HUMANIZED BCMA ANTIBODY AND BCMA-CAR-T CELLS

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

The Sequence Listing is concurrently submitted herewith with the specification as an ASCII formatted text file via EFS-Web with a file name of Sequence Listing.txt with a creation date of January 13, 2020, and a size of 4 kilobytes. The Sequence Listing filed via EFS-Web is part of the specification and is hereby incorporated in its entirety by reference herein.

FIELD OF THE INVENTION

The present invention relates to humanized BCMA antibody (PMC306) and BCMA-CAR-T cells specifically decreasing multiple myeloma tumor growth, which are useful in the field of adoptive immunity gene therapy for tumors.

BACKGROUND OF THE INVENTION

Immunotherapy is emerging as a highly promising approach for the treatment of cancer. T cells or T lymphocytes, the armed forces of our immune system, constantly look for foreign antigens and discriminate abnormal (cancer or infected cells) from normal cells. Genetically modifying T cells with CAR (Chimeric antigen receptor) constructs is the most common approach to design tumor-specific T cells. CAR-T cells targeting tumor-associated antigens (TAA) can be infused into patients (called adoptive cell transfer or ACT) representing an efficient immunotherapy approach [1, 2]. The advantage of CAR-T technology compared with chemotherapy or antibody is that reprogrammed engineered T cells can proliferate and persist in the patient ("a living drug") [1, 2].

CARs typically consist of a monoclonal antibody-derived single-chain variable fragment (scFv) at the N-terminal part, hinge, transmembrane domain and a number of intracellular co-activation domains: (i) CD28, (ii) CD137 (4-1BB), CD27, or other co-stimulatory domains, in tandem with an activation CD3-zeta domain (FIG. 1). The evolution of CARs went from first generation (with no co-stimulation domains) to second generation (with one co-stimulation domain) to third generation CAR (with several co-stimulation domains). Generating CARs with two costimulatory domains (the so-called 3rd generation CAR) have led to increased cytolytic CAR-T cell activity, improved persistence of CAR-T cells leading to its augmented antitumor activity.

BCMA

B cell maturation antigen (BCMA) is a cell surface receptor, also known as CD269 and tumor necrosis factor receptor superfamily member 17 (TNFRSF17), that is encoded by TNFRSF17 gene. This receptor is expressed mainly in mature B lymphocytes and in most cases overexpressed in multiple myeloma (MM) [4]. Current therapies to target BCMA in MM include monoclonal antibodies, bi-specific antibodies and T cellular immunotherapies, CAR-T therapies [4, 5].

The human BCMA protein consists of 184 amino-acids: 1-54-extracellular domain; 55-77-transmembrane domain; 78-184-cytoplasmic domain. The amino-acid sequence of BCMA is shown on FIG. 2. BCMA lacks signaling peptide and resembles other receptors BAFF Receptor and transmembrane activator and cyclophilin ligand interactor and calcium modulator (TACI) [4]. These receptors play major role in B cell maturation and differentiation into plasma cells. Their ligands include BAFF and APRIL which expression is increase in MM patients [4].

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the structures of CAR [3]. The left panel shows the structure of first generation (no costimulatory domains). The middle panel shows the structure of the second generation (one co-stimulation domain of CD28 or 4-BB). The right panel shows the structure of the third generation (two or more co-stimulation domains).

FIG. 2 shows the amino-acid sequence of BCMA protein (SEQ ID NO: 1). Extracellular domain is underlined.

FIG. 3 shows the structure of humanized BCMA CAR construct.

FIG. 4 shows that humanized BCMA-CAR construct was detected by FACS analysis with fluorescently labeled recombinant BCMA protein. Humanized BCMA-CAR-positive cells were detected after transduction of lentiviral humanized BCMA-CAR into T cells.

FIGs. 5A-5B show that humanized BCMA-CAR-T cells killed CHO-BCMA cells but not CHO cells. XCelligence Real-time cytotoxicity assay was used for detection of humanized BCMA-CAR-T cell cytotoxicity. Normalized cell index is shown on Y-axis, and time is shown on X-axis. FIG. 5A shows CHO-BCMA target cells. on the right: From top to bottom: Mock CAR-T cells, T cells, target cells alone, and humanized CAR-T cells. FIG. 5B shows CHO target cells. From top to bottom on the right, Mock CAR-T cells, humanized BCMA CAR-T cells, T cells, and target cells alone.

