(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) **World Intellectual Property Organization**
International Bureau

(43) **International Publication Date**
**31 March 2022 (31.03.2022)**

(10) **International Publication Number**
**WO 2022/061837 A1**

(51) **International Patent Classification:**
*A61K 48/00* (2006.01) *C07K 16/00* (2006.01)
*A61K 39/395* (2006.01) *A61P 35/04* (2006.01)

(21) **International Application Number:**
PCT/CN2020/118184

(22) **International Filing Date:**
27 September 2020 (27.09.2020)

(25) **Filing Language:** English

(26) **Publication Language:** English

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report (Art. 21(3))*

(54) **Title:** FIBRONECTIN EXTRA DOMAIN B (EDB) -SPECIFIC CAR-T FOR CANCER

(57) **Abstract:** Provided is chimeric antigen receptor (CAR) specific for the extra domain B (EDB) of fibronectin, which CAR can be use in immune cells (such as T cells and NK cells) for treating diseases such as cancer and inflammatory disease.

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# FIBRONECTIN EXTRA DOMAIN B (EDB)-SPECIFIC CAR-T FOR CANCER

## BACKGROUND OF THE INVENTION

Chimeric antigen receptors (CARs) are engineered receptors that combine both antigen-binding and immune cell (*e.g.*, T-cell) activating functions into a single receptor, which then confers immune cells having such engineered receptors new ability to target a specific protein.

CARs have recently been used in therapies in cancer therapy, based on modified T cells with newly acquired ability to recognize cancer antigens on cancer cells in order to more effectively target and destroy them. Typically, autologous T cells are harvested from a patient in need of CAR T therapy, before engineered CARs are introduced into the isolated T cells *ex vivo*, before infusing the resulting CAR-T cells back into the patient to attack the tumors bearing antigen recognized by CARs.

CAR-T cells can be either derived from T cells in a patient's own blood (autologous), or more recently derived from the T cells of another healthy donor (allogeneic). For safety, CAR-T cells are preferably engineered to be specific to an antigen expressed on a tumor that is not expressed on healthy cells. Once CAR-T cells are infused into a patient, they act as a "living drug" against cancer cells, in that the CAR-T cells bind to the cancer antigen and become activated, leading to their proliferation and cytotoxicity against the cancer cells.

CAR-T cells can destroy cancer cells through several mechanisms, including extensive stimulated cell proliferation, increasing the degree to which they are toxic to other living cells (cytotoxicity) and by causing the increased secretion of factors that can affect other cells such as cytokines, interleukins and growth factors.

In recent years, CAR-T cell immunotherapy has achieved highly effective results in treating hematological malignancies. Despite significant progress, however, some major challenges still have not been solved in engineered T cells to treat solid tumors and have remain significant barriers to its broader clinical application, especially in terms of specificity, persistence, safety, and immunosuppressive microenvironment. Thus, there is a need for improved CAR-based therapy that is reliable, safe, and effective that can be extended toward the treatment of a broader range of tumors, including solid tumor.

## SUMMARY OF THE INVENTION

One aspect of the invention provides a chimeric antigen receptor (CAR) comprising: (1) an antigen-binding domain specific for the extra domain B (EDB) of fibronectin; (2) a transmembrane (TM) domain of a membrane protein selected from CD3, CD4, CD8, CD28, OX40, or CD137; and, (3) an intracellular ITAM (Immunoreceptor Tyrosine-based Activation Motif) domain of CD3ζ, with or without a costimulatory domain; wherein the CAR, when expressed on the surface of a T cell, is capable of activating the T cell (a) upon binding to a soluble EDB, (b) upon binding to a membrane-bound EDB, and/or (c) upon binding to EDB in extracellular matrices (*e.g.*, those that are part of fibronectin mesh functioning as scaffold for cell attachment).

In certain embodiments, the antigen-binding domain is an scFv, a single chain antibody, a nanobody (*e.g.*, a derivative of VHH (camelid Ig)), a domain antibody (dAb, a derivative of VH or VL domain), a Bispecific T cell Engager (BiTE, a bispecific diabody); a Dual Affinity ReTargeting (DART, a bispecific diabody); an anticalin (a derivative of Lipocalins); an adnectin (10th FN3 (Fibronectin)); a Designed Ankyrin Repeat Proteins (DARPins); or an avimer.

In certain embodiments, the antigen-binding domain is a human scFv or a humanized scFv.

