thereof, preferably a lentiviral vector.

In a second aspect, the present application provides a recombinant lentivirus mixture comprising a recombinant lentivirus which is obtained by transducing mammalian cells with a viral vector comprising a nucleotide sequence encoding a chimeric antigen receptor targeting CD19 and packaging helper plasmids pNHP and pHEF-VSVG and a recombinant lentivirus which is obtained by transducing mammalian cells with a viral vector comprising a nucleotide sequence encoding a chimeric antigen receptor targeting CD22 and packaging helper plasmids pNHP and pHEF-VSVG.

In the present application, the recombinant lentivirus can efficiently immunize cells including T cells to prepare targeting T cells.

According to the present application, the mammalian cell is any one of the group consisting of a 293 cell, a 293T cell and a TE671 cell, or a combination of at least two thereof.

In a third aspect, the present application provides a pharmaceutical composition comprising the immune cell mixture as described in the first aspect and/or the recombinant lentivirus mixture as described in the second aspect.

In a fourth aspect, the present application provides use of the immune cell mixture as described in the first aspect, the recombinant lentivirus mixture as described in the second aspect or the pharmaceutical composition as described in the third aspect for the preparation of chimeric antigen receptor T cells, immune competent cells or tumor therapeutics.

In the present application, the antigen receptor T cells have a good targeting effect and are capable of releasing low dose of immune factors, having a property of low toxic reaction.

Preferably, the tumor is a blood-associated neoplastic disease and/or a solid tumor. The neoplastic disease is selected from, but not limited to, leukemia.

In another aspect, the present application provides a method for treating a tumor comprising administrating to a subject in need thereof a therapeutically effective amount of

a) an immune cell expressing both a chimeric antigen receptor targeting CD19 and a chimeric antigen receptor targeting CD22; or

b) a mixture of an immune cell expressing a chimeric antigen receptor targeting CD19 and an

immune cell expressing a chimeric antigen receptor targeting CD22.

Compared with the prior art, the present application has the following beneficial effects:

(1) The CD19- and CD22-based CAR-T cells obtained by genetically modifying T cells with the chimeric antigen receptors of the present application bind to tumor surface antigens CD19 and CD22, and kill tumors with a stronger effect, achieving a more significant tumor reduction effect;

(2) The two chimeric antigen receptors of the present application specifically recognize tumor surface antigens CD19 and CD22 that are highly expressed in leukemia and lymphoma and has a safer and more significant effect than other chimeric antigen receptors and other tumor antigens, thereby improving the immune effects of CAR-T cells, making CD19 escape not easy to occur, being easier to reach the lesion site, and improving the therapeutic effects for diseases.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram showing the synthetic gene sequence map of the chimeric antigen receptor targeting CD19 and the chimeric antigen receptor targeting CD22 according to the present application;

Figure 2 is a graph showing the results of flow cytometry analysis of CD19 and CD22 of B cell leukemia and lymphoma cell lines;

Figure 3 is a graph showing the results of *in vitro* specific killing of B cell leukemia lymphoma cell lines with CD19-targeted and CD22-targeted chimeric antigen receptor T cells as detected by flow cytometry using Annexin V/PI;

Figure 4 is a graph showing the results of *in vitro* specific killing of B cell leukemia lymphoma cell lines with CD19-targeted and CD22-targeted chimeric antigen receptor T cells as detected by observing green fluorescent target cells under a microscope;

Figure 5 is a flow chart showing the clinical trial using CD19-targeted chimeric antigen receptor T cells combined with CD22-targeted chimeric antigen receptor T cells to treat B cell leukemia/lymphoma;

Figure 6 is a dot-plot showing the results of flow cytometric analysis of primary B-ALL cells stained with anti-CD19 and CD22 antibodies, wherein the samples were obtained from the bone marrow of three B-ALL patients;

Figure 7 shows the statistical analysis of the expression level of CD22 in CD19-positive cells in bone marrow malignant cells of B cell leukemia patients as detected by flow cytometry;

Figure 8 shows the statistical analysis of immunohistochemical staining of tissue sections of B cell lymphoma patients, wherein Fig. 8(a) shows the distribution of CD19 expression, and Fig. 8(b) shows the distribution of CD22 expression;

Figure 9 shows examples of immunohistochemical staining of CD19 and CD22 in tissue sections of patients with B cell lymphoma;

Figure 10 shows the remission rates of 18 patients with B cell leukemia (Fig. 10(a))/lymphoma (Fig. 10(b)) after infusion of CD19 and CD22 CAR-T cells in clinical, wherein PD represents progressive disease, PR represents partial remission, and CR represents complete remission;

Figure 11 shows changes in clones of bone marrow malignant cells and CD19-positive cells in patients with refractory B cell follicular lymphoma after infusion of CD19 CAR-T cells combined with CD22 CAR-T cells in clinical trial;

Figure 12 shows changes in lymph node size in patients after infusion;

Figure 13 shows changes in copy numbers of CD19 and CD22 CAR in peripheral blood of patients after infusion;

Figure 14 shows changes in lymphoma size in patient YXX after infusion as indicated by PET-CT.

DETAILED DESCRIPTION

In order to further illustrate the technical measures adopted by the present application and the effects thereof, the technical solutions of the present application are further described below with reference to the accompanying drawings and specific embodiments, and however, the present application is not limited to the scope of the embodiments. In the examples, techniques or conditions, which are not specifically indicated, are performed according to techniques or conditions described in the literature of the art, or according to product instructions.

The reagents or instruments used herein, which are not indicated with manufacturers, are conventional products that are commercially available from formal sources.

Example 1 Construction of chimeric antigen receptors

(1) The Secretory signal peptide, CD19 or CD22 antigen-binding domain, CD8α and/or CD28 transmembrane domain, CD28 signaling domain, CD27 signaling domain, CD3ζ signaling domain, 2A sequence and Caspase 9 domain, as shown in Figure 1, *i.e.* Secretory-CD19 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9 and Secretory-CD22 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9 were synthesized by whole gene synthesis. Specifically,

Secretory-CD19 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9 had the nucleotide sequence as shown in SEQ ID NO. 5, and

Secretory-CD22 scFv-CD28-CD27-CD3ζ-2A-FBKP.Casp9 had the nucleotide sequence as shown in SEQ ID NO. 7.

Example 2 Lentiviral packaging

(1) 293T cells were used and cultured for 17-18 hours;

(2) Fresh DMEM containing 10% FBS was added;

(3) The following reagents were added to a sterile centrifuge tube: the DMEM taken for each well and helper DNA mix (pNHP, pHEF-VSV-G) and pTYF DNA vector, vortexed and shaken;

(4) Superfect or any transgenic material was added to the centrifuge tube, left for 7-10 minutes at room temperature;

(5) To each culture cells the DNA-Superfect mixture in the centrifuge tube was added, vortexed and mixed;

(6) Incubated in a 37 °C, 3% CO_2 incubator for 4-5 hours ;

(7) The supernatant was removed from the culture medium, the culture was rinsed with 293 cell media, and media was added for further culture;

(8) The plate was placed back into the incubator with 3% CO_2 for overnight incubation. The next morning, transduction efficiency was observed with a fluorescence microscope.

