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(73) Patenthaver: **Abelzeta Inc., 9605 Medical Center Drive, Suite 100, Rockville, MD 20850, USA**

(72) Opfinder: **YAO, Yihong, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina**
HUANG, Jiaqi, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
ZHU, Shigui, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
ZHU, Wei, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
YAO, Xin, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
LI, Zhiyuan, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
ZHANG, Li, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
ZHU, Lin, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
MA, Anyun, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
WEI, Yutian, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
LI, Yanfeng, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
WANG, Qingxia, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina
HE, Jiaping, Level 5 Building 1 333 Guiping Road, Xuhui District, Shanghai 200233, Kina

(74) Fuldmægtig i Danmark: **Budde Schou A/S, Dronningens Tværgade 30, 1302 København K, Danmark**

(54) Benævnelse: **KONSTRUKTION AF KIMÆR ANTIGENRECEPTOR, DER ER MÅLRETTET MOD CD20-ANTIGEN, OG AKTIVITETSIDENTIFIKATION AF GENMANIPULEREDE T-CELLER DERAF**

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DESCRIPTION

Technical field

[0001] The present invention provides a sequence component of chimeric antigen receptor targeting CD20 antigen, and a preparation method for its modified T cells (CART20) and activity identification thereof. The present invention identifies a chimeric antigen receptor structure for treating CD20 positive B cell lymphoma.

Background technique

[0002] Malignant tumors of the blood system account for about 10% of human malignant tumors, and 95% of malignant tumors of the blood system are derived from B lymphocytes. Traditional chemotherapy and radiotherapy play an important role in the treatment of malignant tumors of the blood system. Some patients also have significant effects, but most of them are difficult to cure. New and effective treatments have been a hot topic in this field.

[0003] Adoptive T cell therapy has shown its powerful efficacy and bright prospect in the clinical treatment of malignant tumors. Among them, multiple centers independently using Chimeric Antigen Receptor (CAR)-modified T cells to target recurrent, refractory malignant tumors of CD19-expressed B cell have achieved unprecedented success. In particular, in a clinical trial carried out at the School of Medicine, University of Pennsylvania using CART19 in the treatment of recurrent, refractory acute B-cell lymphoma (R/R B-ALL), up to 94% of patients achieved complete remission. Although the initial response rate of this clinical trial was high, nearly 40% of patients who achieved complete response after 1 month of treatment, had a relapse , and more than 60% of patients with relapse had CD19-negative tumor cells escape. Therefore, there is an urgent need to screen out CART structure that target B cell lymphoma-associated antigens other than CD19 to treat patients with malignant lymphoma.

[0004] CD20 is a glycosylated protein and is the first identified B cell membrane marker. CD20 is also known as B1, and encoded by the MS4A gene. CD20 molecule has four transmembrane hydrophobic regions, and its N-terminal and C-terminal are located on the cytoplasmic side, thereby forming two closed loops outside the cell, which are respectively called big loop and small loop. CD20 is specifically expressed in more than 95% of normal and cancerous B cells. These cells are in the pre-B cell stage and subsequent developmental stages, and CD20 stops expression until the cells differentiated into plasma cells. Therefore, CD20 is an ideal target for immunotherapy of B cell malignancies.

[0005] Rituximab (MabThera®, Rituxan®) is the first generation of chimeric monoclonal antibody targeting CD20 which is firstly approved by the US FDA and the European EMA for treating indolent lymphoma. Rituximab recognizes and binds to the big loop structure of the

extracellular domain of CD20, and it kills tumor cells by ADCC-mediated killing effect. However, Rituximab alone shows limited activity and short duration of response, but its combination with chemotherapy can significantly enhance the efficacy of chemotherapy. Rituximab is used for the treatment of lymphoma, and half of the patients have a complete response (CR) or a partial response (PR).

[0006] Ofatumumab (Arzerra®) is the first completely humanized CD20 therapeutic antibody. Unlike Rituximab, the epitope recognized by Ofatumumab contains parts of the big loop and the small loop of CD20. At the same time, the tumor killing method of Ofatumumab is mainly through the complement-dependent pathway, followed by ADCC-dependent tumor killing effect.

[0007] Obinutuzumab (Gazyvaro ®, Gazyva ®) is a humanized type II CD20 antibody that reduces fucosylation levels and optimizes FcyRIIIa affinity. Obinutuzumab recognizes and binds to the big loop of the extracellular molecule of CD20, and mediates the killing effect on tumor mainly through the ADCC effect. At the same time, the binding of Obinutuzumab to CD20 molecule also has the effect of inducing apoptosis of tumor cells. As for the NHL that does not respond to Rituximab treatment, Obinutuzumab is combined with bendamustine, a nitrogen mustard drug. The phase III clinical trial found that the duration with no deterioration of combination therapy of Obinutuzumab and bendamustine was twice as long as that of bendamustine therapy alone (the former is 29 months and the latter is 14 months). Obinutuzumab has an overall response rate (ORR, including CR and PR) of 77.3%, and Rituximab is 65.7%.

[0008] Compared with therapeutic antibodies, cellular immunotherapy is an emerging and highly effective tumor treatment model, and is a new type of autoimmunolgy treatment for cancer. It is a method for *in vitro* culture and amplification of immune cells collected from a patient using biotechnology and biological agents, and then the cells are transfused back to the patient to stimulate and enhance the body's autoimmune function, thereby achieving the purpose of treating tumors. The skilled in the art have been working to develop new cellular immunotherapy to increase its efficiency and reduce its side effect. Although many therapeutic antibodies as described above have been developed in these years, their clinical therapeutic effects have not reached the same level of therapeutic effects as CART19. Therefore, the development of CART therapy targeting CD20 has great market value and social significance.

[0009] International patent application WO2016/097231 discloses a negative T-cell signal inducing CAR with an anti-CD20 extracellular domain. International patent application WO00/23573, and Lihua Budde et al, PLOS One, 2013, 8(12):1-10, disclose CARs that bind CD20.

Summary of the invention

[0010] In view of the differences in affinity and killing mechanisms of the therapeutic antibodies targeting CD20, we constructed a series of chimeric antigen receptors targeting CD20 using the DNA sequences of the antigen-binding regions of different antibodies, and completed the identification of anti-tumor activity and differential comparison of these chimeric antigen receptor engineering T cells *in vitro.* It is herein described effective methods and preparations for clinical application of CAR-T in the treatment of CD20-positive leukemia and lymphoma.

[0011] It is an object of the present invention to provide a chimeric antigen receptor targeting CD20, a preparation method and application thereof.

[0012] The present invention relates to the construction of a chimeric antigen receptor structure targeting CD20, a preparation method of a chimeric antigen receptor engineered T cell targeting CD20, and activity identification thereof.

[0013] The invention is defined in the appended set of claims.

[0014] In a first aspect of the invention, it provides a chimeric antigen receptor (CAR) wherein the CAR has amino acid sequence SEQ ID NO: 5.

[0015] In a second aspect of the invention, a nucleic acid molecule is provided, encoding the chimeric antigen receptor (CAR) of the first aspect of the invention.

[0016] In a preferred embodiment, the nucleic acid molecule is isolated.

[0017] In a third aspect of the invention, it provides a vector, comprising the nucleic acid molecule of the second aspect of the invention.

[0018] In another preferred embodiment, the vector is a lentiviral vector.

[0019] In a fourth aspect of the invention, it provides a host cell comprising the vector of the third aspect of the invention or having the exogenous nucleic acid molecule of the second aspect of the invention integrated into its genome.

[0020] In another preferred embodiment, the cell is an isolated cell, and/or the cell is a genetically engineered cell.

[0021] In another preferred embodiment, the cell is a mammalian cell.