FIG. 6 shows that humanized BCMA-CAR-T cells, secreted high level of IFN-gamma with CHO-BCMA-positive cells, but not with BCMA-negative CHO control cells. $p<0.05$ IFN-gamma secretion in CHO-BCMA cells of BCMA-CAR-T cells versus T cells and Mock CAR-T cells.

FIG. 7 shows humanized BCMA-CAR-T cells secreted high level of IFN-gamma against multiple myeloma cells but not against BCMA-negative K562 control cells. $*p<0.05$, IFN-gamma secretion in multiple myeloma cells of BCMA-CAR-T cells versus T cells and Mock-CAR-T cells.

FIG. 8A shows humanized BCMA-CAR-T cells significantly decreased RPMI8226 xenograft tumor growth. CAR-T cells were injected at day 7 and 20 by i.v 1x10^7 cells/mice. Bars show average tumor volume +/- standard errors. $*p<0.05$, BCMA CAR-T cells vs Mock T cells.

FIG. 8B shows that humanized BCMA-CAR-T cells did not decrease mouse body weight. Bars show average mice body weight+/- standard deviations.

FIG. 8C shows that humanized BCMA-CAR-T cells, but not Mock-Car-T-cells, were detected in the mouse blood by FACS with BCMA recombinant protein. The peripheral blood cells were analyzed by flow cytometry at the end of the study for binding to human BCMA protein and antibodies specific for human T (CD4+/CD8+) cells. The percentage of cells binding to the CD4 antibody is shown on the left panel of FIG. 8C, and the percentage of those human T cells that also bound to the BCMA protein is shown on the right panel of FIG. 8C.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, a "chimeric antigen receptor (CAR)" is a receptor protein that has been engineered to give T cells the new ability to target a specific protein. The receptor is chimeric because they combine both antigen-binding and T-cell activating functions into a single receptor. CAR is a fused protein comprising an extracellular domain capable of binding to an antigen, a transmembrane domain, and at least one intracellular domain. The "chimeric antigen receptor (CAR)" is sometimes called a "chimeric receptor", a "T-body", or a "chimeric immune receptor (CIR)." The "extracellular domain capable of binding to an antigen" means any oligopeptide or polypeptide that can bind to a certain antigen. The

"intracellular domain" means any oligopeptide or polypeptide known to function as a domain that transmits a signal to cause activation or inhibition of a biological process in a cell.

As used herein, a "domain" means one region in a polypeptide which is folded into a particular structure independently of other regions.

As used herein, “humanized antibodies” are antibodies derived from non-human species whose protein sequences have been modified to increase their similarity to antibody variants produced naturally in humans. For example, after a mouse antibody is developed, the DNA coding for that antibody can be sequenced. The DNA sequence corresponding to the antibody CDRs can then be determined. The CDR sequences can be inserted into a construct containing the DNA for a human antibody variant to prepare humanized antibodies.

As used herein, a "single chain variable fragment (scFv)" means a single chain polypeptide derived from an antibody which retains the ability to bind to an antigen. An example of the scFv includes an antibody polypeptide which is formed by a recombinant DNA technique and in which Fv regions of immunoglobulin heavy chain (H chain) and light chain (L chain) fragments are linked via a spacer sequence. Various methods for engineering an scFv are known to a person skilled in the art.

As used herein, a "tumor antigen" means a biological molecule having antigenicity, expression of which causes cancer.

The inventors have engineered humanized BCMA scFv starting from heavy and light chain variable regions of mouse monoclonal antibody derived from a mouse monoclonal antibody, clone 4C8A. Mouse 4C8A antibody exhibits strong and selective binding to human BCMA [6]. The inventors have produced BCMA-CAR-T cells based on humanized BCMA antibody to target cancer cells overexpressing BCMA tumor antigen. The BCMA-CAR-T cells of the present invention have high cytotoxic activity against several cancer cell lines.

The present invention is directed to a humanized anti-human BCMA antibody comprising V_H having the amino acid of SEQ ID NO: 3 and V_L having the amino acid of SEQ ID NO: 5.