Example 3 Purification and Concentration of Lentivirus

1) Virus purification

Cell debris were removed by centrifuging at 1000 g for 5 minutes to obtain virus supernatant. The virus supernatant was filtered with a 0.45 μm low protein-binding filter, and the virus was divided into small portions and stored at -80 °C;

Typically, lentiviral vectors at a titer of 10^6 to 10^7 transducing units can be produced by transduced cells per ml media.

2) Concentration of lentivirus with a Centricon filter or the like

(1) The virus supernatant was added to the Centricon filter tube or the like, then centrifuged at 2500g for 30 minutes;

(2) The filter tube was shaken, then centrifuged at 400 g for 2 minutes, and the concentrated virus was collected to a collection cup. Finally, the virus was collected from all tubes into a single centrifuge tube.

Example 4 Transduction of CAR-T cells

The activated T cells were seeded into a culture dish, and concentrated lentiviruses containing target genes were added, centrifuged at a centrifugal force of 100 g for 100 minutes, then cultured at 37 °C for 24 hours, and AIM-V media containing cell culture factors were added, after 2-3 days of culture, the cells were harvested and counted to produce available CD19 CAR-T cells and CD22 CAR-T cells.

Example 5 *In vitro* killing of malignant B cell lines with CD19 CAR-T cells combined with CD22 CAR-T cells

Prior to the killing test, the expression of CD19 and CD22 on malignant B cell lines was analyzed. Flow cytometry analysis was performed on three malignant B cell lines: RS4;11, Daudi, and BLCL. The results were shown in Figure 2. It can be seen that both CD19 and CD22 were highly expressed in all of the three cell lines, wherein the expression of CD19 was 88%. 93% and 60%, respectively, and the expression of CD22 was 100%, 100% and 99%, respectively. This result demonstrated the prevalence of CD19 and CD22 in malignant B cell lines and was used as a basis for the killing test.

CD19 CAR-T cells and CD22 CAR-T cells were co-cultured with RS4;11 (RS4;11 wassort) target cells transduced with green fluorescence proteins with a ratio of CAR-T cells to target cells of 2:1. After one hour of culture, cells were stained with Annexin V/PI, and the percentage of viable cells and dead cells were analyzed to obtain the killing efficiency of CAR-T cells.

It can be seen from Fig. 3 that the proportion of dead cells of RS4;11 which was not co-cultured with CAR-T cells was less than 10%, and the proportions of dead cells in all groups

co-cultured with CAR-T cells were more than 40%, wherein the proportion of dead cells caused by CD19 and CD22 CAR-T cells was higher than 70%, which was higher than CD19 or CD22 CAR-T cells alone. The results of long-term observation of the killing test were shown in Figure 4, in which T cells and GD2 CAR-T cells were used as a background control group and a non-specific killing control group, respectively. The second day after the killing test, most of the green fluorescence disappeared. By the third day of killing, it can be seen that the number of fluorescent cells in the group in which CD19 and CD22 CAR-T cells were simultaneously used was the least, compared to CD19 or CD22 CAR-T cells alone. After the fifth day, the green fluorescence almost completely disappeared, showing the high efficiency of CAR-T cells and the feasibility of combining CD19 and CD22 CAR-T cells *in vitro*.

Example 6 Clinical Application of CD19 and CD22 CAR-T Cells

Figure 5 is a flow chart showing the clinical trial using CD19-targeted chimeric antigen receptor T cells combined with CD22-targeted chimeric antigen receptor T cells to treat B cell leukemia/lymphoma. Specific steps were as follows.

(1) First, patients were evaluated by the hospital and laboratory, and autologous or donor white blood cells were collected if the enrollment conditions were met. Then following the standard protocol, CD3-positive T cells were screened from PBMC, activated and transduced with 4SCAR19 and 4SCAR22 (as described herein) to prepare CD19 CAR-T cells and CD22 CAR-T cells. The patients were pretreated with cyclophosphamide and fludarabine prior to infusion. On average, 1.05×10^6 cells per kilogram of body weight were infused. The quality of white blood cells and CAR-T cells, the gene transduction rate, the expansion of CAR-T cells and the number of effective infused CAR-T cells were evaluated and recorded.

(2) After the infusion of CAR-T cells into patients, the immune factor storm (cytokine release syndrome, CRS) and CAR copy numbers were closely monitored within one month. Conclusions about treatment toxicity and final therapeutic effects were obtained by long-term clinical and laboratory evaluations. Toxicity evaluation was performed based on the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE v4.03).

Specifically, the clinical results were as follows.

Figure 6 is a dot-plot showing the results of flow cytometric analysis of primary B-ALL cells

stained with anti-CD19 and CD22 antibodies, wherein the samples were obtained from the bone marrow of three B-ALL patients. The expression of CD19 and CD22 in the three patients was higher than 60%, and was nearly 100% in patient 3. According to the flow cytometry dot plot, CD19 and CD22 double-positive cells also exceeded 60% of malignant cells. This figure showed the prevalence of CD19 and CD22 in B-ALL patients and high proportions of the same in malignant cells, so CD22 can be a very good adjuvant target for CAR-T treatment of B-ALL.

Figure 7 shows the scatter diagram of CD22 expression in the CD19-positive cell population in the bone marrow of 15 B-ALL patients as detected by flow cytometry staining. The results were shown in Table 1 below:

Table 1

B-ALL patient	CD22 percentage
#1	97%
#2	100%
#3	96%
#4	93%
$5	98%
#6	97%
#7	99%
#8	99%
#9	98%
#10	94%
#11	100%
#12	85%
#13	76%
#14	37%
#15	57%

Of the 15 patients, 11 patients showed more than 90% of double-positive expression of CD19 and CD22, indicating the prevalence and importance of CD22 as a second target.

Analysis was also performed on patients with B-cell lymphoma, and the results were as follows.

Figure 8 (a) - Figure 8 (b) shows the statistical analysis on immunohistochemical staining of CD19 and CD22 in tissue sections of 28 patients with B cell lymphoma. In CD19-positive patients, the staining intensity was well-distributed, with from 1+ to 4+ each accounting for a quarter of the total number of positive patients. In patients with positive CD22 staining, the number of patients with a staining intensity of 3+ were the most, which again proved that CD22 was a good auxiliary target also in the CAR-T treatment of B-cell lymphoma.

Figure 9 shows the results of immunohistochemical staining of three different B cell lymphoma patients. Both CD19 and CD22 were consistently highly expressed in follicular lymphoma, diffuse large B lymphoma, and primary mediastinal large B-cell lymphoma.

Through the above clinical trial procedure, 18 patients with B-cell leukemia/B-cell lymphoma were treated, and the remission rate results were shown in Fig. 10(a) - Fig. 10(b). In the treatment of 7 patients with B-cell leukemia, 3 of them received complete remission and 1 received partial remission, *i.e.,* 57% patients had response to the treatment. In the treatment of 11 patients with B-cell lymphoma, 2 of them received complete remission and 5 received partial remission, *i.e.,* 64% patients had response to the treatment.