[0022] In another preferred embodiment, the cell is a T cell.

[0023] In a fifth aspect of the invention, it provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and the chimeric antigen receptor of the first aspect of the invention, the nucleic acid molecule of the second aspect of the invention, the vector of the third aspect of the invention, or the cell of the fourth aspect of the invention.

[0024] In a sixth aspect of the invention, it provides the chimeric antigen receptor of the first aspect of the invention, the nucleic acid molecule of the second aspect of the invention, the vector of the third aspect of the invention, or the cell of the fourth aspect of the invention for use in treating a tumor.

[0025] In another preferred embodiment, the tumor comprises CD20 positive tumor.

[0026] In an further aspect of the invention, it provides a method for preparing a CAR-T cell (CAR-modified T cell) expressing the chimeric antigen receptor of the first aspect of the invention,
comprising the steps of: transducing the nucleic acid molecule of the second aspect of the invention or the vector of the third aspect of the invention into a T cell, thereby obtaining the CAR-T cell.

Description of Drawings

[0027]

Figure 1 shows the structure of the chimeric antigen receptor targeting CD20. Each element of the designed CAR structure is shown in the figure, and the listed elements include: a leader sequence, an antigen recognition sequence (Ofatumumaband, Obinutuzumab, Rituximab), a hinge region, a transmembrane region, a co-stimulatory factor signal region, and a CD3ζ signaling region. CAR-T20.14, CAR-T20.13 and CAR-T20.16 are CAR structures constructed based on the antibody variable region sequences of Ofatumumab, Obinutuzumab and Rituxmab, respectively. CAR-T20.19 and CAR-20.20 are the mutant form of CAR-T20.14, having L235E-N297Q mutation in IgG4 Hinge-CH2-CH3 linker region. CAR-T20.20 is a third generation chimeric antigen receptor structure with coding sequences of both CD28 and 4-1BB co-stimulatory signaling molecule.

Figure 2 shows detection of transfection efficiency of engineered T cell with chimeric antigen receptors targeting CD20. The expression level of the CAR gene-encoded protein on the surface of the T cell membrane in CAR-T20s cells cultured on day 7 (A) and day 11 (B) was identified by the Protein L method.

Figure 3. $1^{*}10^{5}$ of NT, CART-20.13, CART-20.14 and CAR-T20.16 cells (cultured on day 6) were co-cultured respectively with CD20-positive RAJI and RAMOS tumor cell lines, and CD20-negative MOLT-4 tumor cell line in 200 μl GT-551 medium for 18h in a ratio of 1:1. Then the expression level of CD137 on the surface of T cell membrane (A) and the secretion level of IFNγ in the co-culture supernatant (B) were detected.

Figure 4 shows detection of apoptosis levels of tumor cells induced by CART-20. $1^{*}10^{4}$ of CFSE-labeled CD20-negative (MOLT-4) or CD20-positive (RAJI, RAMOS) tumor cell lines were co-cultured respectively with NT, CART-20.13, CART-20.14 and CAR-T20.16 cells (cultured on

day 11) in 200 μl GT-551 medium for 4h according to the ratio as shown in figure. Then the cell pellet was collected by centrifugation. The cells were washed twice with PBS and stained for 30 min with Annexin V-APC dye in a ratio of 1:50 in 100 μl of dyeing solution. After washing with PBS for 1 time, the proportion of Annexin V positive cells in CFSE positive cells was analyzed on a flow cytometry. The results in figure show the statistical analysis of Annexin V positive cells in the corresponding co-culture samples.

Figure 5 shows identification of the activation ability *in vitro* of the third generation chimeric antigen receptor and the chimeric antigen receptor with mutation in hinge region (which are constructed based on the sequence of Ofatumumaband antibody). The expression level of the CAR gene-encoded protein (A) on the surface of the T cell membrane in CAR-T20.14, CAR-T20.19 and CAR-T20.20 cells cultured on day 7 was identified by the Protein L method. $1*10^5$ of NT, CART-20.14, CART-20.19 and CAR-T20.20 cells (cultured on day 7) were cultured respectively with K562, K562 stable transfected cells of CD19 single positive, CD20 single positive, CD19 and CD20 double positive, and RAJI target cell in 200 μl GT-551 medium for 18h in a ratio of 1:1. Then the expression level of CD137 on the surface of T cell membrane (B) and the secretion level of IFNγ in the culture supernatant (C) were detected, respectively.

Figure 6 shows the detection results of the ability of CAR-T20 cells to scavenge CD20-positive cells *in vivo.* The results indicate that CAR-T20.19 can effectively inhibit the *in vivo* expansion of CD20-positive tumor cells.

Modes for Carrying Out the Present Invention

[0028] After extensive and intensive studies, the inventors have obtained a chimeric antigen receptor targeting CD20 and the preparation and application thereof. The extracellular antigen binding domain of the chimeric antigen receptor includes the antibody heavy chain variable region shown in SEQ ID NO: 1 and the antibody light chain variable region shown in SEQ ID NO: 2. The experimental results show that the chimeric antigen receptor provided by the present invention shows extremely high killing ability against tumor cells.

[0029] In view of the differences in affinity, killing mechanism of therapeutic antibodies targeting CD20, as well as the significant effects of different transmembrane domains and intracellular domains on the activity of chimeric antigen receptor, a series of chimeric antigen receptors targeting CD20 were constructed by combining various transmembrane and intracellular components with the amino acid sequences of the variable regions in various anti-CD20 antibodies. The expression of such chimeric antigen receptors in primary T cells was completed. The detection method of receptor expression intensity was established. The ability of the CAR-T cells to recognize CD20 antigen *in vitro* and *in vivo,* as well as the difference in the activity of scavenging malignant tumors carrying CD20 antigen *in vitro* and *in vivo* were identified, providing a new effective method and preparation for the clinical application of CAR

T in treating CD20 positive leukemia and lymphoma.

Chimeric antigen receptor

[0030] The invention provides a chimeric antigen receptor (CAR) comprising an extracellular domain, a transmembrane domain, and an intracellular domain. The extracellular domain comprises a target-specific binding element (also known as an antigen binding domain). The intracellular domain includes a co-stimulatory signaling region and a ζ chain moiety. The co-stimulatory signaling region refers to a part of the intracellular domain that includes a co-stimulatory molecule. The co-stimulatory molecule is a cell surface molecule required for efficient response of lymphocytes to antigens, rather than an antigen receptor or its ligand.

[0031] The extracellular domain of the CAR provided by the invention comprises an antigen binding domain targeting CD20. When the CAR of the present invention is expressed in T cell, antigen recognition can be performed based on antigen binding specificity. When it binds to its cognate antigen, it affects a tumor cell so that the tumor cell fails to grow, is prompted to die, or otherwise is affected so that the tumor burden in a patient is diminished or eliminated. The antigen binding domain is preferably fused with an intracellular domain from one or more of a co-stimulatory molecule and a ζ chain. Preferably, the antigen binding domain is fused with an intracellular domain of a combination of a 4-1BB signaling domain and/or a CD28 signaling domain, and a CD3ζ signaling domain.

[0032] In one embodiment, the CAR targeting CD20 of the invention comprises the specific signaling domain of the invention (the transmembrane region of CD8, the intracellular signal domains of CD137 and CD3ζ are made in series). The signaling domain of the invention significantly increases anti-tumor activity and *in vivo* persistence of CAR-T cells compared to an otherwise identical CAR targeting CD20.