In certain embodiments, the CAR further comprises a hinge / spacer domain between the antigen-binding domain and the TM domain.

In certain embodiments, the hinge / spacer domain and the TM domain originate from the same protein.

In certain embodiments, the same protein is CD8α, and wherein the hinge / spacer domain is the extracellular domain of CD8α.

In certain embodiments, (3) comprises the costimulatory domain.

In certain embodiments, the costimulatory domain is from CD28.

In certain embodiments, (3) comprises two costimulatory domains.

In certain embodiments, the two costimulatory domains comprises a costimulatory domain from CD28, and/or a costimulatory domain from CD27, 4-1BB, or OX-40.

In certain embodiments, the CAR comprises the scFv of residues 21-236 of SEQ ID NO: 1, a CD8α extracellular and transmembrane domain, a 4-1BB intracellular domain, and a CD3zeta intracellular domain.

In certain embodiments, the CAR further comprises an N-terminal signal peptide sequence (such as the hIL-2 signal peptide sequence, or residues 1-20 of SEQ ID NO: 1).

In certain embodiments, the CAR comprises a polypeptide of SEQ ID NO: 1.

Another aspect of the invention provides polynucleotide encoding the CAR of the invention. For example, the polynucleotide may be SEQ ID NO: 2.

In certain embodiments, the polynucleotide is codon-optimized for expression in a human cell.

Another aspect of the invention provides a vector comprising the polynucleotide of the invention.

In certain embodiments, the vector is a viral vector capable of infecting and/or expressing said CAR in T cells, macrophages, and/or NK cells, such as primary human T cells, macrophages, or NK cells.

In certain embodiments, the vector is a viral vector capable of infecting and/or expressing said CAR in peripheral monocytes, monocyte derived dendritic cells, hematopoietic stem cells, and/or induced PSC (pluripotent stem cell).

In certain embodiments, the vector is a lentiviral vector.

In certain embodiments, the lentiviral vector is a self-inactivating lentiviral vector.

Another aspect of the invention provides a cell expressing the CAR of the invention, comprising the polynucleotide of the invention, or the vector of the invention.

In certain embodiments, the cell is an immune cell.

In certain embodiments, the cell is a T cell. In certain embodiments, the cell is an NK cell. In certain embodiments, the cell is a monocyte or a macrophage.

In certain embodiments, the cell is a primary cell isolated from a patient.

In certain embodiments, the cell is from an established cell line, such as an allogeneic cell line with respect to a patient to whom the cell is to be administered.

In certain embodiments, the cell expresses a cytokine.

In certain embodiments, the cytokine comprises IL-2, IL-7, IL-12, IL-15, or IL-21.

In certain embodiments, expression of the cytokine is under the control of a promoter that is activated by activation of the immune cell.

In certain embodiments, the cell further comprises a safety switch for down-regulating the activity of the immune cell.

In certain embodiments, the safety switch comprises a coding sequence for an iCaspase9 (inducible caspase-9) monomer that can be activated by dimerization with, *e.g.*, FKBP, to trigger apoptosis of the immune cell.

Another aspect of the invention provides a method of inhibiting angiogenesis in a subject having a disease or condition treatable by angiogenesis inhibition, the method comprising administering to the subject a therapeutically effective amount of an immune cell expressing a chimeric antigen receptor (CAR) comprising: (1) an antigen-binding domain specific for the extra domain B (EDB) of fibronectin; (2) a transmembrane (TM) domain of a membrane protein selected from CD3, CD4, CD8, CD28, OX40 or CD137; and, (3) an intracellular ITAM (Immunoreceptor Tyrosine-based Activation Motif) domain of CD3ζ, with or without a costimulatory domain.

In certain embodiments, the CAR is any one of the CAR described herein.

In certain embodiments, the disease or condition is a solid tumor or a chronic inflammatory condition.

In certain embodiments, cancer cells from the solid tumor do not express EDB on cell surface.

In certain embodiments, the disease or condition is a solid tumor, and wherein the method further comprises administering an immune checkpoint inhibitor such as a PD-1 inhibitor (*e.g.* pembrolizumab, nivolumab, and cemiplimab), a PD-L1 inhibitor (*e.g.* atezolizumab, avelumab, and durvalumab), a CTLA-4 targeting agents (*e.g.* ipilimumab), or an immunomodulating agent (*e.g.* thalidomide and lenalidomide).