In one embodiment, the humanized anti-human BCMA antibody is a single-chain variable fragment (scFv). ScFv can be V_H -linker-V_L or V_L-linker- V_H.

The present invention is also directed to a chimeric antigen receptor fusion protein comprising from N-terminus to C-terminus: (i) a single-chain variable fragment (scFv) against BCMA in which V_H has the amino acid sequence of SEQ ID NO 3, and V_L has the amino acid of SEQ ID NO: 5, (ii) a transmembrane domain, (iii) at least one co-stimulatory

domains, and (iv) an activating domain.

In one embodiment, the CAR structure is shown in FIG. 2.

In one embodiment, the co-stimulatory domain is selected from the group consisting of CD28, 4-1BB, GITR, ICOS-1, CD27, OX-40 and DAP10. A preferred the co-stimulatory domain is CD28.

A preferred activating domain is CD3 zeta (CD3 Z or CD3ζ).

The transmembrane domain may be derived from a natural polypeptide, or may be artificially designed. The transmembrane domain derived from a natural polypeptide can be obtained from any membrane-binding or transmembrane protein. For example, a transmembrane domain of a T cell receptor α or β chain, a CD3 zeta chain, CD28, CD3ε., CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, ICOS, CD154, or a GITR can be used. The artificially designed transmembrane domain is a polypeptide mainly comprising hydrophobic residues such as leucine and valine. It is preferable that a triplet of phenylalanine, tryptophan and valine is found at each end of the synthetic transmembrane domain. Optionally, a short oligopeptide linker or a polypeptide linker, for example, a linker having a length of 2 to 10 amino acids can be arranged between the transmembrane domain and the intracellular domain. In one embodiment, a linker sequence having a glycine-serine continuous sequence can be used.

The present invention provides a nucleic acid encoding the BCMA-CAR. The nucleic acid encoding the CAR can be prepared from an amino acid sequence of the specified CAR by a conventional method. A base sequence encoding an amino acid sequence can be obtained from the aforementioned NCBI RefSeq IDs or accession numbers of GenBank for an amino acid sequence of each domain, and the nucleic acid of the present invention can be prepared using a standard molecular biological and/or chemical procedure. For example, based on the base sequence, a nucleic acid can be synthesized, and the nucleic acid of the present invention can be prepared by combining DNA fragments which are obtained from a cDNA library using a polymerase chain reaction (PCR).

A nucleic acid encoding the CAR of the present invention can be inserted into a vector, and the vector can be introduced into a cell. For example, a virus vector such as a retrovirus vector (including an oncoretrovirus vector, a lentivirus vector, and a pseudo type vector), an adenovirus vector, an adeno-associated virus (AAV) vector, a simian virus vector, a vaccinia virus vector or a sendai virus vector, an Epstein-Barr virus (EBV) vector, and a HSV vector can be used. A virus vector lacking the replicating ability so as not to self-

replicate in an infected cell is preferably used.

For example, when a retrovirus vector is used, a suitable packaging cell based on a LTR sequence and a packaging signal sequence possessed by the vector can be selected for preparing a retrovirus particle using the packaging cell. Examples of the packaging cell include PG13 (ATCC CRL-10686), PA317 (ATCC CRL-9078), GP+E-86 and GP+envAm-12, and Psi-Crip. A retrovirus particle can also be prepared using a 293 cell or a 293T cell having high transfection efficiency. Many kinds of retrovirus vectors produced based on retroviruses and packaging cells that can be used for packaging of the retrovirus vectors are widely commercially available from many companies.

A CAR-T cell binds to a specific antigen via the CAR, thereby a signal is transmitted into the cell, and as a result, the cell is activated. The activation of the cell expressing the CAR is varied depending on the kind of a host cell and an intracellular domain of the CAR, and can be confirmed based on, for example, release of a cytokine, improvement of a cell proliferation rate, change in a cell surface molecule, or the like as an index. For example, release of a cytotoxic cytokine (a tumor necrosis factor, lymphotoxin, etc.) from the activated cell causes destruction of a target cell expressing an antigen. In addition, release of a cytokine or change in a cell surface molecule stimulates other immune cells, for example, a B cell, a dendritic cell, a NK cell, and a macrophage.