[0033] The amino acid sequence of the chimeric antigen receptor (CAR) provided by the invention is as follows:

CAR-T20.19(SEQ ID NO:5)

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MALPVTALLL PLALLLHAAR PEVQLVESGG GLVQPGRSLR LSCAASGFTF NDYAMHWVRQ  60
APGKGLEWVS TISWNSGSIG YADSVKGRFT ISRDNAKKSL YLQMNSLRAE DTALYYCAKD  120
IQYGNYYYGM DVWGQGTTVT VSSGGGGSGG GGSGGGGSEI VLTQSPATLS LSPGERATLS  180
CRASQSVSSY LAWYQQKPGQ APRLLIYDAS NRATGIPARF SGSGSGTDFT LTISSLEPED  240
FAVYYCQQRS NWPITFGQGT RLEIKESKYG PPCPPCPAPE FEGGPSVFLF PPKPKDTLMI  300
SRTPEVTCVV VDVSQEDPEV QFNWYVDGVE VHNAKTKPRE EQFQSTYRVV SVLTVLHQDW  360
LNGKEYKCKV SNKGLPSSIE KTISKAKGQP REPQVYTLPP SQEEMTKNQV SLTCLVKGFY  420
PSDIAVEWES NGQPENNYKT TPPVLDSDGS FFLYSRLTVD KSRWQEGNVF SCSVMHEALH  480
NHYTQKSLSL SLGKIYIWAP LAGTCGVLLL SLVITLYCKR GRKKLLYIFK QPFMRPVQTT  540
QEEDGCSCRF PEEEEGGCEL RVKFSRSADA PAYKQGQNQL YNELNLGRRE EYDVLDKRRG  600
RDPEMGGKPR RKNPQEGLYN ELQKDKMAEA YSEIGMKGER RRGKGHDGLY QGLSTATKDT  660
YDALHMQALP PR  672
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[0034] The DNA sequence encoding CAR-T20.19 (SEQ ID NO: 6) is as follows:

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atggccttac cagtgaccgc cttgctcctg ccgctggcct tgctgctcca cgccgccagg  60
ccggaaglgc agctgglgga glclgggggа ggcllgglac agcclggcag glcccigaga  120
ctctcctgtg cagcctctgg attcaccttt aatgattatg ccatgcactg ggtccggcaa  180
gctccaggga agggcctgga gtgggtctca actattagtt ggaatagtgg ttccataggc  240
tatgcggact ctgtgaaggg ccgattcacc atctccagag acaacgccaa gaagtccctg  300
tatctgcaaa tgaacagtct gagagctgag gacacggcct tgtattactg tgcaaaagat  360
atacaglacg gcaactacta clacggtatg gacgtctggg gccaagggac cacggtcacc  420
gtctcctcag gtggcggtgg ctcgggcggt ggtgggtcgg gtggcggcgg atctgaaatt  480
gtgttgacac agtctccagc caccctgtct ttgtctccag gggaaagagc cacctctcc   540
tgcagggcca gtcagagtgt tagcagctac ttagcctggt accaacagaa acctggccag  600
gctcccaggc tcctcatcta tgatgcatcc aacagggcca ctggcatccc agccaggttc  660
agtggcagtg ggtctgggac agacttcact ctcaccatca gcagcctaga gcctgaagat  720
tttgcagttt attactgtca gcagcgtagc aactggccga tcaccttcgg ccaagggaca  780
cgactggaga ttaaagagag caagtacgga ccgccctgcc ccccttgccc tgcccccgag  840
ttcgagggcg gacccagcgt gttcctgttc ccccccaagc ccaaggacac cctgatgatc  900
agccggaccc ccgaggtgac ctgcgtggtg gtggacgtga gccaggaaga tcccgaggtc  960
cagttcaatt ggtacgtgga cggcgtggaa gtgcacaacg ccaagaccaa gcccagagag  1020
gaacagttcc aaagcaccta ccgggtggtg tctgtgctga ccgtgctgca ccaggactgg  1080

ctgaacggca aagaatacaa gtgcaaggtg tccaacaagg gcctgcccag cagcatcgaa  1140
aagaccatca gcaaggccaa gggccagcct cgcgagcccc aggtgtacac cctgcctccc  1200
tcccaggaag agatgaccaa gaaccaggtg tccctgacct gcctggtgaa gggcttctac  1260
cccagcgaca tcgccgtgga gtgggagagc aacggccagc ctgagaacaa ctacaagacc  1320
acccctcccg tgctggacag cgacggcagc ttcttcctgt acagccggct gaccgtggac  1380
aagagccggt ggcaggaagg caacgtcttt agctgcagcg tgatgcacga ggccctgcac  1440
aaccactaca cccagaagag cctgagcctg tccctgggca agatctacat ctgggcgccc  1500
ttggccggga cttgtggggt ccttctcctg tcactggtta tcacccttta ctgcaaacgg  1560
ggcagaaaga aactcctgta tatattcaaa caaccattta tgagaccagt acaaactact  1620
caagaggaag atggctgtag ctgccgattt ccagaagaag aagaaggagg atgtgaactg  1680
agagtgaagt tcagcaggag cgcagacgcc cccgcgtaca agcagggcca gaaccagctc  1740
tataacgagc tcaatctagg acgaagagag gagtacgatg ttttggacaa gagacgtggc  1800
cgggaccctg agatgggggg aaagccgaga aggaagaacc ctcaggaagg cctgtacaat  1860
gaactgcaga aagataagat ggcggaggcc tacagtgaga ttgggatgaa aggcgagcgc  1920
cggaggggca aggggcacga tggcctttac cagggtctca gtacagccac caaggacacc  1980
tacgacgccc ttcacatgca ggccctgccc cctcgctag  2019
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[0035] Other CAR sequences are described herein, but are not part of the present invention:

CAR-T20.13 (amino acid sequence SEQ ID NO:29 and DNA sequence SEQ ID NO: 30)

CAR-T20.14 (amino acid sequence SEQ ID NO:1 and DNA sequence SEQ ID NO: 2):

CAR-T20.16 (amino acid sequence SEQ ID NO:3 and DNA sequence SEQ ID NO: 4)

CAR-T20.20 (amino acid sequence SEQ ID NO:31 and DNA sequence SEQ ID NO: 32)

Antigen binding domain

[0036] The CAR of the invention comprises a target-specific binding element referred to as

antigen binding domain. The antigen binding domain of the CAR of the invention is a specific binding element targeting CD20.

[0037] The antigen binding domain comprises a heavy chain variable region and a light chain variable region of an anti-CD20 antibody.

Hinge region and transmembrane region

[0038] As for the hinge region and the transmembrane region (transmembrane domain), the CAR can be designed to comprise a transmembrane domain fused to the extracellular domain of the CAR. In one embodiment, a transmembrane domain that is naturally associated with one of the domains in the CAR is used. In some embodiments, transmembrane domains may be selected or modified by amino acid substitutions to avoid binding such domains to the transmembrane domain of the same or different surface membrane proteins, thereby minimizing the interaction with other members of the receptor complexes.