Example 7 Actual Clinical Application Example of CD19 and CD22 CAR-T Cells

In the case of complete remission mentioned above, a patient was specifically proposed and discussed as a case wherein excellent treatment results were obtained by using a combination of CD19 and CD22 CAR-T cells in clinical.

Sample: a 31-year-old male patient with relapsed and refractory stage IV follicular lymphoma. No therapeutic effect was achieved and the disease was progressed after several R-CHOP chemotherapies and second-line R-ESHAP chemotherapies. The patient's malignant cells expressed CD20, CD22, FMC7, CD79b and CD45, and a part thereof expressed CD19 and CD23.

CAR-T cells were prepared according to the above procedures, and 1×10^6/kg CAR-T cells were infused. Only responses less than grade 1 CRS were observed in the patient after infusion.

Changes in clones of bone marrow malignant cells and CD19-positive cells after infusion were shown in Figure 11.

As can be seen from Figure 11, 100 days after the infusion, the bone marrow malignant cells completely disappeared, which was continued for more than 234 days, and the clones of CD19-positive cells also decreased significantly after the second infusion. Figure 12 shows changes in the size of swollen lymph nodes caused by malignant cell proliferation after infusion of CAR-T cells. After the first infusion, the lymph nodes were significantly and continuously reduced. After the second infusion, up to 300 days after the reinfusion, the malignant swollen lymph nodes had disappeared. Figure 13 shows the respective amplification of CD19 and CD22 CAR-T cells in peripheral blood of patients. On the 14th and 21st day after the first reinfusion, significant CAR-T cell amplification was observed. Within 10 days after the second reinfusion, significant CAR-T cell amplification was observed. Figure 14 shows changes in lymphoma size as indicated by PET-CT under long-term follow-up. As observed at day 62, 141 and 285 after the reinfusion, the lymphoma was continuously reduced, and no visible lymphoma was seen after nine months, suggesting that a sustained complete remission was achieved.

In summary, the two chimeric antigen receptors of the present application specifically recognize tumor surface antigens CD19 and CD22. Compared to using other single-targeted chimeric antigen receptor T cells, using two types of CAR-T cells in combination achieves better therapeutic effects, makes CD19 escape not easy to occur, and allows the disease to be easily relieved.

The applicant states that detailed methods of the present application are demonstrated in the present application through the above embodiments, however, the present application is not limited to the above detailed methods, and does not mean that the present application must rely on the above detailed methods to implement. It should be apparent to those skilled in the art that, for any improvement of the present application, the equivalent replacement of the raw materials of the present application, the addition of auxiliary components, and the selection of specific modes, etc., will all fall within the protection scope and the disclosure scope of the present application.

CLAIMS

1. An immune cell mixture comprising an immune cell genetically modified with a chimeric antigen receptor targeting CD19 and an immune cell genetically modified with a chimeric antigen receptor targeting CD22.

2. The immune cell mixture according to claim 1, wherein the chimeric antigen receptor targeting CD19 and the chimeric antigen receptor targeting CD22 each comprises an antigen-binding domain, a transmembrane domain, a costimulatory signaling region, a CD3ζ signaling domain, and an inducible suicide fusion domain in tandem arrangement.

3. The immune cell mixture according to claim 2, wherein in the case of a chimeric antigen receptor targeting CD19, the antigen-binding domain is a single chain antibody against tumor surface antigen CD19; and in the case of a chimeric antigen receptor targeting CD22, the antigen-binding domain is a single chain antibody against tumor surface antigen CD22.

4. The immune cell mixture according to claim 3, wherein the single chain antibody against tumor surface antigen CD19 has an amino acid sequence selected from any one of the group consisting of

(I) the amino acid sequence as shown in SEQ ID NO.1;

(II) an amino acid sequence that shares ≥90%, preferably ≥95%, more preferably ≥98%, most preferably ≥99% homology with the amino acid sequence as shown in SEQ ID NO. 1;

(III) an amino acid sequence that is obtained by modifying, substituting, deleting or adding one or several amino acids to the amino acid sequence as shown in SEQ ID NO. 1; and

the amino acid sequence has the activity of a single chain antibody against the tumor surface antigen CD19.

5. The immune cell mixture according to claim 3, wherein the single chain antibody against tumor surface antigen CD22 has an amino acid sequence selected from any one of the group consisting of

(I) the amino acid sequence as shown in SEQ ID NO. 2;

(II) an amino acid sequence that shares ≥90%, preferably ≥95%, more preferably ≥98%, most preferably ≥99% homology with the amino acid sequence as shown in SEQ ID NO. 2;

(III) an amino acid sequence that is obtained by modifying, substituting, deleting or adding one or several amino acids to the amino acid sequence as shown in SEQ ID NO. 2; and

the amino acid sequence has the activity of a single chain antibody against the tumor surface antigen CD22.

6. The immune cell mixture according to any one of claims 2-5, wherein the transmembrane domain is a CD28 transmembrane domain and/or a CD8α transmembrane domain;

preferably, the costimulatory signaling region is any one selected from the group consisting of a CD28 signaling domain, a CD27 signaling domain or a CD137 signaling domain, or a combination of at least two thereof;

preferably, the inducible suicide fusion domain comprises a caspase 9 domain;

preferably, the caspase 9 domain has the amino acid sequence as shown in SEQ ID NO. 3;

preferably, the inducible suicide fusion domain is connected in tandem with the CD3ζ signaling domain via a 2A sequence.

7. The immune cell mixture according to any one of claims 1-6, wherein the chimeric antigen receptor targeting CD19 and the chimeric antigen receptor targeting CD22 each comprises a signal peptide, an antigen-binding domain, a transmembrane domain, a costimulatory signaling region, a CD3ζ signaling domain, a 2A sequence and an inducible suicide fusion domain in tandem arrangement;

preferably, the chimeric antigen receptor targeting CD19 is obtained by connecting a Secretory signal peptide, a CD19 antigen-binding domain, CD8α and/or CD28 transmembrane domain(s), a CD28 signaling domain, a CD27 signaling domain, a CD3ζ signaling domain, a 2A sequence and a caspase 9 domain in tandem; and the chimeric antigen receptor targeting CD22 is obtained by connecting a Secretory signal peptide, a CD22 antigen-binding domain, CD8α and/or CD28 transmembrane domain(s), a CD28 signaling domain, a CD27 signaling domain, a CD3ζ signaling domain, a 2A sequence and a caspase 9 domain in tandem;

preferably, the chimeric antigen receptor targeting CD19 is Secretory-CD19 scFv-CD28-CD27-CD3ζ-2A-FBKP. Casp9; and the chimeric antigen receptor targeting CD22 is Secretory-CD22 scFv-CD28-CD27-CD3ζ-2A-FBKP. Casp9;

preferably, the chimeric antigen receptor targeting CD19 has the amino acid sequence as shown in SEQ ID NO. 4 or an amino acid sequence that shares more than 90% homology therewith;

preferably, the chimeric antigen receptor targeting CD22 has the amino acid sequence as

shown in SEQ ID NO. 6 or an amino acid sequence that shares more than 90% homology therewith.