The cell expressing the CAR can be used as a therapeutic agent for a disease. The therapeutic agent comprises the cell expressing the CAR as an active ingredient, and it may further comprise a suitable excipient.

The inventors have generated CAR-T cells based on humanized BCMA ScFv sequence specifically targeting BCMA. The inventors have produced humanized BCMA-CAR-T cells to target cancer cells overexpressing BCMA tumor antigen. The humanized BCMA-CAR-T cells of the present invention secreted high level of cytokines against multiple myeloma cancer cells and kill CHO-BCMA-positive target cells but not control parental CHO cells.

The advantages of the humanized BCMA-ScFv of the present invention over the corresponding mouse ScFv include less immunogenicity to human due to the humanized BCMA scFv sequence. Thus, the humanized BCMA antibody of the present invention is highly potent and advantageous as therapeutic agents in many clinical applications.

The present humanized BCMA ScFv can be used for immunotherapy applications: toxin/drug-conjugated antibody, monoclonal therapeutic antibody, and CAR-T cell immunotherapy.

Humanized BCMA-CAR-T cells using the present humanized BCMA ScFv effectively target BCMA antigen in BCMA-positive cancer cell lines such as ovarian, colon, pancreatic, melanoma, cervical cancer, and other BCMA-positive cancers.

Humanized BCMA-CAR-T cells can be used in combination with different chemotherapy: checkpoint inhibitors, targeted therapies, small molecule inhibitors, and antibodies.

Humanized BCMA-CAR-T cells can be used clinically for BCMA-positive cancer cells.

Modifications of co-activation domains such as CD28, 4-1BB and others can be used to increase the efficacy of CAR-T cells. Tag-conjugated humanized BCMA scFv can be used for CAR generation.

Humanized BCMA-CAR-T cells can be used with different safety switches such as t-EGFR, RQR (Rituximab-CD34-Rituximab), inducible caspase-9 and other.

Third generation CAR-T or other co-activation signaling domains can be used with humanized BCMA-scFv to prepare BCMA-CAR-T.

The humanized BCMA CAR can be combined with CARs targeting other tumor antigens or tumor microenvironment, e.g., VEGFR-1-3, PDL-1. Bi-specific antibodies against BCMA and CD3, or other antigens can be generated for therapy.

The humanized BCMA-CAR can be used for generating other types of cells such as CAR-natural killer (NK) cells, BCMA-CAR-macrophages, allogenic CAR-T cells, gene-edited T cells, and other BCMA-CAR hematopoietic cells, which can target BCMA-positive cancers.

The present invention provides T cells, NK cells, macrophages, or hematopoietic cells, modified to express BCMA-CAR.

BCMA-CAR-T cells can be used against cancer stem cells and circulating tumor stem cells that are most resistant against chemotherapy and form aggressive tumors.

BCMA-CAR-T cells, BCMA-NK cells, BCMA-macrophages, and other cells can be used for targeting different types of cancers.

BCMA-CAR-T cells can be delivered intra-tumorally to patients for increased safety.

The following examples further illustrate the present invention. These examples are intended merely to be illustrative of the present invention and are not to be construed as being limiting.

EXAMPLES

Example 1. Humanized BCMA VH and VL and scFv SEQUENCES

The BCMA scFv was derived from hybridoma clones 4C8A (WO2019/195017). The sequences of heavy and light chain variable regions of mouse clone 4C8A were determined and used to construct a humanized scFv.

The structure of humanized BCMA (PMC306) scFv is: V_H-linker-V_L. Linker is G4Sx3.

The bold highlights the nucleotide sequence of humanized BCMA PMC306 ScFv clone: V_H; the underlined highlights the nucleotide sequence of V_L; in between (italicized) is the nucleotide sequence encoding a linker.