[0039] In one aspect, the hinge region comprises the following amino acid sequence (IgG4 Hinge-CH2-CH3 hinge region):

ESKYGPPCPP CPAPEFLGGP SVFLFPPKPK DTLMISRTPE VTCVVVDVSQ EDPEVQFNWY 60
VDGVEVHNAK TKPREEQFNS TYRVVSVLTV LHQDWLNGKE YKCKVSNKGL PSSIEKTISK 120
AKGQPREPQV YTLPPSQEEM TKNQVSLTCL VKGFYPSDIA VEWESNGQPE NNYKTTPPVL 180

DSDGSFFLYS RLTVDKSRWQ EGNVFSCSVM HEALHNHYTQ KSLSLSLGK 229 (SEQ ID NO:17)

[0040] Its coding DNA sequence is as follows:

GAGAGCAAGT ACGGACCGCC CTGCCCCCCT TGCCCTGCCC CCGAGTTCCT GGGCGGACCC 60
AGCGTGTTCC TGTTCCCCCC CAAGCCCAAG GACACCCTGA TGATCAGCCG GACCCCCGAG 120
GTGACCTGCG TGGTGGTGGA CGTGAGCCAG GAAGATCCCG AGGTCCAGTT CAATTGGTAC 180
GTGGACGGCG TGGAAGTGCA CAACGCCAAG ACCAAGCCCA GAGAGGAACA GTTCAACAGC 240
ACCTACCGGG TGGTGTCTGT GCTGACCGTG CTGCACCAGG ACTGGCTGAA CGGCAAAGAA 300
TACAAGTGCA AGGTGTCCAA CAAGGGCCTG CCCAGCAGCA TCGAAAAGAC CATCAGCAAG 360
GCCAAGGGCC AGCCTCGCGA GCCCCAGGTG TACACCCTGC CTCCCTCCCA GGAAGAGATG 420
ACCAAGAACC AGGTGTCCCT GACCTGCCTG GTGAAGGGCT TCTACCCCAG CGACATCGCC 480
GTGGAGTGGG AGAGCAACGG CCAGCCTGAG AACAACTACA AGACCACCCC TCCCGTGCTG 540
GACAGCGACG GCAGCTTCTT CCTGTACAGC CGGCTGACCG TGGACAAGAG CCGGTGGCAG 600
GAAGGCAACG TCTTTAGCTG CAGCGTGATG CACGAGGCCC TGCACAACCA CTACACCCAG 660
AAGAGCCTGA GCCTGTCCCT GGGCAAG 687 (SEQ ID NO:18);

or, the hinge region comprises the following amino acid sequence (IgG4 Hinge-CH2-CH3 (L235E, N297Q)):

ESKYGPPCPP CPAPEFEGGP SVFLFPPKPK DTLMISRTPE VTCVVVDVSQ EDPEVQFNWY 60
VDGVEVHNAK TKPREEQFQS TYRVVSVLTV LHQDWLNGKE YKCKVSNKGL PSSIEKTISK 120
AKGQPREPQV YTLPPSQEEM TKNQVSLTCL VKGFYPSDIA VEWESNGQPE NNYKTTPPVL 180
DSDGSFFLYS RLTVDKSRWQ EGNVFSCSVM HEALHNHYTQ KSLSLSLGK 229 (SEQ ID NO:19)

[0041] Its coding DNA sequence is as follows:

GAGAGCAAGT ACGGACCGCC CTGCCCCCCT TGCCCTGCCC CCGAGTTCGA GGGCGGACCC 60
AGCGTGTTCC TGTTCCCCCC CAAGCCCAAG GACACCCTGA TGATCAGCCG GACCCCCGAG 120
GTGACCTGCG TGGTGGTGGA CGTGAGCCAG GAAGATCCCG AGGTCCAGTT CAATTGGTAC 180
GTGGACGGCG TGGAAGTGCA CAACGCCAAG ACCAAGCCCA GAGAGGAACA GTTCCAAAGC 240
ACCTACCGGG TGGTGTCTGT GCTGACCGTG CTGCACCAGG ACTGGCTGAA CGGCAAAGAA 300
TACAAGTGCA AGGTGTCCAA CAAGGGCCTG CCCAGCAGCA TCGAAAAGAC CATCAGCAAG 360
GCCAAGGGCC AGCCTCGCGA GCCCCAGGTG TACACCCTGC CTCCCTCCCA GGAAGAGATG 420
ACCAAGAACC AGGTGTCCCT GACCTGCCTG GTGAAGGGCT TCTACCCCAG CGACATCGCC 480
GTGGAGTGGG AGAGCAACGG CCAGCCTGAG AACAACTACA AGACCACCCC TCCCGTGCTG 540
GACAGCGACG GCAGCTTCTT CCTGTACAGC CGGCTGACCG TGGACAAGAG CCGGTGGCAG 600
GAAGGCAACG TCTTTAGCTG CAGCGTGATG CACGAGGCCC TGCACAACCA CTACACCCAG 660
AAGAGCCTGA GCCTGTCCCT GGGCAAG 687 (SEQ ID NO:20).

[0042] In one aspect, the amino acid sequence of the transmembrane region derived from CD8 (CD8TM) is as follows:
IYIWAPLAGT CGVLLLSLVI TLYC 24 (SEQ ID NO:21)

[0043] The coding DNA sequence thereof is as follows:

ATCTACATCT GGGCGCCCTT GGCCGGGACT TGTGGGGTCC TTCTCCTGTC ACTGGTTATC 60
ACCCTTTACT GC 72 (SEQ ID NO:22)

[0044] In another aspect, the amino acid sequence of the transmembrane region derived from CD28 (CD28TM) is as follows:

FWVLVVVGGV LACYSLLVTV AFIIFWV 27 (SEQ ID NO:37);

The DNA sequence encoding the transmembrane region derived from CD28 (CD28TM) is as follows:

TTTTGGGTGC TGGTGGTGGT TGGTGGAGTC CTGGCTTGCT ATAGCTTGCT AGTAACAGTG 60
GCCTTTATTA TTTTCTGGGT G 81 (SEQ ID NO:38).

Intracellular domain

[0045] The intracellular domain in the CAR described herein comprises the signaling domain of 4-1BB and the signaling domain of CD3ζ.

[0046] Preferably, the intracellular signaling domain of 4-1BB comprises the following amino acid sequence:
KRGRKKLLYI FKQPFMRPVQ TTQEEDGCSC RFPEEEEGGC EL 42 (SEQ ID N0:23)

[0047] The coding DNA sequence thereof is as follows:

AAACGGGGCA GAAAGAAACT CCTGTATATA TTCAAACAAC CATTTATGAG ACCAGTACAA 60
ACTACTCAAG AGGAAGATGG CTGTAGCTGC CGATTTCCAG AAGAAGAAGA AGGAGGATGT 120

GAACTG 126 (SEQ ID NO:24)

[0048] Preferably, the intracellular signaling domain derived from CD28 comprises the following amino acid sequence:
RSKRSRLLHS DYMNMTPRRP GPTRKHYQPY APPRDFAAYR S 41 (SEQ ID N0:39)

[0049] The coding DNA sequence thereof is as follows:
AGGAGTAAGA GGAGCAGGCT CCTGCACAGT GACTACATGA ACATGACTCC CCGCCGCCCC 60
GGGCCCACCC GCAAGCATTA CCAGCCCTAT GCCCCACCAC GCGACTTCGC AGCCTATCGC 120
TCC 123 (SEQ ID NO:40)

[0050] Preferably, the intracellular signaling domain of CD3ζ comprises the following amino acid sequence:
RVKFSRSADA PAYQQGQNQL YNELNLGRRE EYDVLDKRRG RDPEMGGKPQ RRKNPQEGLY 60
NELQKDKMAE AYSEIGMKGE RRRGKGHDGL YQGLSTATKD TYDALHMQAL PPR 113 (SEQ ID NO:25)

[0051] The coding DNA sequence thereof is as follows:
AGAGTGAAGT TCAGCAGGAG CGCAGACGCC CCCGCGTACA AGCAGGGCCA GAACCAGCTC 60
TATAACGAGC TCAATCTAGG ACGAAGAGAG GAGTACGATG TTTTGGACAA GAGACGTGGC 120
CGGGACCCTG AGATGGGGGG AAAGCCGAGA AGGAAGAACC CTCAGGAAGG CCTGTACAAT 180
GAACTGCAGA AAGATAAGAT GGCGGAGGCC TACAGTGAGA TTGGGATGAA AGGCGAGCGC 240
CGGAGGGGCA AGGGGCACGA TGGCCTTTAC CAGGGTCTCA GTACAGCCAC CAAGGACACC 300
TACGACGCCC TTCACATGCA GGCCCTGCCC CCTCGC 336 (SEQ ID NO:26)

Vector

[0052] The invention also provides a DNA construct encoding the CAR sequences of the invention.