8. The immune cell mixture according to any one of claims 1-7, wherein the chimeric antigen receptor is transduced into T cells by nucleic acid sequence encoding the same for expression;

preferably, the transduction is performed by transduction into T cells via any one of the group consisting of a viral vector, an eukaryotic expression plasmid and an mRNA sequence, or a combination of at least two thereof, preferably by transduction into T cells via a viral vector;

preferably, the viral vector is any one of the group consisting of a lentiviral vector and a retroviral vector, or a combination of at least two thereof, preferably a lentiviral vector.

9. A recombinant lentivirus mixture, comprising a recombinant lentivirus which is obtained by transducing mammalian cells with a viral vector comprising a nucleotide sequence encoding a chimeric antigen receptor targeting CD19 and packaging helper plasmids pNHP and pHEF-VSVG, and a recombinant lentivirus which is obtained by transducing mammalian cells with a viral vector comprising a nucleotide sequence encoding a chimeric antigen receptor targeting CD22 and packaging helper plasmids pNHP and pHEF-VSVG.

10. The recombinant lentivirus mixture according to claim 9, wherein the mammalian cell is any one of the group consisting of a 293 cell, a 293T cell and a TE671 cell, or a combination of at least two thereof.

11. A pharmaceutical composition comprising the immune cell mixture according to any one of claims 1-8 and/or the recombinant lentivirus mixture according to claim 9 or 10.

12. Use of the immune cell genetically mixture according to any one of claims 1-8, the recombinant lentivirus mixture according to claim 9 or 10 or the pharmaceutical composition according to claim 11 for the preparation of chimeric antigen receptor T cells, immune competent cells or tumor therapeutics.

13. The use according to claim 12, wherein the tumor is a blood-associated neoplastic disease.

14. The use according to claim 13, wherein the blood-associated neoplastic disease is leukemia or lymphoma.

15. A method for treating a tumor comprising administrating to a subject in need thereof a therapeutically effective amount of

a) an immune cell expressing both a chimeric antigen receptor targeting CD19 and a chimeric

antigen receptor targeting CD22; or

b) a mixture of an immune cell expressing a chimeric antigen receptor targeting CD19 and an immune cell expressing a chimeric antigen receptor targeting CD22.