In certain embodiments, the chronic inflammatory condition is psoriasis, rheumatoid arthritis, Crohn's disease, psoriatic arthritis, ulcerative colitis, osteoarthritis, asthma, pulmonary fibrosis, IBD, inflammation-induced lymphangiogenesis, obesity, diabetes, retinal neovascularization (RNV), diabetic retinopathy, choroidal neovascularization (CNV), age-related macular degeneration (AMD), metabolic syndrome-associated disorder, prolonged peritoneal dialysis, juvenile arthritis, or atherosclerosis.

In certain embodiments, the method further comprises administering a second therapeutic agent effective to inhibit angiogenesis.

In certain embodiments, the second therapeutic agent comprises axitinib, bevacizumab, cabozantinib, everolimus, lenalidomide, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, thalidomide, vandetanib, and/or ziv-aflibercept.

In certain embodiments, the immune cell is produced by introducing *in vitro* a vector of the invention into a primary immune cell isolated from the subject, and optionally culturing and/or expanding *in vitro* the primary immune cells introduced by the vector.

In certain embodiments, the method further comprises administering a reagent that suppresses cytokine release syndrome (CRS), such as an anti-IL-6 monoclonal antibody (*e.g.*, tocilizumab); and/or immunoglobulin therapy.

It should be understood that any one embodiment of the invention described herein, including any one embodiment described only in the examples or claims, can be combined with any one or more other embodiments of the invention, unless expressly disclaimed or otherwise improper.

## BRIEF DESCRIPTION OF THE DRAWINGS

**FIG. 1** shows expression of EDB-CAR on lentivirus-transduced human T cells in flow cytometry analysis. M1: mock transduction T cells. T: untransduced T cells.

**FIG. 2A** shows production of IFN-γ by EDB-CAR T cells in the presence of recombinant EDB protein.

**FIG. 2B** shows lysis of U87-MG cells after 2-24 hours co-culturing with EDB-CAR T cells at effector to target (E:T) ratio of 5:1. Cell lysis was determined using LDH assay. N=3, and each data point reflects the mean SEM of triplicates (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-tailed Student's *t*-test).

**FIGS. 3A-3B** show levels of expression of EDB in multiple cell lines detected at protein level by Western blotting (FIG. 3A) and at mRNA level by qPCR (FIG. 3B).

**FIGS. 4A-4B** show cytotoxicity of EDB-CAR T cells to human or murine cancer cells and HUVEC cells after co-culturing for 24 hours at various E:T ratios. Lysis of target cells was determined using LDH assay. N=3; two-tailed Student's *t*-test.

**FIG. 5** shows *in vitro* IFN-γ production by EDB-CAR T cells in the presence of tumor cells. EDB CAR-T cells were incubated for 24 hours with cancer cells at various E:T ratios for 24 hours. Supernatants were assayed for IFN-γ. N=3; two-tailed Student's *t*-test.

**FIG. 6** shows TNF-α production by EDB-CAR T cells in the presence of tumor cells. EDB-CAR T cells were incubated for 24 hours with the cancer cells at various E:T ratios for 24 hours. Supernatants were assayed for TNF-α. N=3; two-tailed Student's *t*-test.

**FIG. 7** shows expression of EDB-CAR on NK-92 after transduction by flow

cytometry analysis.

**FIG. 8A** shows cytotoxicity of EDB-CAR NK-92 cells, and **FIG. 8B** shows release of IFN-γ to the supernatant by EDB-CAR NK-92 cells after co-culturing with U87-MG cells at various E:T ratios for 24 hours. N=3; two-tailed Student's *t*-test.

**FIG. 9** shows the results of histopathological analysis of murine organ tissues by hematoxylin and eosin staining, demonstrating the lack of pathological changes / toxicity in normal mice injected with very high doses of CAR-T cells specific for EDB. The images were taken through Leica Aperio VERSA 8 slice scanner under magnification × 20. Each scale bar represents 100 μm.

**FIGs. 10A-10B** show purity and EDB-CAR-expression of $CD14^+$ monocytes. Specifically, FIG. 10A shows the purity of the $CD14^+$ monocytes / macrophages (as evidenced by flow cytometry) isolated from PBMCs using CD14 MicroBeads. FIG. 14B shows expression of EDB-CAR in lentiviral-transduced human monocytes, as analyzed by flow cytometry. M1 stands for mock transduction negative control. Transduction efficiency was also shown.