[0053] The nucleic acid sequences coding for the desired molecules can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the gene of interest can be produced synthetically.

[0054] The present invention also provides vectors in which the DNA construct of the present invention is inserted. Vectors derived from retroviruses such as the lentivirus are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the advantage over vectors derived from onco-retroviruses such as murine leukemia viruses in that they can transduce non-proliferating cells, such as hepatocytes. They also have the advantage of low immunogenicity.

[0055] In brief summary, the expression of natural or synthetic nucleic acids encoding CARs is typically achieved by operably linking a nucleic acid encoding the CAR polypeptide or portions thereof to a promoter, and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration in eukaryotes. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

[0056] The expression constructs of the present invention may also be used for nucleic acid immune and gene therapy, using standard gene delivery protocols. Methods for gene delivery are known in the art. See, e.g., U.S, Pat. Nos. 5,399,346, 5,580,859, 5,589,466. In another embodiment, the invention provides a gene therapy vector,

The nucleic acid can be cloned into a number of types of vectors. For example, the nucleic acid can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors,

Further, the expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook et al, (2001 , Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO 01/29058; and U.S, Pat. No. 6,326, 193).

[0057] A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo.* A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

[0058] Additional promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter,

it appears that individual elements can function either cooperatively or independently to activate transcription.

[0059] One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable promoter is Elongation Growth Factor-1α (EF- 1α). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the invention should not be limited to the use of constitutive promoters, inducible promoters are also contemplated as part of the invention. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionein promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

[0060] In order to assess the expression of a CAR polypeptide or portions thereof, the expression vector to be introduced into a ceil can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

[0061] Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., 2000 FEBS Letters 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

[0062] Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

[0063] Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). A preferred method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

[0064] Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat, Nos. 5,350,674 and 5,585,362.

[0065] Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (e.g., an artificial membrane vesicle).

[0066] In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (*in vitro*, *ex vivo* or *in vivo*). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

[0067] In the case where a non-viral delivery system is utilized, genome editing technique is

exemplarily employed to complete the invention, for example CRISPR-Cas9, ZFN or TALEN.

[0068] In a preferred embodiment of the invention, the vector is a lentiviral vector.

[0069] In a preferred embodiment of the invention, the DNA construct further comprises a signal peptide coding sequence. Preferably, the signal peptide sequence is ligated upstream of the nucleic acid sequence of antigen binding domain. Preferably the signal peptide is a human CD8a signal peptide.

[0070] Preferably, the amino acid sequence of the signal peptide is as follows:
The amino acid sequence of CD8 leader sequence is :
MALPVTALLL PLALLLHAAR P 21 (SEQ ID N0:27)

[0071] The DNA sequence encoding CD8 leader sequence is :
ATGGCCTTAC CAGTGACCGC CTTGCTCCTG CCGCTGGCCT TGCTGCTCCA CGCCGCCAGG 60
CCG 63 (SEQ ID N0:28)

Therapeutic Application

[0072] The present invention encompasses a cell (e.g., T cell) transduced with a lentiviral vector (LV) encoding the CAR of the invention. The transduced T cell can elicit a CAR-mediated T-cell response.

[0073] Thus, it is also described a method for stimulating a T cell-mediated immune response to a target cell population or tissue in a mammal comprising the step of administering to the mammal a T cell that expresses the CAR of the invention.

[0074] In one embodiment, the present invention includes a type of cellular therapy where T cells are genetically modified to express the CAR of the invention and the CAR-T cell is infused to a recipient in need thereof. The infused cell is able to kill tumor cells in the recipient. Unlike antibody therapies, CAR-T cells are able to replicate *in vivo* resulting in long-term persistence that can lead to sustained tumor control.

[0075] In one embodiment, the CAR-T cells of the invention can undergo robust *in vivo* T cell expansion and can persist for an extended amount of time. In addition, the CAR mediated immune response may be part of an adoptive immunotherapy approach in which CAR-modified T cells induce an immune response specific to the antigen binding moiety in the CAR. For example, an anti-CD20 CAR-T cell elicits an immune response specific against cells expressing CD20.

[0076] Adaptation diseases that may be treated include CD20 positive tumors. CD20 positive tumors may include CD20 positive non-solid tumors (such as hematological tumors, for

example, leukemias and lymphomas) or solid tumors. Types of tumors or cancers to be treated with the CARs of the invention include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies e.g., sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included.

[0077] Hematologic cancers are cancers of the blood or bone marrow. Examples of hematological (or hematogenous) cancers include leukemias, including acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblasts, promyeiocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

[0078] Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors can be benign or malignant. Different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and lymphomas). Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, mesothelioma, malignant lymphoma, pancreatic cancer and ovarian cancer.

[0079] The CAR-modified T cells of the invention may also serve as a type of vaccine for *ex vivo* immunization and/or *in vivo* therapy in a mammal. Preferably, the mammal is a human.

[0080] With respect to *ex vivo* immunization, at least one of the following occurs *in vitro* prior to administering the cell into a mammal: i) expansion of the cells, ii) introducing a nucleic acid encoding a CAR to the cells, and/or iii) cryopreservation of the cells.

[0081] *Ex vivo* procedures are well known in the art and are discussed more fully below. Briefly, cells are isolated from a mammal (preferably a human) and genetically modified (i.e., transduced or transfected *in vitro*) with a vector expressing a CAR disclosed herein. The CAR-modified cell can be administered to a mammalian recipient to provide a therapeutic benefit. The mammalian recipient may be a human and the CAR-modified cell can be autologous with respect to the recipient. Alternatively, the cells can be allogeneic, syngeneic or xenogeneic with respect to the recipient.

[0082] In addition to using a cell-based vaccine in terms of *ex vivo* immunization, the present disclosure also provides compositions and methods for *in vivo* immunization to elicit an immune response directed against an antigen in a patient.

[0083] Generally, the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised. In particular, the CAR-modified T cells of the invention are used in the treatment of CCL. In

certain embodiments, the cells of the invention are used in the treatment of patients at risk for developing CCL. Thus, it is herein described the use for the treatment or prevention of CCL comprising administering to a subject in need thereof, a therapeutically effective amount of the CAR-modified T cells of the invention.

[0084] The CAR-modified T cells of the present invention may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as IL-2, IL-17 or other cytokines or cell populations. Briefly, pharmaceutical compositions of the present invention may comprise a target cell population as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the present invention are preferably formulated for intravenous administration.

[0085] Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

[0086] When "an immunologically effective amount", "an anti-tumor effective amount", "an tumor-inhibiting effective amount", or "therapeutic amount" is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the T cells described herein may be administered at a dosage of 10^4 to 10^9 cells/kg body weight, preferably 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. T cell compositions may also be administered multiple times at these dosages. The cells can be administered by using infusion techniques that are commonly known in immunotherapy (see, e.g., Rosenberg et al,, New Eng. J. of Med. 319: 1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

[0087] The administration of the subject compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermaliy, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. In one embodiment, the T cell compositions of the present invention are administered to a patient by intradermal or subcutaneous injection. In another embodiment, the T cell compositions of the present invention are preferably

administered by i.v. injection. The compositions of T cells may be injected directly into a tumor, lymph node, or site of infection.