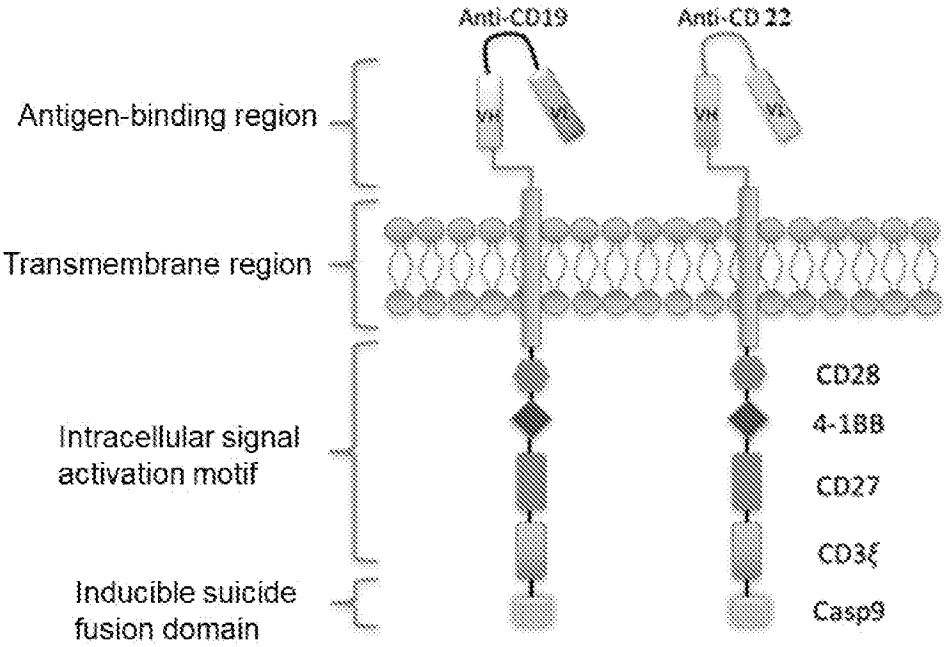

Figure 1

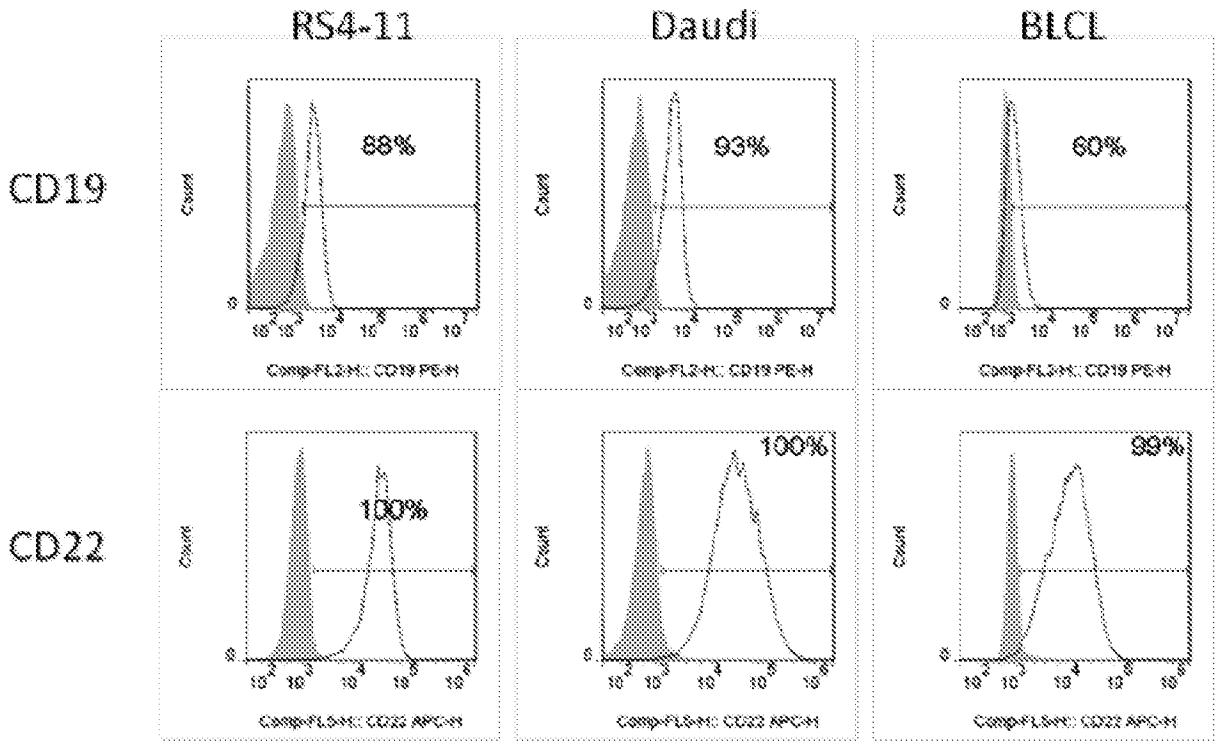

Figure 2

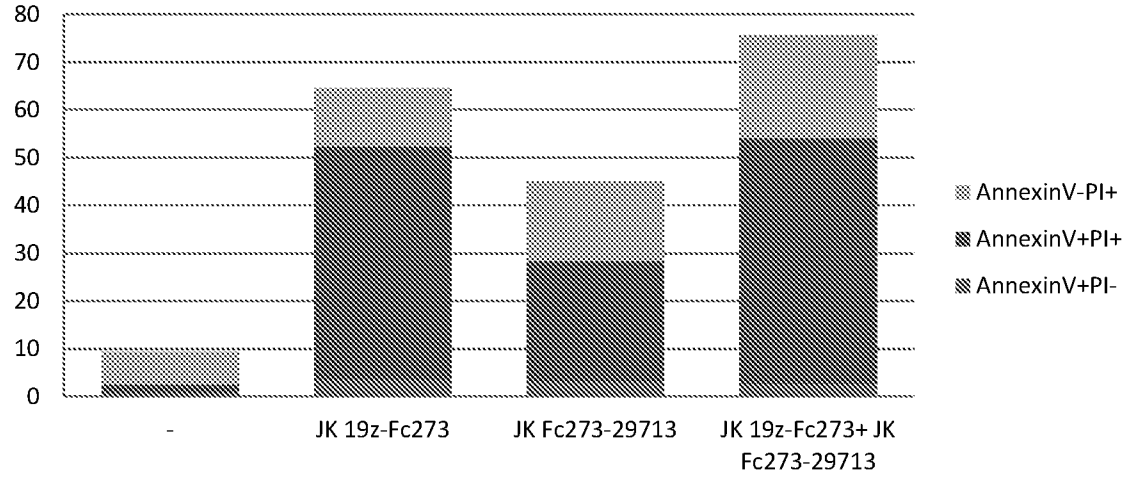

Figure 3

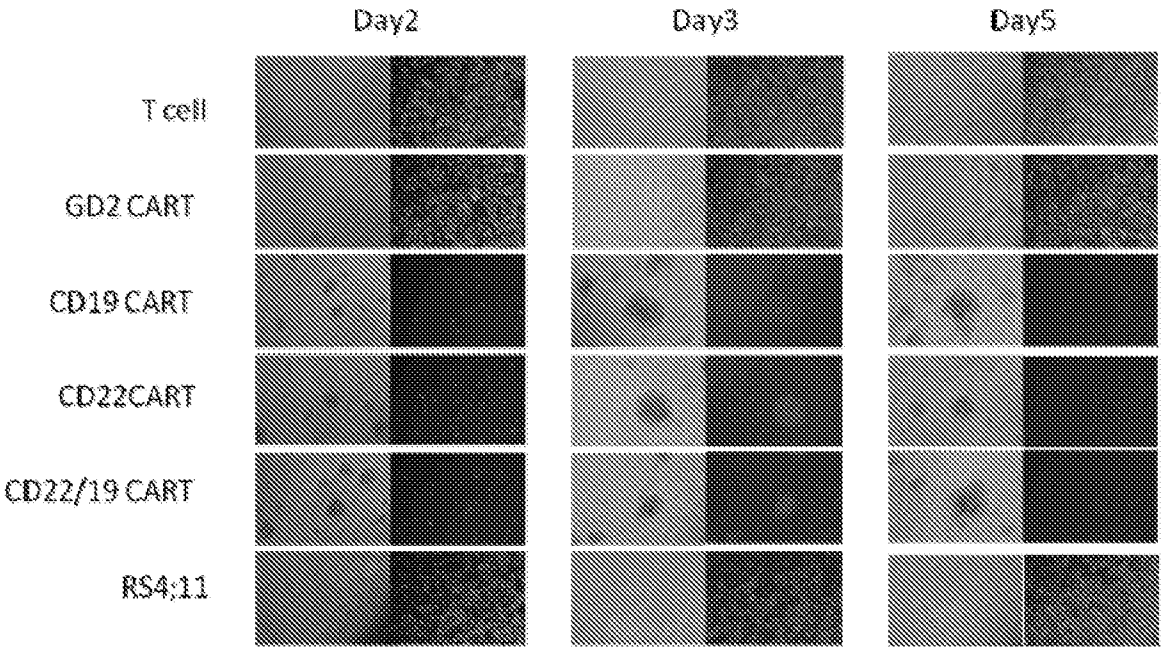

Figure 4

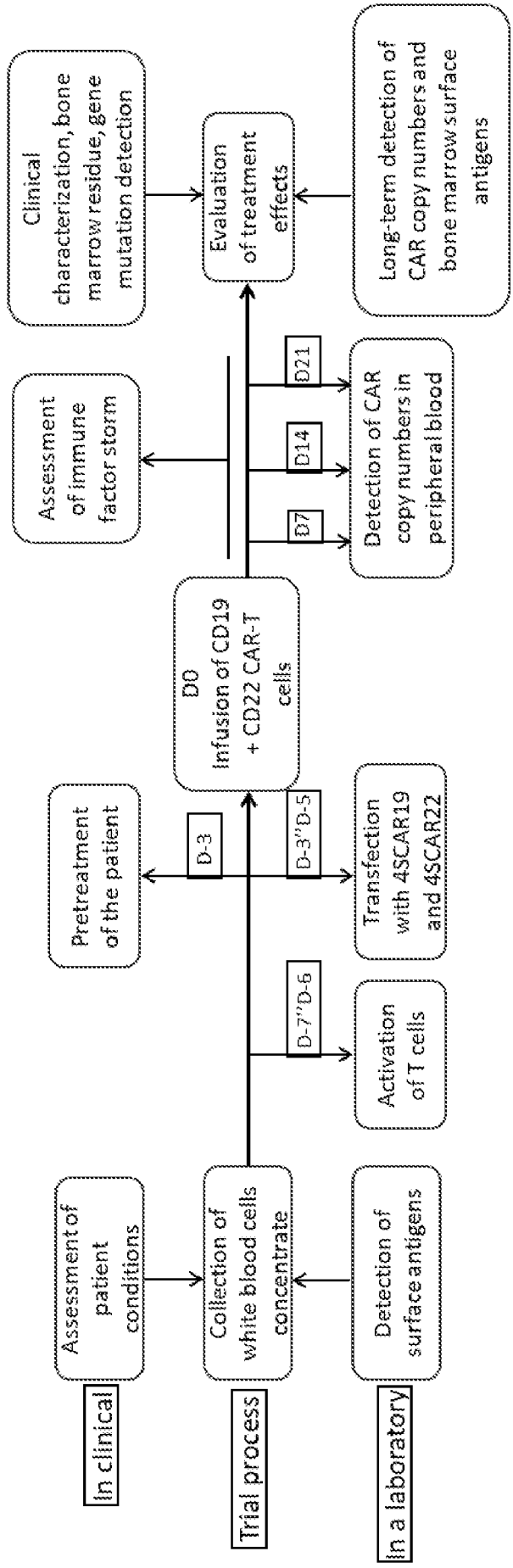
In clinical
Trial process
In a laboratory
Assessment of patient conditions
Collection of white blood cells concentrate
Detection of surface antigens
Pretreatment of the patient
D-3
D-7''D-6
D-3''D-5
Activation of T cells
Transfection with 4SCAR19 and 4SCAR22
D0 Infusion of CD19 + CD22 CAR-T cells
Assessment of immune factor storm
D7
D14
D21
Detection of CAR copy numbers in peripheral blood
Clinical characterization, bone marrow residue, gene mutation detection
Evaluation of treatment effects
Long-term detection of CAR copy numbers and bone marrow surface antigens