**FIGs. 11A-11J** show characterization of EDB-Targeted CAR monocytes. Specifically, EDB-CAR-monocytes / macrophages were incubated with various EDB-expressing cell lines at the various effector:target (E:T) ratios (FIGs. 11A-11F) or 5 μg/mL EDB protein (FIGs. 11G-11J) for 24 hours. Culture supernatants were harvested at 24 hrs after cocultured cells were assayed for TNF-α (FIGs. 11C, 11F, and 11J), IL-12 (FIGs. 11B, 11E, and 11H), and IFN-γ (FIGs. 11A, 11D, and 11G) expression. Data are representative of three independent experiments. Each data point reflects the mean SEM of triplicates (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; two-tailed Student *t* test).

## DETAILED DESCRIPTION OF THE INVENTION

### *1. Overview*

The invention described herein is partly based on the discovery that certain antibodies or antigen-binding fragments thereof specific for the EDB domain of fibronectin can be used to construct CAR (chimeric antigen receptor) constructs that not only recognizes membrane bound EDB, or EDB in extracellular matrix (these deposite in tumor tissues), but also soluble form of EDB in solution.

The invention described herein is also partly based on the surprising discovery that immune cells bearing the subject EDB-specific CAR (such as CAR T cells) are cytotoxic *in*

*vitro* against normal human umbilical vein endothelial cells (HUVECs), yet very large amounts of such CAR-bearing immune cells (*e.g.*, T cells) injected *in vivo* to mice do not elicit expected toxicity. As is known in the art, a prominent barrier to widespread use of CAR T-cell therapy is toxicity, primarily cytokine release syndrome (CRS) and neurologic toxicity. For example, earlier attempts for solid tumor treatment using CAR against Her2 or carboxyanhydrase IX were unsuccessful due to on-target toxicity towards healthy tissues, leading to uncontrolled inflammatory-driven tissue damages or even death. Manifestations of CRS include fevers, hypotension, hypoxia, end organ dysfunction, cytopenias, coagulopathy, and hemophagocytic lymphohistiocytosis. Neurologic toxicities are diverse and include encephalopathy, cognitive defects, dysphasias, seizures, and cerebral edema. Yet such symptoms appear to be absent using the subject CAR constructs.

Thus, the subject CAR constructs have been found to be able to support CAR-based immune therapy using, *e.g.*, CAR T or CAR NK cells, to treat diseases in which angiogenesis is a pathological condition. Such diseases include cancer and inflammatory diseases.

Thus in one aspect, the invention provides a chimeric antigen receptor (CAR) comprising: (1) an antigen-binding domain specific for the extra domain B (EDB) of fibronectin; (2) a transmembrane (TM) domain of a membrane protein, such as one selected from CD3, CD4, CD8, CD28, OX40 or CD137; and, (3) a signaling domain such as an intracellular ITAM (Immunoreceptor Tyrosine-based Activation Motif) domain of CD3ζ, with or without a costimulatory domain; wherein the CAR, when expressed on the surface of a T cell, is capable of activating the T cell (a) upon binding to a soluble EDB, (b) upon binding to a membrane-bound EDB, and/or (c) upon binding to EDB in extracellular matrices (*e.g.*, those that are part of fibronectin mesh functioning as scaffold for cell attachment). A representative CAR of the invention is SEQ ID NO: 1.

Another aspect of the invention provides a polynucleotide encoding the CAR of the invention, such as SEQ ID NO: 2.

Another aspect of the invention provides a vector comprising the polynucleotide of the invention, such as a lentiviral vector comprising SEQ ID NO: 2.

Another aspect of the invention provides a cell, such as an immune cell, comprising the CAR of the invention, the polypeptide of the invention, and/or a vector of the invention. The cell may be a T cell, or an NK cell.

Another aspect of the invention provides a method of inhibiting angiogenesis in a subject having a disease or condition treatable by angiogenesis inhibition, the method

comprising administering to the subject a therapeutically effective amount of an immune cell expressing a chimeric antigen receptor (CAR) comprising: (1) an antigen-binding domain specific for the extra domain B (EDB) of fibronectin; (2) a transmembrane (TM) domain of a membrane protein selected from CD3, CD4, CD8, CD28, OX40 or CD137; and, (3) an intracellular ITAM (Immunoreceptor Tyrosine-based Activation Motif) domain of CD3ζ, with or without a costimulatory domain.