[0088] In certain aspects, cells activated and expanded using the methods described herein, or other methods known in the art where T cells are expanded to therapeutic levels, are to be administered to a patient in conjunction with (e.g., before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or natalizumab treatment for MS patients or efalizumab treatment for psoriasis patients or other treatments for PML patients. In further embodiments, the T cells of the invention may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunotherapeutic agents. In a further embodiment, the cell compositions of the present invention are administered to a patient in conjunction with (e.g., before, simultaneously or following) bone marrow transplantation, or the use of chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgery.

[0089] The dosage of the above treatments to be administered to a patient will vary with the precise nature of the condition being treated and the recipient of the treatment. The scaling of dosages for human administration can be performed according to art-accepted practices. In general, 1×10^6 to 1×10^{10} of the modified T cells of the invention (e.g., CAR-T 20 cells) can be applied to patients by means of, for example, intravenous infusion each treatment or each course of treatment.

The advantages of the present invention include:

[0090]

1. (1) As for the chimeric antigen receptor of the present invention, the extracellular antigen binding domain thereof is a specific anti-CD20 scFv. The CAR formed by binding the specific anti-CD20 scFv to a specific hinge region and an intracellular domain shows a great capability of killing tumor cells with low cytotoxicity and low side effects.
2. (2) The chimeric antigen receptor provided by the invention can achieve stable expression and membrane localization of CAR protein after T cells is infected by lentivirus carrying CAR gene.
3. (3) The CAR-modified T cell of the present invention has a longer survival time *in vivo* and strong anti-tumor efficacy. The optimized CAR with the IgG4 Hinge-CH2-CH3 linker region can avoid the binding of the Fc receptor and the subsequent ADCC effect (antibody-dependent cytotoxicity).

Example 1 Construction of lentiviral expression vector

[0091] The full-length DNA synthesis and cloning construction of coding plasmids were commissioned by Shanghai Boyi Biotechnology Co., Ltd. Different anti-CD20 scFv coding sequences were used in each plasmid. The cloning vector was selected as pWPT lentiviral vector. The cloning sites were BamH I and Sal I sites. The specific sequence structure is shown in Figure 1. The amino acid and nucleotide sequences of each element are as described above.

[0092] In the following examples, CAR-T20.13, CAR-T20.14, CAR-T20.16, CAR-T20.19, CAR-T20.20 with better effects are taken as examples.

Example 2 Preparation of CAR-T cell

[0093]

1. (1) After taking venous blood from healthy people, mononuclear cells (PBMCs) were isolated by density gradient centrifugation.
2. (2) On day 0, PBMCs were cultured in GT-T551 cell culture medium containing 2% human albumin, and the final concentration of cells was adjusted to 2×10^6 cells/mL. The cells were seeded in a cell culture flask previously coated with Retronectin (purchased from TAKARA) at a final concentration of 10 μg/mL and CD3 monoclonal antibody (OKT3) at a final concentration of 5 μg/mL. Recombinant human interleukin 2 (IL-2) was added to the culture medium at a final concentration of 1000 U/mL. The cells were cultured in an incubator with a saturated humidity and 5% CO_2 at 37 °C.
3. (3) On day 2, fresh medium, concentrated and purified CAR20 lentivirus solution, protamine sulfate (12 μg/ml), and IL-2 (at a final concentration of 1000 U/mL) were added. After 12 hours of infection in a 5% CO_2 incubator at 37 °C, the culture medium was discarded, fresh medium was added, and cultivation was continued in a 5% CO_2 incubator at 37 °C.
4. (4) Starting from day 6, CART20 cells can be taken for the corresponding activity assay.

[0094] In the present invention, the preparation process of CAR-modified T cell targeting CD20 antigen is improved, and GT-551 serum-free medium supplemented with 2% human albumin was selected to culture lymphocytes *in vitro.*

Example 3 Detection of the integration rate of the CAR gene in the T cell genome and

the expression level of the encoded protein thereof on the membrane surface.

[0095] 0.5×10^{6} of CART-20 cell samples cultured on day 7 (Fig. 2A and Fig. 5A) and day 11 (Fig. 2B) in Example 2 were taken, respectively. The expression level of CAR20 protein on the surface of T cell membrane was analyzed by flow cytometry after Protein L staining. The results showed that, except for CAR-T20.13, all of the CAR structures designed in this study can detect the chimeric antigen receptor localization on the cell membrane surface of the corresponding modified T cells using Protein L.

Example 4 Detection of the *in vitro* activation ability of CAR-T20s

[0096] The deCAR-T20 cells cultured on day 6 in Example 2 were co-cultured with target cells. Then the up-regulated level of CD137 and the secretion level of IFNγ in the culture supernatant were examined. 1×10^{5} of CART-20 cells (cultured on day 6) were cultured respectively with CD20-positive RAJI and RAMOS tumor cell lines, and CD20-negative MOLT-4 tumor cell line, or without tumor cells, in 200 μl GT-551 medium for 18h in a ratio of 1:1. Then the expression level of CD137 on the surface of T cell membrane was detected by flow cytometry (Fig. 3A) and the secretion level of IFNγ in the culture supernatant was detected by ELISA (Fig. 3B).

[0097] From the results in Figure 3, we could concluded that the CAR based on Obinutuzumab also achieved expression and membrane surface localization in the corresponding modified cells, but the CAR structure based on the Ofatumumab sequence showed better *in vitro* activation ability and specificity targeting antigen when compared with the CAR constructed based on Obinutuzumab and Rituximab.

Example 5 Detection of the CAR-T20s cells induced early apoptosis activity of tumor cells

[0098] CART-20.13, CART-20.14 and CAR-T20.16 cells (cultured on day 11) from Example 2 were co-cultured respectively with 1×10^{4} of CFSE-labeled CD20-negative (MOLT-4) or CD20-positive (RAJI, RAMOS) tumor cell lines in 200 μl GT-551 medium for 4h. Then the cell pellet was collected by centrifugation. The cells were washed twice with PBS and stained for 30 min with Annexin V-APC dye in a ratio of 1:50 in 100 μl of dyeing solution. After washing with PBSonce, the proportion of Annexin V positive cells in CFSE positive cells was analyzed on a flow cytometry.

[0099] The results in Figure 4 show that the CAR structure based on the Ofatumumab sequence shows better ability to induce early apoptosis of CD20 target cells *in vitro* when

compared with the CAR constructed based on Obinutuzumab and Rituximab.

Example 6 Identification of the *in vitro* activation ability of the third generation chimeric antigen receptor and the chimeric antigen receptor with mutation in hinge region

[0100]

1. (1) Under the condition that the transfection rate was basically equal (Fig. 5A), the CAR-T20s cells (prepared by the method of Example 2) cultured on the day 7 were cultured respectively with K562, K562 stable transfected cells of CD19 single positive, CD20 single positive, CD19 and CD20 double positive, and RAJI target cell (each taking 1×10^5 cells) in 200 μl GT-551 medium for 18h in a ratio of 1:1. Then the up-regulated level of CD137 (Fig. 5B) and the secretion level of IFNγ in the culture supernatant (Fig. 5C) were detected.
2. (2) The results shown in Figure 5 indicate that the *in vitro* activation ability (CD137 and IFNg) of the chimeric antigen receptor CAR-T20.14 and CAR-T20.19 (having a mutation in the hinge region) is substantially equivalent, in the case of substantially identical infection efficiency. The third generation CAR structure CAR-T20.20 shows better *in vitro* activation capacity (CD137 and IFNγ) than the second generation CAR-T20.14 and CAR-T20.19.