caggtgcagctggtgcagagcggcgcggaagtgaaaaaaccgggcagcagcgtgaaagtg
agctgcaaagcgagcggctatacctttaccagctatgtgatgcattgggtgcgccaggcg
ccgggccagggcctggaatggatgggctatattattccgtataacgatgcgaccaaatat
aacgaaaaatttaaaggccgcgtgaccattaccgcggataaaagcaccagcaccgcgtat
atggaactgagcagcctgcgcagcgaagataccgcggtgtattattgcgcgcgctataac
tatgatggctattttgatgtgtggggccagggcaccctggtgaccgtgagcagc*ggcggc*
*ggcggcagcggcggcggcggcagcggcggcggcggcagc*gaaattgtgctgacccagagc
ccggcgaccctgagcctgagcccgggcgaacgcgcgaccctgagctgccgcgcgagccag
agcattagcgattatctgcattggtatcagcagaaaccgggccaggcgccgcgcctgctg
atttattatgcgagccagagcattaccggcattccggcgcgctttagcggcagcggcagc
ggcaccgattttaccctgaccattagcagcctggaaccggaagattttgcggtgtattat
tgccagaacggccatagctttccgccgacctttggcggcggcaccaaagtggaaattaaa
(SEQ ID NO: 2)

PMC306 V_H amino acid sequence: (SEQ ID NO: 3)

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTSYVMHWVRQAPGQGLEWMGYIIPYN
DATKYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARYNYDGYFDVWGQ
GTLVTVSS

Linker amino acid sequence (SEQ ID NO: 4)

GGGGSGGGGSGGGGS

PMC306 V_L amino acid sequence: (SEQ ID NO: 5)

EIVLTQSPATLSLSPGERATLSCRASQSISDYLHWYQQKPGQAPRLLIYYASQSITGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQNGHSFPPTFGGGTKVEIK

Humanized BCMA (PMC306) scFv Protein: (SEQ ID NO: 6)

Q V Q L V Q S G A E V K K P G S S V K V S C K A S G Y T F T S Y V M H W V R Q A P G Q G L E W M G Y I I P Y N D A T K Y N E K F K G R V T I T A D K S T S T A Y M E L S S L R S E D T A V Y Y C A R Y N Y D G Y F D V W G Q G T L V T V S S
G G G G S G G G G S G G G G S E I V L T Q S P A T L S L S P G E R A T L S C R A S Q S I S D Y L H W Y Q Q K P G Q A P R L L I Y Y A S Q S I T G I P A R F S G S G S G T D F T L T I S S L E P E D F A V Y Y C Q N G H S F P P T F G G G T K V E I K

Example 2. Humanized BCMA-CAR Sequences

The scheme of humanized (PMC306) BCMA-CAR construct is shown on FIG. 3. Lentiviral vector with EF1a promoter was used for cloning of humanized scFv CAR sequences.

The BCMA-CAR structure includes human CD8 signaling peptide, humanized BCMA scFv (V_H-Linker-V_L), CD8 hinge, CD28 transmembrane, activation domains CD3 zeta (FIG. 3).

The nucleotide sequences and some of the amino acid sequences of CD8 signaling - BCMA scFv (V_H-Linker -V_L)-CD8 hinge-CD28 TM-CD28-CD3-zeta are shown below.

<CD8 leader>

Nucleotide
ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCG CCAGGCCG (SEQ ID NO: 7)

Amino Acid
MALPVTALLLPLALLLHAARP (SEQ ID NO: 8)

<Nhe I site>
gctagc

<Humanized BCMA, PMC 306 scFv>

V_H-linker-V_L, see Example 1 for nucleic acid sequences and amino acid sequences.

<XhoI restriction site>
CTCGAG

<CD8 hinge>
Nucleotide
AAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGCG TCGCAGCCCCTGTCCCTGCGCCCAGAGGCGAGCCGGCCAGCGGCGGGGGGCGCA GTGCACACGAGGGGGCTGGACTTCGCCAGTGAT (SEQ ID NO: 9)

Amino Acid
KPTTTPAPRPPTPAPTIASQPLSLRPEASRPAAGGAVHTRGLDFASD (SEQ ID NO: 10)

<aagccc>

<CD28 transmembrane >
Nucleotide
TTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAA
CAGTGGCCTTTATTATTTTCTGGGTG (SEQ ID NO: 11)

Amino Acid
FWVLVVVGGVLACYSLLVTVAFIIFWV (SEQ ID NO: 12)

<CD28 co-stimulatory>
Nucleotide
AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGC
CGCCCCGGGCCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCG
CAGCCTATCGCTCC (SEQ ID NO: 13)

Amino acid
RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS (SEQ ID NO: 14)