The disease or condition may be a solid tumor or a chronic inflammatory condition.

With the general aspects of the invention described herein, the following section provide further details regarding the different aspects of the invention.

## *2. EDB of Fibronectin (FN)*

Fibronectin is a high-molecular weight glycoprotein of the extracellular matrix (ECM) that binds to membrane-spanning receptor proteins integrins and ECM components such as collagen, fibrin, and heparan sulfate proteoglycans. Fibronectin exists as a protein dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds. Fibronectin is encoded by a single gene, but alternative splicing of its pre-mRNA leads to the creation of at least 20 different isoforms in humans (see a general discussion of FN function by White and Muro, "Fibronectin splice variants: understanding their multiple roles in health and disease using engineered mouse models." IUBMB Life. 63(7):538-546, 2011 (incorporated herein by reference).

The FN monomers are each about 250 kDa in size, and are linked together by disulfide bonds near the C-terminus. FNs are made of repeating units of three different types of homologies: type I, II, and III, having about 40, 60, and 90 amino acids, respectively. Many of these independently folded domains are also present in different ECM proteins. Among them, the type III modules are the most abundant modules in the FN molecule, and are also found in many different proteins across a wide range of species, whereas type I modules are found only in vertebrates.

In human, FN protein diversity is obtained by alternative splicing of two type III exons, known as Extra Domains A and B (also called EIIIA and EIIIB), respectively, and of a segment connecting two other type III repeats - type III connecting segment (IIICS). EDA and EDB splicing is similar in all species (either total inclusion or exclusion), whereas that of the IIICS region is species-specific (five variants in humans, three in rodents, and two in

chickens).

FN is found either as a soluble dimer in plasma, secreted by hepatocytes directly into circulation (plasma FN, or pFN), or deposited as insoluble fibrils in the ECM of tissues (cellular FN, or cFN). The two FN isoforms differ in the presence of the EDA and EDB domains: (a) pFN lacks the alternatively spliced EDA and EDB sequences and (b) cFN contains variable proportions of these domains.

As used herein, the term "EDB," "EIIIB," "EDB domain" or "ED-B-domain" refers to the extra-domain B of (human) fibronectin. In human, EDB is a type III homology domain with about 91 residues. EDB is essentially undetectable in healthy adult tissues, but is highly abundant in the vasculature of many aggressive solid tumors, thus making EDB a suitable target for anti-cancer and/or anti-inflammatory therapy of the invention.

In one embodiment, the antigen recognized by the subject CAR is a splice isoform of fibronectin, such as the ED-B domain of FN.

## *3. Antibody and Antigen-Binding Fragment of EDB*

In certain embodiments, the CAR binds to the EDB-domain of fibronectin exhibits a high binding affinity, *e.g.*, with a $K_D$ value of nanomolar or subnanomolar. Affinity can be measured using any art-recognized methods, such as by Bilayer Interferometry (BLI), surface plasmon resonance (SPR) or BIACORE, or other methods.

In certain embodiments, the antigen-binding portion of the CAR is based on EDB-specific antibodies or antigen-binding fragments thereof, such as those described in WO99/058570 (all incorporated herein by reference).

In certain embodiments, the EDB-specific antibody or antigen-binding fragments thereof is based on CAA06864.2 (incorporated herein by reference).

In certain embodiments, the EDB-specific antibody or antigen-binding fragments thereof is based on at least one CDR sequence of the L19 antibody.

In certain embodiments, the EDB-specific antibody or antigen-binding fragments thereof is based on huBC1, which is a humanized antibody that targets a cryptic sequence of the human ED-B-containing fibronectin isoform, B-FN, present in the subendothelial extracellular matrix of most aggressive tumors. B-FN is oncofetal and angiogenesis-associated.

In some embodiments, the antigen-binding portion of the CAR comprises an amino acid sequence sharing at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98%, or 99% identity with any of the antigen-binding portion of the CAR amino acid sequences provided herein. In some embodiments, the antigen-binding portion of the CAR may comprise up to 5 (*e.g.*, 4, 3, 2, or 1) amino acid residue variations in one or more of the CDR regions of one of the antibodies exemplified herein, and binds the same epitope of EDB with substantially similar affinity (*e.g.*, having a $K_D$ value in the same order or magnitude). In certain embodiments, the amino acid residue variations are conservative amino acid residue substitutions. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution that does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made.