Example 7. Detection of the ability of CAR-T20 cells to scavenge CD20 positive cells *in vivo*

[0101]

1. (1) Raji-Luc cells expressing luciferase were injected into NCG mice (5×10^5/ mouse) through the tail vein. One week after the inoculation, the *in vivo* expansion of the tumor cells was observed by *in vivo* imaging and recorded as Day 0. NT and CAR-T20.19 cells were injected into Day 0 mice (5×10^6/mouse) through the tail vein. On Day0, Day7, Day14, Day21, the expansion of tumor cells in mice was observed by *in vivo* imaging and analyzed based on changes in fluorescence intensity and body weight changes of mice.
2. (2) The results shown in Figure 6 indicate that CAR-T20.19 can effectively inhibit the *in vivo* expansion of CD20-positive tumor cells.

REFERENCES CITED IN THE DESCRIPTION

Cited references

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- **SAMBROOK et al.**Molecular Cloning: A Laboratory ManualCold Spring Harbor Laboratory20010000 [0056] [0063]
- **UI-TEI et al.**FEBS Letters, 2000, vol. 479, 79-82 [0061]
- **ROSENBERG et al.**New Eng. J. of Med., 1988, vol. 319, 1676- [0086]

Patentkrav

1. Kimær antigenreceptor (CAR), hvor CAR'en har aminosyresekvensen SEQ ID NO: 5.

2. Nukleinsyremolekyle, der koder for den kimære antigenreceptor (CAR) ifølge krav 1.

3. Vektor, der omfatter nukleinsyremolekylet ifølge krav 2.

4. Værtscelle, der omfatter vektoren ifølge krav 3 eller har et eksogent nukleinsyremolekyle ifølge krav 2 integreret i sit genom.

5. Farmaceutisk sammensætning, der omfatter en farmaceutisk acceptabel bærer og den kimære antigenreceptor ifølge krav 1, nukleinsyremolekylet ifølge krav 2, vektoren ifølge krav 3 eller cellen ifølge krav 4.

6. Farmaceutisk sammensætning ifølge krav 5 til anvendelse ved behandling af en tumor.

7. Farmaceutisk sammensætning til anvendelse ifølge krav 6, hvor tumoren er en CD20-positiv leukæmi eller et CD20-positivt lymfom.

8. *In vitro*-fremgangsmåde til fremstilling af en CAR-T-celle (CAR-modificeret T-celle), der udtrykker den kimære antigenreceptor ifølge krav 1, hvilken fremgangsmåde omfatter et trin til: transduktion af nukleinsyremolekylet ifølge krav 2 eller vektoren ifølge krav 3 ind i en T-celle, hvorved CAR-T-cellen opnås.

DRAWINGS

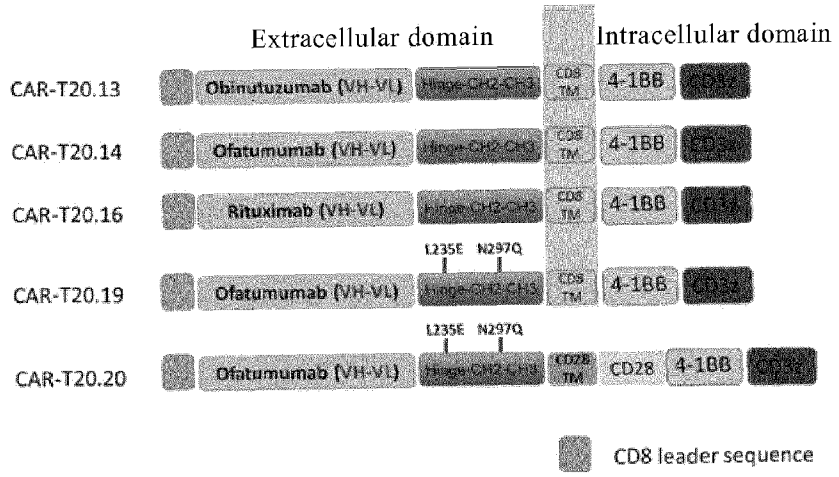


Fig. 1

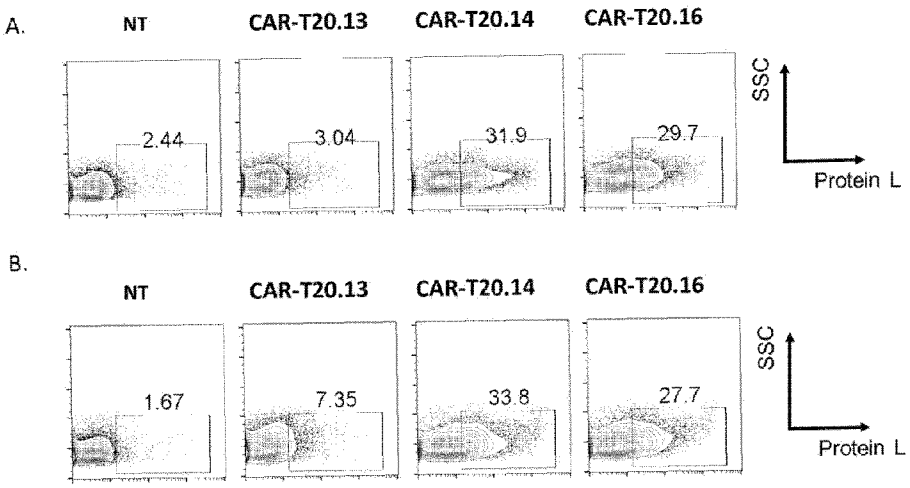
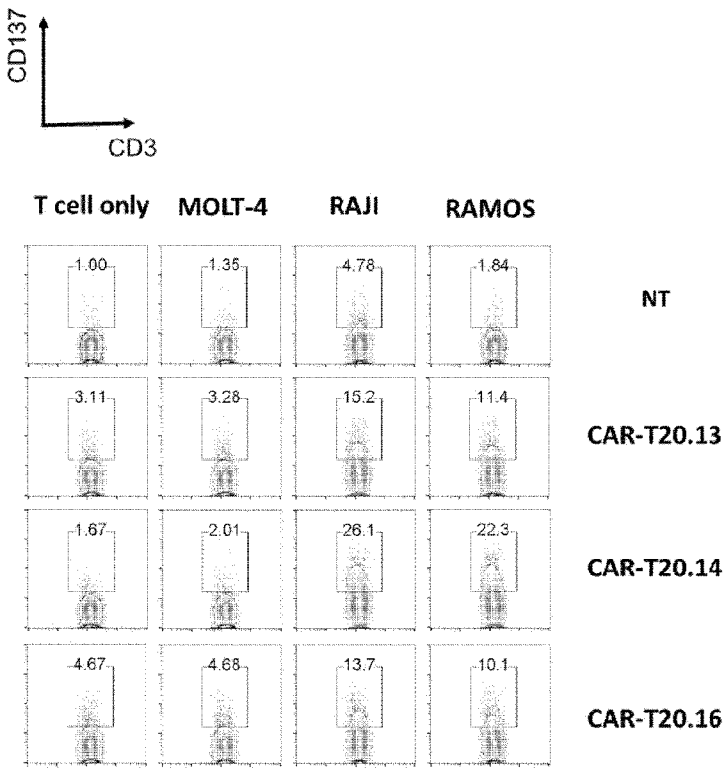


Fig. 2

A.


CD137
CD3
T cell only
MOLT-4
RAJI
RAMOS
1.00
1.35
4.78
1.84
NT
3.11
3.28
15.2
11.4
CAR-T20.13
1.67
2.01
26.1
22.3
CAR-T20.14
4.67
4.68
13.7
10.1
CAR-T20.16

B.