Figure 5

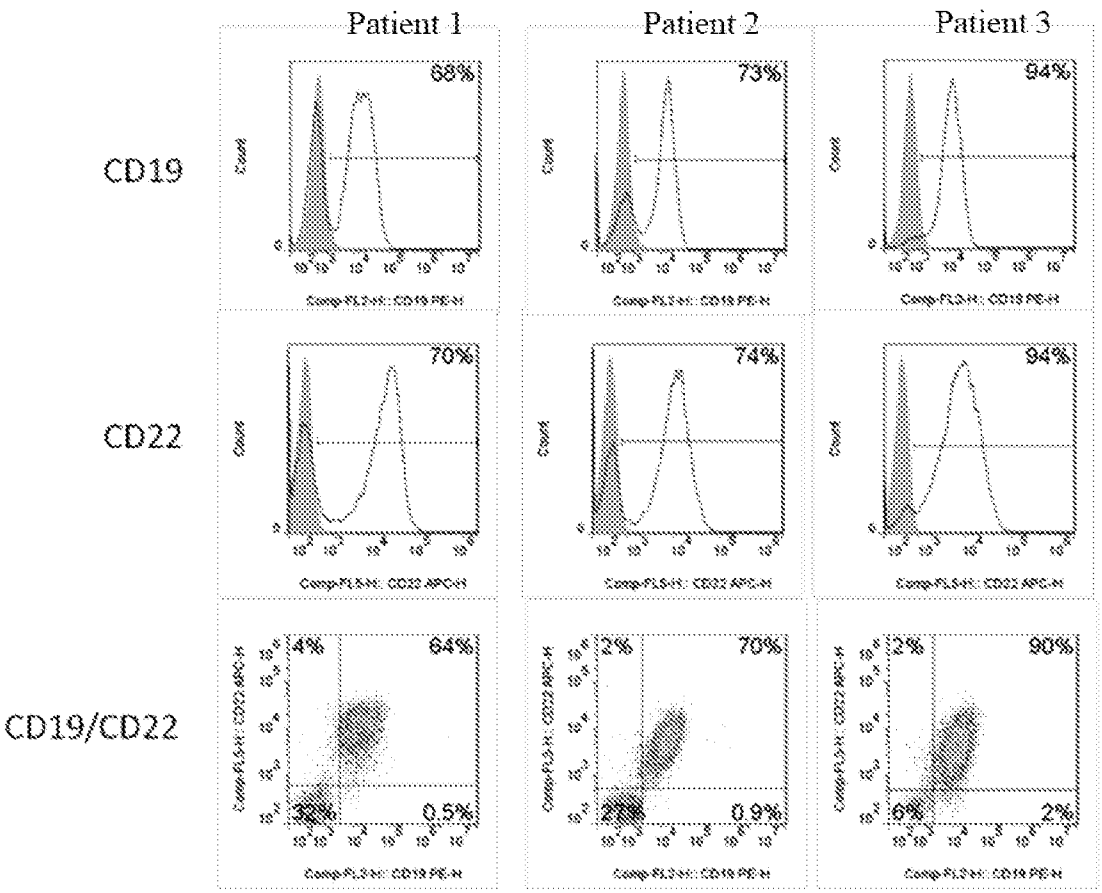

Figure 6

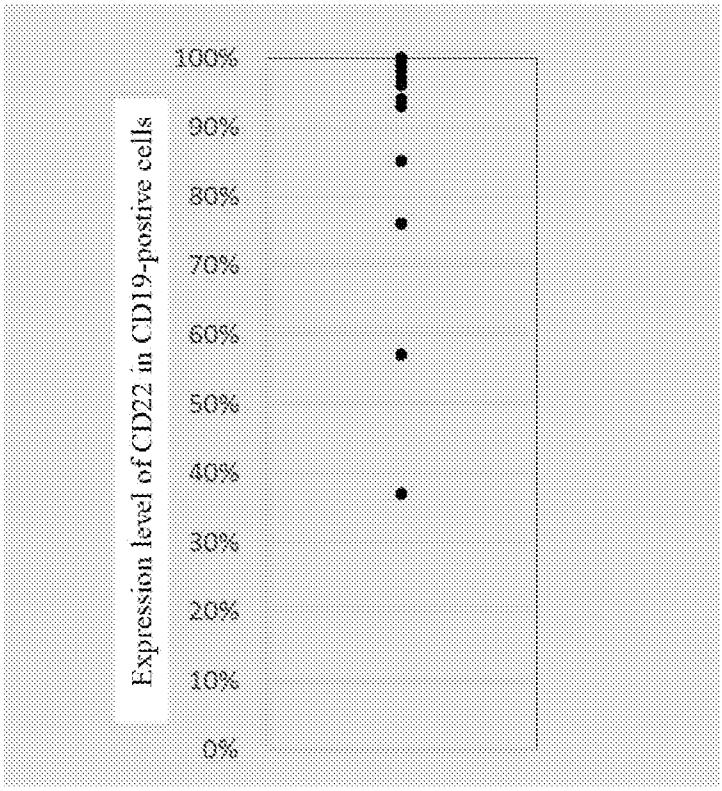

Figure 7

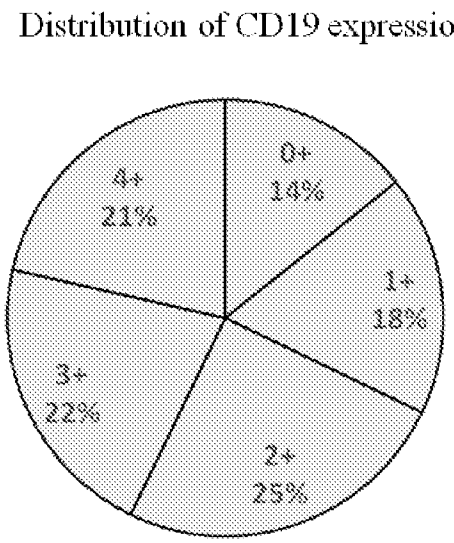

Figure 8 (a)