As used herein, "antibody" or "immunoglobulin (Ig)" generally comprises four polypeptide chains, two heavy chains (HCs) and two light chains (LCs), but also includes equivalent Ig homologues such as camelid (*e.g.*, alpaca) nanobody (which comprises only a heavy chain), single domain antibody (dAb) (which can be derived either from a heavy or a light chain), and also includes full length or functional mutants, variants, or derivatives thereof (including, but not limited to, murine, chimeric, humanized and fully human antibodies, which retain the essential epitope binding features of an Ig molecule, and including dual specific, bispecific, multispecific, and dual variable domain immunoglobulins. Antibody or immunoglobulin can be of any class, *e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY (a type of immunoglobulin which is the major antibody in bird, reptile, and lungfish blood, as well as being in high concentrations in chicken egg yolk), or subclass, *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2 and allotype.

"Humanized" antibody or antigen-binding fragment thereof results from replacing one or more amino acid residues in the amino acid sequence of the naturally occurring non-human antibody or fragment thereof, such as VHH sequence (and, in particular, in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a VH domain from a conventional four-chain antibody from a human being. Methods for humanization are well known. Humanized antibody or antigen-binding fragment thereof may have several advantages, such as a reduced immunogenicity, compared to a corresponding naturally occurring non-human antibody or domain thereof.

"Humanization" can be performed by providing a nucleotide sequence that encodes a naturally occurring antibody, and then changing one or more codons in the nucleotide

sequence in such a way that the new nucleotide sequence encodes a "humanized" version thereof. This nucleic acid can then be expressed to provide the humanized antibody or fragment. Alternatively, based on the amino acid sequence of a naturally occurring non-human sequence, humanized version can be designed and then synthesized *de novo* using techniques for peptide synthesis. The skilled artisan may also combine one or more parts of one or more naturally occurring sequences (such as one or more FR sequences or CDR sequences), and/or one or more synthetic or semi-synthetic sequences, in a suitable manner, so as to provide a nucleotide sequence or nucleic acid encoding the humanized antibody or fragment thereof. Optionally, the humanized sequence is also codon-optimized for expression in an immune cell of the host, such as human T cell, NK cell, monocyte or macrophage.

An "antibody derivative or antigen-binding fragment," as used herein, includes a molecule comprising at least one polypeptide chain derived from an antibody that is not full length, including, but not limited to (i) a Fab fragment, which is a monovalent fragment consisting of the variable light (VL), variable heavy (VH), constant light (CL) and constant heavy 1 (CH1) domains; (ii) a $F(ab')_2$ fragment, which is a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a heavy chain portion of a Fab (Fd) fragment, which consists of the VH and CH1 domains; (iv) a variable fragment (Fv) fragment, which consists of the VL and VH domains of a single arm of an antibody, (v) a domain antibody (dAb) fragment, which comprises a single variable domain; (vi) an isolated complementarity determining region (CDR); (vii) a single chain Fv Fragment (scFv); (viii) a diabody, which is a bivalent, bispecific antibody in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with the complementarity domains of another chain and creating two antigen binding sites; and (ix) a linear antibody, which comprises a pair of tandem Fv segments (VH-CH1-VH-CH1) which, together with complementarity light chain polypeptides, form a pair of antigen binding regions; and (x) other non-full length portions of immunoglobulin heavy and/or light chains, or mutants, variants, or derivatives thereof, alone or in any combination.

In certain embodiments, the antigen-binding domain is an scFv, a single chain antibody, a nanobody (*e.g.*, a derivative of VHH (camelid Ig)), a domain antibody (dAb, a derivative of VH or VL domain), a Bispecific T cell Engager (BiTE, a bispecific diabody); a Dual Affinity ReTargeting (DART, a bispecific diabody); an anticalin (a derivative of

Lipocalins); an adnectin (10th FN3 (Fibronectin)); a Designed Ankyrin Repeat Proteins (DARPins); or an avimer.

In certain embodiments, the antigen-binding domain is a human scFv or a humanized scFv.

In any case, said derivative or fragment retains or substantially retains target binding properties (*e.g.*, $K_D$ that is less than 5%, 10%, 20%, 30%, 40%, 50%, 80%, 2-fold, 3-fold, 5-fold, 7-fold, 8-fold, or 10-fold higher than that of the full-length antibody) of the full-length antibody.