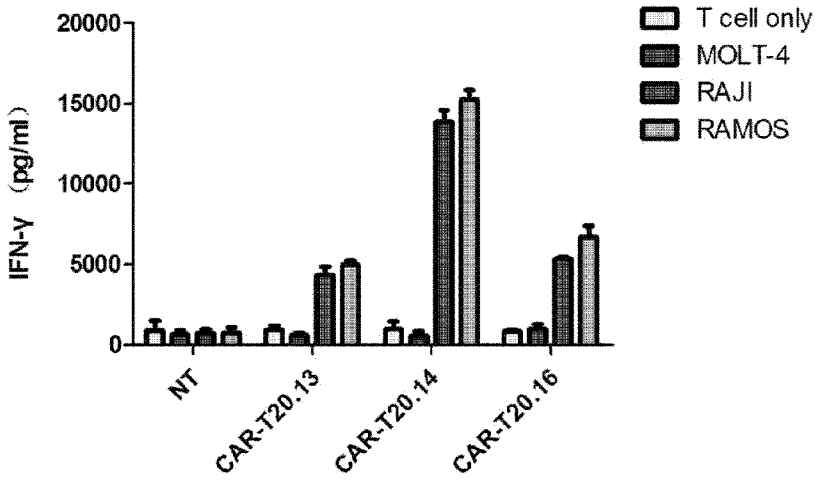

IFN-γ (pg/ml)
20000
15000
10000
5000
0
T cell only
MOLT-4
RAJI
RAMOS
NT
CAR-T20.13
CAR-T20.14
CAR-T20.16

Fig. 3

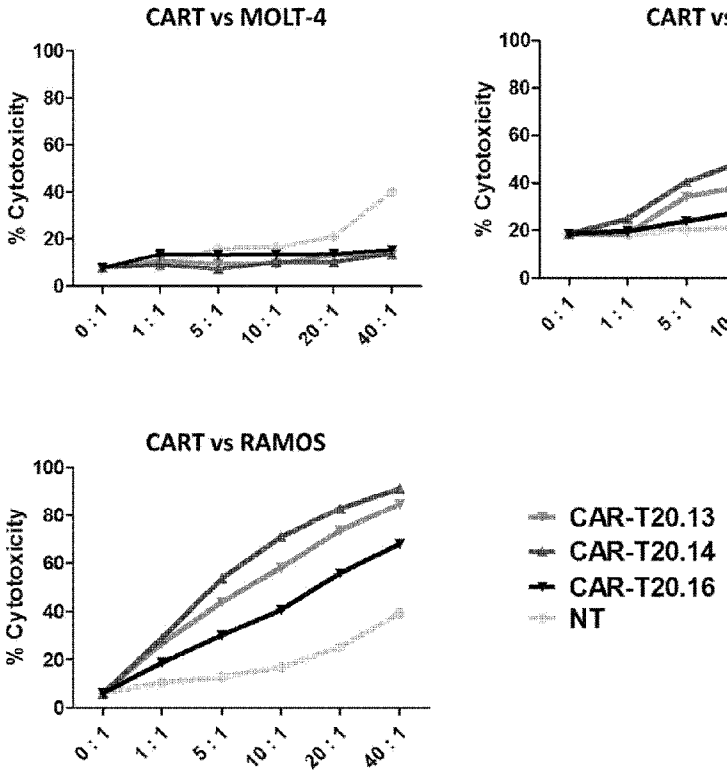
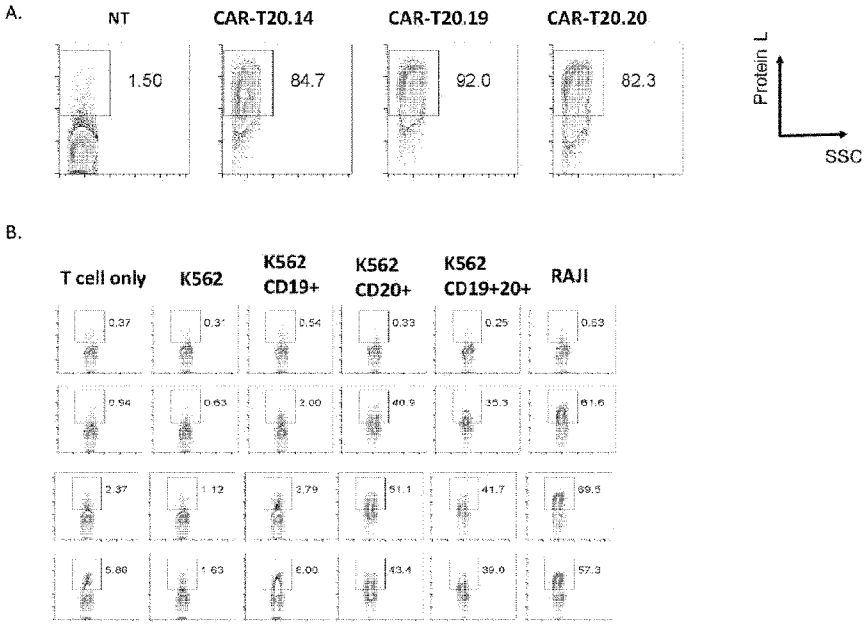

CART vs MOLT-4
% Cytotoxicity
100
80
60
40
20
0
0 : 1
1 : 1
5 : 1
10 : 1
20 : 1
40 : 1
CART vs RAJI
% Cytotoxicity
100
80
60
40
20
0
0 : 1
1 : 1
5 : 1
10 : 1
20 : 1
40 : 1
CART vs RAMOS
% Cytotoxicity
100
80
60
40
20
0
0 : 1
1 : 1
5 : 1
10 : 1
20 : 1
40 : 1
CAR-T20.13
CAR-T20.14
CAR-T20.16
NT

Fig. 4


A.
NT
CAR-T20.14
CAR-T20.19
CAR-T20.20
1.50
84.7
92.0
82.3
Protein L
SSC
B.
T cell only
K562
K562 CD19+
K562 CD20+
K562 CD19+20+
RAJI

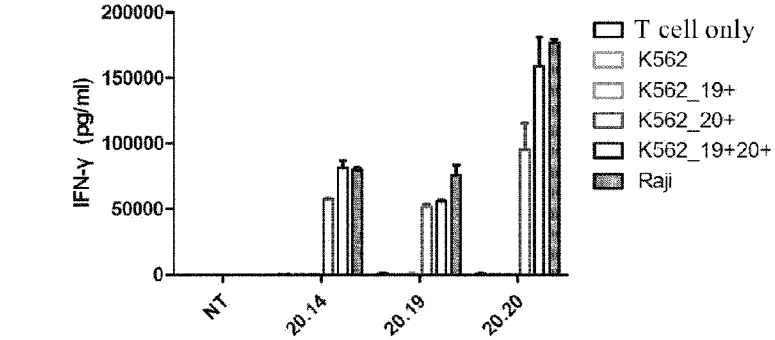

C.
IFN-γ (pg/ml)
200000
150000
100000
50000
0
NT
20.14
20.19
20.20
T cell only
K562
K562_19+
K562_20+
K562_19+20+
Raji

Fig. 5

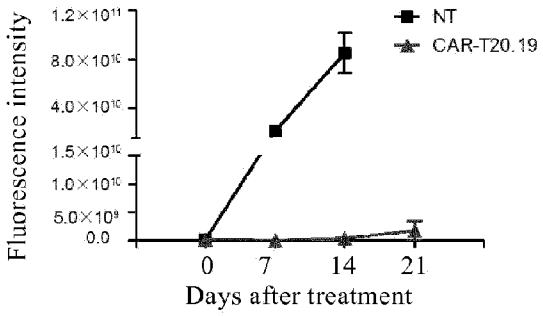

Fluorescence intensity
1.2×10^11
8.0×10^10
4.0×10^10
1.5×10^10
1.0×10^10
5.0×10^9
0.0
0
7
14
21
Days after treatment
NT
CAR-T20.19

Fig. 6

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.