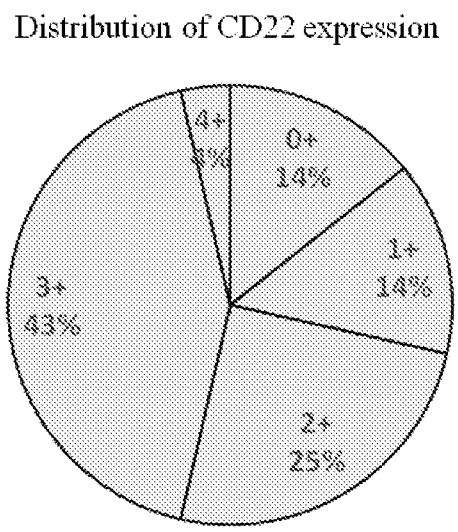

Figure 8 (b)

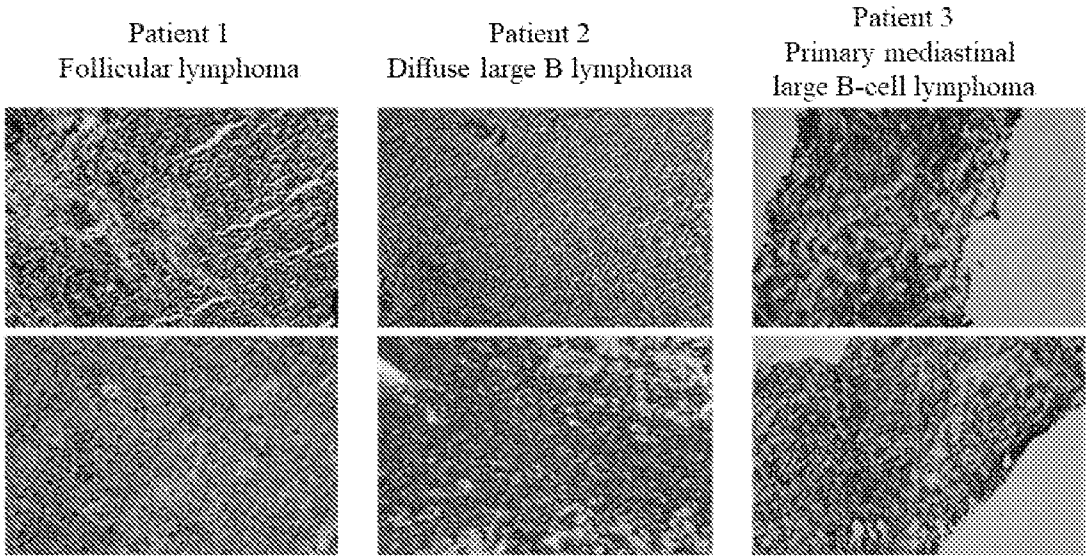

Figure 9

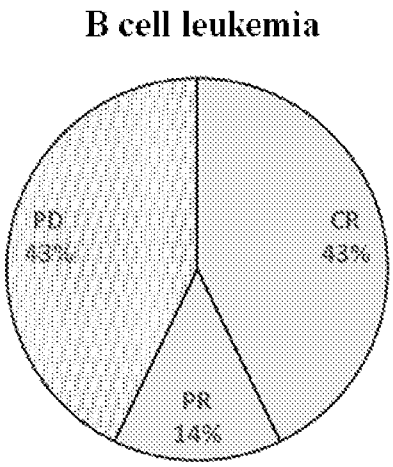

Figure 10 (a)

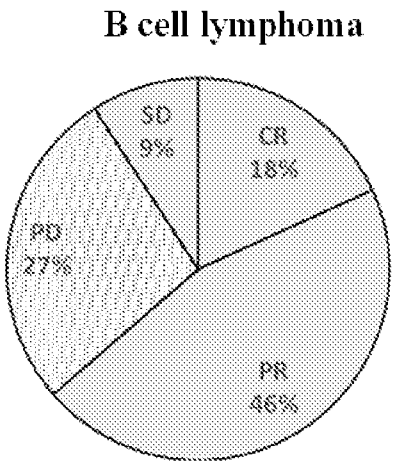

Figure 10 (b)