In certain embodiments, the antigen-binding fragment of the invention also includes "antibody-based binding protein," which as used herein, refers to any protein that contains at least one antibody-derived VH (heavy chain variable region), VL (light chain variable region), or CH (heavy chain constant region) immunoglobulin domain in the context of other non-immunoglobulin, or non-antibody derived components. Such antibody-based proteins include, but are not limited to (i) Fc-fusion proteins of binding proteins, including receptors or receptor components with all or parts of the immunoglobulin CH domains, (ii) binding proteins, in which VH and or VL domains are coupled to alternative molecular scaffolds, or (iii) molecules, in which immunoglobulin VH, and/or VL, and/or CH domains are combined and/or assembled in a fashion not normally found in naturally occurring antibodies or antibody fragments.

In certain embodiments, the antigen-binding fragment of the invention also includes "modified antibody format," which as used herein, encompasses antibody-drug-conjugates, Polyalkylene oxide-modified scFv, Monobodies, Diabodies, Camelid (*e.g.*, alpaca) Antibodies, Domain Antibodies, bi- or tri-specific antibodies, IgA, or two IgG structures joined by a J chain and a secretory component, shark antibodies, new world primate framework + non-new world primate CDR, IgG4 antibodies with hinge region removed, IgG with two additional binding sites engineered into the CH3 domains, antibodies with altered Fc region to enhance affinity for Fc gamma receptors, dimerized constructs comprising CH3+VL+VH, and the like.

In certain embodiments, the antigen-binding fragment of the invention also includes "antibody mimetic," which as used herein, refers to proteins not belonging to the immunoglobulin family, and even non-proteins such as aptamers, or synthetic polymers. Some types have an antibody-like beta-sheet structure. Potential advantages of "antibody mimetics" or "alternative scaffolds" over antibodies are better solubility, higher tissue

penetration, higher stability towards heat and enzymes, and comparatively low production costs. Some antibody mimetics can be provided in large libraries, which offer specific binding candidates against every conceivable target. Just like with antibodies, target specific antibody mimetics can be developed by use of High Throughput Screening (HTS) technologies as well as with established display technologies, just like phage display, bacterial display, yeast or mammalian display. Currently developed antibody mimetics encompass, for example, ankyrin repeat proteins (called DARPins), C-type lectins, A-domain proteins of *S. aureus*, transferrins, lipocalins, 10th type III domains of fibronectin, Kunitz domain protease inhibitors, ubiquitin derived binders (called affilins), gamma crystallin derived binders, cysteine knots or knottins, thioredoxin A scaffold based binders, SH-3 domains, stradobodies, "A domains" of membrane receptors stabilized by disulfide bonds and $Ca^{2+}$, CTLA4-based compounds, Fyn SH3, and aptamers (peptide molecules that bind to a specific target molecules).

The antigen-binding portion of the CAR specifically recognizing EDB fibronectin, in particular the scFv based on CAA06864.2, can be employed in various antibody formats as described herein. For example, other than scFv, antibody formats based on Fab, $(Fab')_2$, diabody, minibody, or nanobody format may be used, based on the CDR sequences of CAA06864.2. In certain embodiments, the antigen-binding fragment thereof is scFv format. In one further embodiment, the heavy and the light chain are connected by a peptide linker.

In certain embodiments, the CAR comprises the sequences according to SEQ ID NO: 1.

## *4. CAR Specific for EDB*

One aspect of the invention provides chimeric antigen receptor (CAR) with an antigen-binding portion specific for the EDB of fibronectin, wherein the CAR, when expressed on the surface of a T cell, is capable of activating the T cell (a) upon binding to a soluble EDB, (b) upon binding to a membrane-bound EDB, and/or (c) upon binding to EDB in extracellular matrices (*e.g.*, those that are part of fibronectin mesh functioning as scaffold for cell attachment).

In certain embodiments, the chimeric antigen receptor comprises an extracellular antigen binding domain, a transmembrane (TM) region, one or more co-stimulatory domain, and an intracellular signal transduction domain. In certain embodiments, the CAR further

comprises a hinge / spacer domain between the antigen-binding domain and the TM domain. The hinge and TM domains may originate from the same protein, or from different proteins.