<CD3 zeta> stop codons underlined
Nucleotide
AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGAAC
CAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGAC
AAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGCAGAGAAGGAAGAA
CCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTA
CAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCC
TTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCA
GGCCCTGCCCCCTCGCTAAtag (SEQ ID NO: 15)

Amino acid
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNP
QEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQA
LPPR (SEQ ID NO: 16)

<EcoRI restriction site>
gaattc

Translated amino-acid sequence of humanized BCMA-CAR protein (SEQ ID NO: 17)

M A L P V T A L L L P L A L L L H A A R P A S Q V Q L V Q S G A E V K K P G S S V
K V S C K A S G Y T F T S Y V M H W V R Q A P G Q G L E W M G Y I I P Y N D A T
K Y N E K F K G R V T I T A D K S T S T A Y M E L S S L R S E D T A V Y Y C A R Y
N Y D G Y F D V W G Q G T L V T V S S G G G G S G G G G S G G G G S E I V L T Q S
P A T L S L S P G E R A T L S C R A S Q S I S D Y L H W Y Q Q K P G Q A P R L L I Y
Y A S Q S I T G I P A R F S G S G S G T D F T L T I S S L E P E D F A V Y Y C Q N G H
S F P P T F G G G T K V E I K L E K P T T T P A P R P P T P A P T I A S Q P L S L R P E
A S R P A A G G A V H T R G L D F A S D K P F W V L V V V G G V L A C Y S L L V
T V A F I I F W V R S K R S R L L H S D Y M N M T P R R P G P T R K H Y Q P Y A P P

R D F A A Y R S R V K F S R S A D A P A Y Q Q G Q N Q L Y N E L N L G R R E E Y D V L D K R R G R D P E M G G K P Q R R K N P Q E G L Y N E L Q K D K M A E A Y S E I G M K G E R R R G K G H D G L Y Q G L S T A T K D T Y D A L H M Q A L P P R (SEQ ID NO: 17)

Example 3. CAR lentivirus Production

Lentivirus was produced by the standard procedure using 293T cells as described in [7] The inventors generated humanized BCMA-ScFv-CAR constructs inside lentiviral vector cloned into Xba I and Eco R I sites of lentiviral vector. pCD510-FMC63-28z lentiviral CAR construct containing the humanized BCMA ScFv-CD28-CD3zeta insert – between the Xba I and Eco RI cloning sites.

The lentiviruses were generated in 293T cells and the titers were established by RT-PCR. Then equal dose of lentiviruses was used for transduction of T cells.

Example 4. Peripheral blood mononuclear cell (PBMC) isolation from whole blood

Whole blood (Stanford Hospital Blood Center, Stanford, CA) was collected from individual or mixed donors (depending on the amount of blood required) in 10 mL Heparin vacutainers (Becton Dickinson). Approximately 10 ml of whole anti-coagulated blood was mixed with sterile phosphate buffered saline (PBS) buffer for a total volume of 20ml in a 50ml centrifuge tube (PBS, pH 7.4, without Ca+2 and Mg+2). The blood/PBS (20 ml) was layered on top of 15 mL of Ficoll-Paque PLUS (GE Healthcare) in a conical centrifuge tube gently, and the sample was centrifuged at 400xg for 30-40min at room temperature. The layer of cells containing peripheral blood mononuclear cells (PBMC) at the diluted plasma/Ficoll interface was removed, washed, and centrifuged at 200xg for 10min at room temperature. Cells were counted with a hemocytomter. The PBMC were washed once with CAR-T media (AIM V-AlbuMAX(BSA) (Life Technologies), with 5% AB serum and 1.25 μg/mL amphotericin B (Gemini Bioproducts, Woodland, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin) and used for experiments or were frozen at -80°C.

Example 5. T-Cell Activation from PBMC

The isolated PBMC cells are resuspended in CAR-T medium with 300U/mL huIL2 (from a 1000x stock; Invitrogen) and mixed with CD3-CD28 beads at a 1:1 bead-to-cell ratio. The cells are incubated at 37°C in the presence of CO2 for 24 hours before viral transduction.