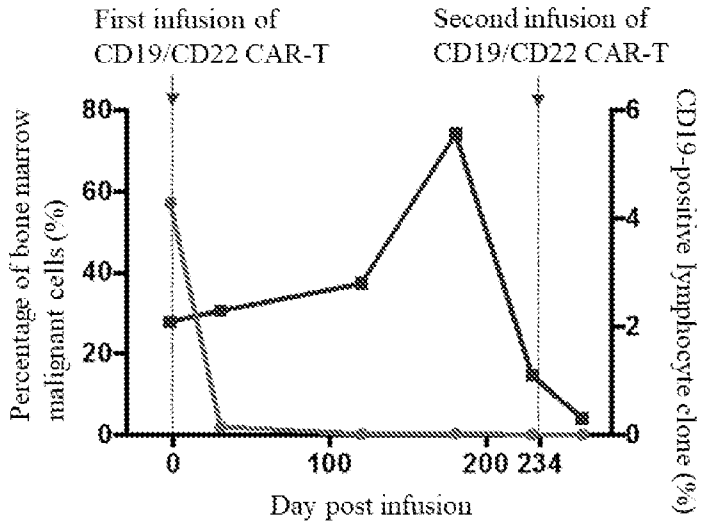

Figure 11

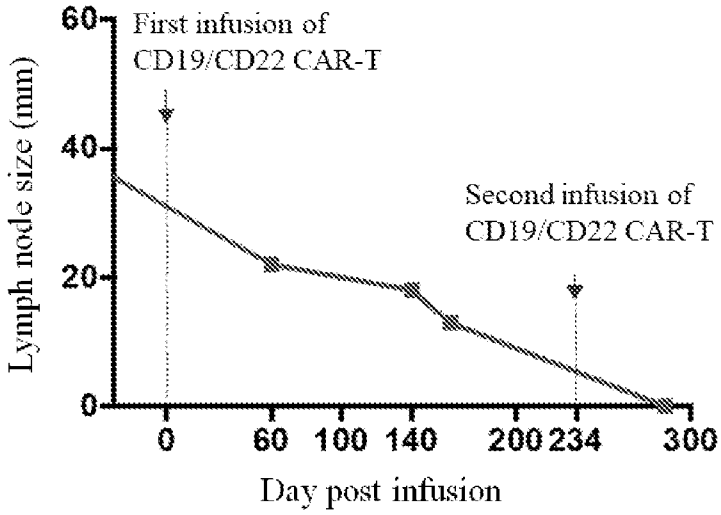

Figure 12

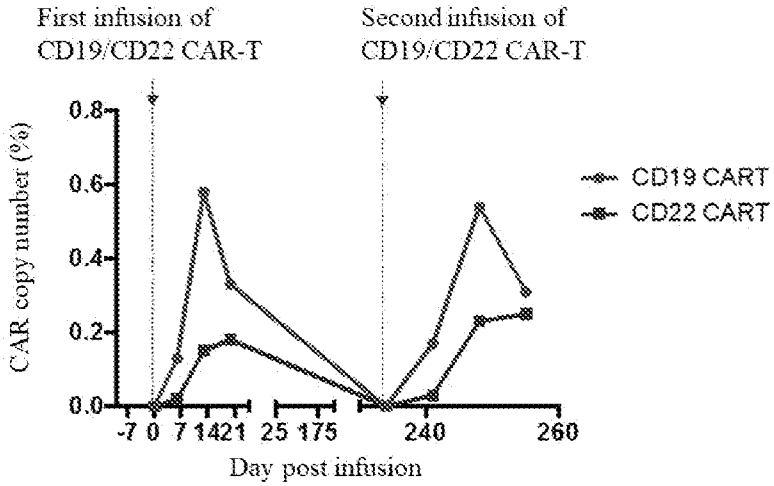

Figure 13

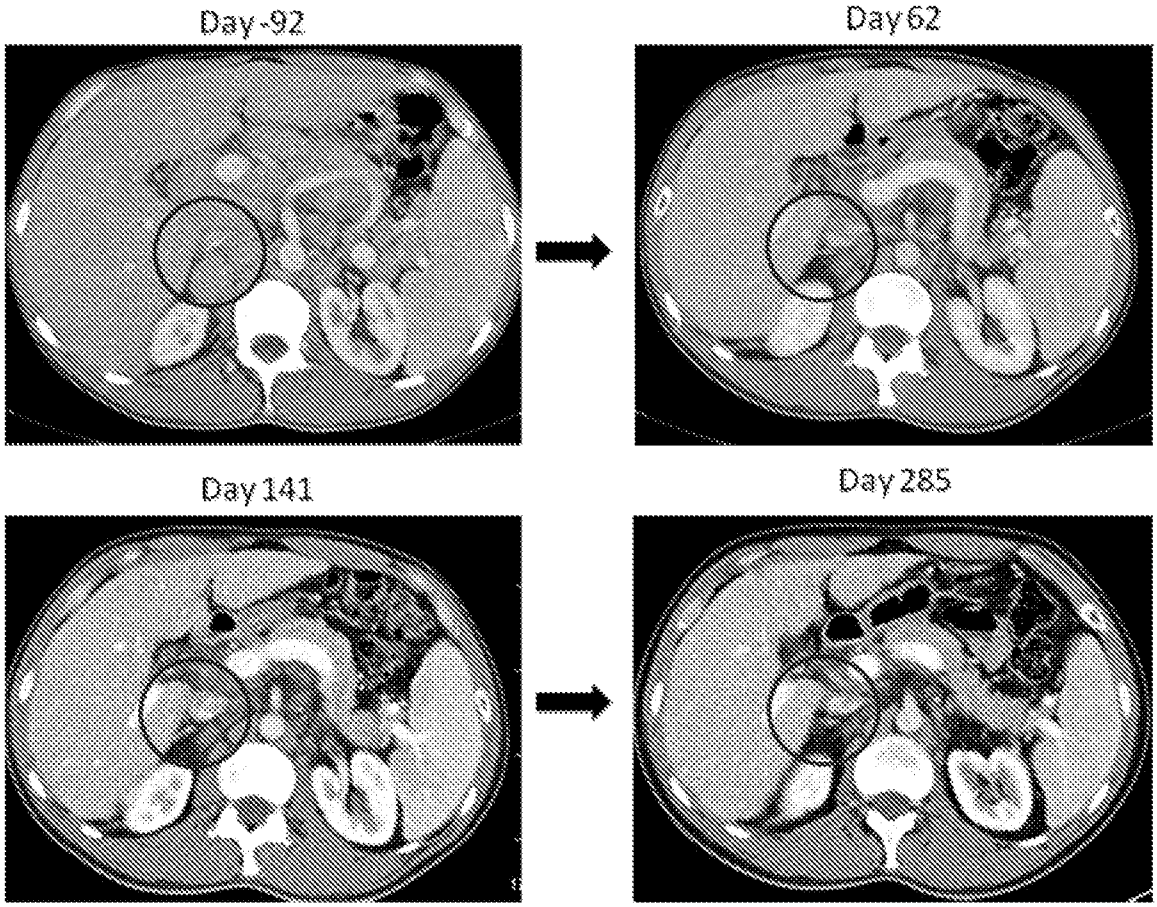

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2019/122162

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/10(2006.01)i; C12N 15/867(2006.01)i; C12N 7/01(2006.01)i; C07K 16/28(2006.01)i; C07K 16/30(2006.01)i; A61K 35/17(2015.01)i; A61P 35/00(2006.01)i; A61P 35/02(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)

C12N; 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)

CNABS, VEN, WOTXT, USTXT, EPTXT, DWPI, CNKI, PubMed, ISI Web of Knowledge, GenBank, EBI-EMBL:MEIKANG, CAR-T, chimeric antigen receptor, CD22, CD19, Double CD19/CD22, Modified T Cells, CARTs, Dual Specific CAR T Cells, Bivalent, SEQ ID NOs: 1-7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	CN 109517799 A (BEIJING MEIKANG GENO-IMMUNE BIOTECHNOLOGY CO., LTD.) 26 March 2019 (2019-03-26) claims 1-10	1-15
PX	CN 109678965 A (CHINESE PLA GENERAL HOSPITAL) 26 April 2019 (2019-04-26) claims 1-10	1, 15
X	WO 2016149578 A1 (U.S.A DEPARTMENT OF HEALTH AND HUMAN SERVICES) 22 September 2016 (2016-09-22) claims 1-23, description, paragraphs [0061]-[0085], example 1	1, 8, 11-15
Y	WO 2016149578 A1 (U.S.A DEPARTMENT OF HEALTH AND HUMAN SERVICES) 22 September 2016 (2016-09-22) claims 1-23, description, paragraphs [0061]-[0085], example 1	2-14
Y	CN 108383914 A (BEIJING MEIKANG GENO-IMMUNE BIOTECHNOLOGY CO. LTD.) 10 August 2018 (2018-08-10) claims 1-10, SEQ ID NOs:1-4	2-14

☑ 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
10 February 2020	**21 February 2020**

Name and mailing address of the ISA/CN	Authorized officer
National Intellectual Property Administration, PRC **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing** **100088** **China**	**CHEN,Ying**
Facsimile No. **(86-10)62019451**	Telephone No. **86-(010)-53961975**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2019/122162

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CN 107312098 A (SHENZHEN GENO-IMMUNE MEDICAL INST.) 03 November 2017 (2017-11-03) claims 1-10, SEQ ID NOs:1-4	2-14
A	CN 108715859 A (INST. OF HEMATOLOGY BLOOD DISEASES HOSPITAL OF CHINESE ACADEMY OF MEDICAL SCIENCES) 30 October 2018 (2018-10-30) the whole document	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2019/122162

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: **15**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] The subject matter of claim 15 relates to a method of treating a tumor in a subject, and therefore does not warrant an international search according to the criteria set out in PCT Rule 39.1(iv). An international search is still carried out on the basis of the use of immune cell mixture for the manufacturing of a medicament for the treatment of tumor.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2019/122162

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
CN	109517799	A	26 March 2019	None			
CN	109678965	A	26 April 2019	None			
WO	2016149578	A1	22 September 2016	US	2018111992	A1	26 April 2018
CN	108383914	A	10 August 2018	WO	2019161796	A1	29 August 2019
CN	107312098	A	03 November 2017	None			
CN	108715859	A	30 October 2018	None			