Example 6. T-Cell Transduction and Expansion

Following activation of PBMC, cells were incubated for 24 hours at 37°C, 5% CO_2. To each well of $1x10^6$ cells, $5x10^6$ lentivirus and 2 µL/mL of media of Transplus (*Alstem,* Richmond, CA) (a final dilution of 1:500) were added. Cells were incubated for an additional 24 hours before repeating the addition of virus. Cells were then grown in the continued presence of 300 U/ML of IL-2 fresh medium with IL-2 for a period of 12-14 days (total incubation time was dependent on the final umber of CAR-T cells required). Cells concentrations were analyzed every 2-3 days, with media being added at that time to dilute the cell suspension to $1x10^6$ cells/mL.

Example 7. Humanized BCMA-CAR-T cells expressed BCMA scFv

We designed humanized BCMA-CAR-T cells with humanized BCMA-CAR construct shown in Example 2. We used Mock scFv with unrelated ScFv and generated Mock-CAR-T cells as a negative control. Humanized BCMA-CAR-positive cells were detected after transduction of lentiviral humanized BCMA CAR into T cells. (FIG. 4).

Example 8. Humanized BCMA-CAR-T cells killed CHO-BCMA cells but not CHO cells.

We incubated humanized BCMA-CAR-T cells with target CHO-BCMA target cells and CHO (BCMA-negative) control cells. Humanized BCMA-CAR-T cells specifically killed CHO-BCMA cells (FIG. 5A) but not CHO cells (FIG. 5B). The results demonstrate high specificity of humanized BCMA-CAR-T cells to target BCMA antigen and to kill BCMA-positive cells.

Example 9. Humanized CAR-T cells secreted IFN-gamma against target CHO-BCMA cells significantly but not against CHO cells.

We collected supernatant after co-incubation of humanized BCMA-CAR-T cells and target CHO-BCMA and parental CHO cells and performed IFN-gamma assay. BCMA-CAR-T cells secreted IFN-gamma with CHO-BCMA cells but not with negative control CHO cells (FIG. 6). The results confirm specificity of humanized BCMA-CAR-T cells.

Example 10. Humanized CAR-T cells secreted high levels of IFN-gamma against BCMA-positive RPMI8226 multiple myeloma cells but not against BCMA-negative K562 leukemia cells.

We incubated BCMA-CAR-T cells with multiple myeloma cancer cells RPMI8266, and BCMA-negative K562 cells (chronic myelogenous leukemia cells) and performed ELISA with IFN-gamma using kit from Fisher, according to manufacturer's protocol. Humanized BCMA-CAR-T cells secreted high level of IFN-gamma against BCMA-positive multiple myeloma cancer cells but not against BCMA-negative K562 cells (FIG. 7). The level of killing and secretion of IFN-gamma was significantly higher with BCMA-CAR-T cells than with T cells and Mock CAR-T cells. This confirms specificity of humanized BCMA-CAR-T cells against hematological BCMA-positive cells.

Example 11. Humanized BCMA-CAR-T cells significantly decreased RPMI8226 xenograft tumor growth in mouse model *in vivo*.

Multiple myeloma RPMI8226 cells were injected subcutaneously into NSG mice (1x10^7 cells/mice), and then humanized BCMA-CAR-T cells were injected twice by i.v. (1x10^7 CAR-T cells/mice). Humanized BCMA-CAR-T cells significantly decreased RPMI8226 tumor growth in mice (FIG. 8A). Mice treated with humanized BCMA-CAR-T cells did not cause decreased mice body weight suggesting that CAR-T cells were not toxic to mice (FIG. 8B). No behavior or visual changes were observed during the study.

The peripheral blood cells were analyzed by flow cytometry at the end of the study for binding to human BCMA protein and antibodies specific for human T (CD4+/CD8+) cells. The percentage of cells binding to the CD4 mAb is shown on the left panel of FIG. 8C, and the percentage of those human T cells that also bound to the BCMA protein is shown on the right panel of FIG. 8C. The results show that humanized BCMA-CAR-T cells, but not Mock-Car-T-cells, were detected in the mouse blood by FACS with BCMA recombinant protein.

References

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SEQUENCE LISTING

<110> Caribou Biosciences, Inc.

<120> HUMANIZED BCMA ANTIBODY AND BCMA-CAR-T CELLS

<130> CBI038.10

<150> PCT/US20/13662
<151> 2020-01-15

<150> US 62/793,274
<151> 2019-01-16

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Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile Tyr Tyr Ala Ser
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Gln Ser Ile Thr Gly Ile Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly
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Lys Asp Thr Tyr Asp Ala Leu His Met Gln Ala Leu Pro Pro Arg
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WHAT IS CLAIMED IS:

1. An anti-human BCMA single-chain variable fragment (scFv), comprising:
   a heavy chain variable region (V_H) having the amino acid of SEQ ID NO: 3; and
   a light chain variable region (V_L) having the amino acid of SEQ ID NO: 5.

2. The scFv of claim 1, further comprising a linker in between the V_H and the V_L.

3. The scFv of Claim 2, wherein the linker has the amino acid sequence of SEQ ID NO: 4.

4. An anti-BCMA chimeric antigen receptor (CAR) comprising:
   an anti-BCMA single-chain variable fragment (scFv) comprising
      a heavy chain variable region (V_H) having the amino acid of SEQ ID NO: 3; and
      a light chain variable region (V_L) having the amino acid of SEQ ID NO: 5.
   a transmembrane domain;
   a co-stimulatory domain; and
   an activating domain.

5. The CAR of Claim 4, wherein the transmembrane domain is a transmembrane domain selected from the group consisting of a T cell receptor α chain, a T cell receptor β chain, a CD3 zeta chain, a CD28, a CD3ε, a CD45, a CD4, a CD5, a CD8, a CD9, a CD16, a CD22, a CD33, a CD37, a CD64, a CD80, a CD86, a CD134, a CD137, an ICOS, a CD154, and a GITR, optionally wherein the transmembrane domain comprises a transmembrane domain of a CD8.

6. The CAR of Claim 4, wherein the co-stimulatory domain is a co-stimulatory domain selected from the group consisting of a CD28, a 4-1BB, a GITR, an ICOS-1, a CD27, an OX-40, and a DAP10, optionally wherein the co-stimulatory domain comprises a 4-1BB co-stimulatory domain.

7. The CAR of Claim 4, wherein the activation domain comprises a CD3 zeta activating domain.

8. The CAR of Claim 4, having the amino acid sequence of SEQ ID NO: 17.

9. A nucleic acid, having a 5' end and a 3' end, encoding the CAR of claim 4.

10. A vector, comprising the nucleic acid of Claim 9.

11. The vector of Claim 10, wherein the vector is a virus vector.

12. An isolated CAR-T cell, comprising the CAR of Claim 4 or Claim 8.

13. Use of a humanized CAR-T cell in the preparation of a medicament for the treatment of a BCMA-positive cancer in a patient in need thereof, the humanized CAR-T cell comprising an anti-BCMA chimeric antigen receptor (CAR) comprising:

an anti-BCMA single-chain variable fragment (scFv) comprising

a heavy chain variable region (V_H) having the amino acid sequence of SEQ ID NO: 3, and

a light chain variable region (V_L) having the amino acid sequence of SEQ ID NO: 5;

a transmembrane domain;

a co-stimulatory domain; and

an activating domain.

14. The use of Claim 13, wherein the medicament is formulated for intra-tumoral administration.

15. The use of Claim 13, wherein the BCMA-positive cancer cells comprise multiple myeloma cancer cells.

16. An in vitro method of making an anti-BCMA CAR-T cell, comprising:

introducing into a T cell a nucleic acid encoding an anti-BCMA chimeric antigen receptor (CAR) comprising

an anti-BCMA single-chain variable fragment (scFv) comprising

a heavy chain variable region (V_H) having the amino acid sequence of SEQ ID NO: 3, and

a light chain variable region (V_L) having the amino acid sequence of SEQ ID NO: 5;

a transmembrane domain;

a co-stimulatory domain; and

an activating domain.

17. The method of Claim 16, wherein the nucleic acid is introduced into the T cell by a virus vector.

18. The method of Claim 17, wherein the virus vector is selected from the group consisting of a retrovirus vector, an adenovirus vector, an adeno-associated virus (AAV) vector, a simian virus vector, a vaccinia virus vector, a Sendai virus vector, an Epstein-Barr virus (EBV) vector, and a HSV vector.