For example, in certain embodiments, the CAR comprises (1) an antigen-binding domain specific for the extra domain B (EDB) of fibronectin (see above); (2) a transmembrane (TM) domain of a membrane protein, such as that from CD3, CD4, CD8, CD28, OX40 or CD137; and, (3) an intracellular ITAM (Immunoreceptor Tyrosine-based Activation Motif) domain of CD3ζ, with or without a costimulatory domain.

In certain embodiments, the extracellular antigen binding region may be an sc-Fv, Fab, scFab or scIgG fragment thereof.

In certain embodiments, the transmembrane region comprises the transmembrane region of CD3ζ, CD4, CD8, CD28, OX40 or CD137.

In some embodiments, the transmembrane region comprises the transmembrane region of a CD28 transmembrane domain.

In some embodiments, the transmembrane region comprises the transmembrane region of a CD8 transmembrane domain, such as CD8α transmembrane domain (*e.g.*, the CD8α hinge region included in SEQ ID NO: 1).

In some embodiments, the CAR further comprises a hinge region between the extracellular antigen binding domain and the transmembrane domain. In certain embodiments, the hinge region is from a CD8 hinge region, such as the CD8α hinge region included in SEQ ID NO: 1.

In certain embodiments, the hinge region and the TM region can be from the same protein, *e.g.*, both from the CD8 protein.

In certain embodiments, the hinge region and the TM region can be from different proteins, *e.g.*, the hinge region may be from the CD8α protein, while the TM region can be from the TM region of CD3 or CD28, *etc*.

In certain embodiments, the length of the hinge region in the CAR is substantially the same as that of the hinge region in SEQ ID NO: 1. For example, the hinge region may be longer or shorter than the hinge region in SEQ ID NO: 1 by no more than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 residue(s).

In certain embodiments, the CAR comprises one or more signal transduction domain(s) capable of activating the immune cell in which the CAR is expressed.

In certain embodiments, the CAR comprises one or more (*e.g.*, two) signal

transduction domain(s) capable of stimulating T-cell activation. In certain embodiments, the one or more signal transduction domain(s) can include, without limitation, one or more of TCRζ, FcRγ, FcRβ, FcRε, CD3γ, CD3δ, CD3ε, CD3ζ, signal transduction domain of CD5, CD22, CD79a, CD79b, and CD66d. In some embodiments, the CAR comprises a CD3ζ signal transduction domain, such as the CD3ζ signal transduction domain in SEQ ID NO: 1.

In certain embodiments, the two costimulatory domains comprise a costimulatory domain from CD28, and/or a costimulatory domain from CD27, 4-1BB, or OX-40.

In some embodiments, the CAR further comprises one or more co-stimulatory domain from one or more of: CD2, CD3, CD4, CD5, CD7, CD27, CD28, CD30, CD40, CD83, CD86, CD127, CD134, CD137 / 4-1BB, 4-1BBL, OX-40, PD-1, LFA-1, Lck, DAP10, LIGHT, NKG2C, B7-H3, CD3ζ, or ICOS. In certain embodiments, the one or more co-stimulatory domain comprises an intracellular signal transduction region from CD3ζ, FcεRIγ, PKCθ, or ZAP70. In some embodiments, the CAR comprises a CD28 co-stimulatory domain. In certain embodiments, the CAR comprises an ITAM from 4-1BB (CD137), which acts as the costimulatory signaling domain of the CAR, and serves to enhance antigen activation and increase potency. In certain other embodiments, the CAR comprises an ITAM from the costimulatory domain of CD28, which also increases CAR-mediated T cell activation.

In certain embodiments, a leader sequence or signal peptide is fused N-terminal to the CAR to promote CAR secretion. In certain embodiments, the leader sequence of the GM-CSF receptor may be used. In certain embodiments, the leader sequence is that of the human IL-2.

In some embodiments, the CAR further comprises a reporter molecule, such as GFP, for display or tracking CAR expression.

In one embodiment, the CAR comprises the scFv based on CAA06864.2, fused to the CD8α extracellular and transmembrane domains, the 4-1BB intracellular domain, and the CD3zeta intracellular domain. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 1. In certain embodiments, the entire EDB CAR is expressed with a signal peptide, such as the human interleukin-2 signal peptide, for directing it to the plasma membrane.

*5. Polynucleotide*

Another aspect of the invention provides a polynucleotide encoding the CAR of the invention described herein. In one embodiment, the polynucleotide comprises SEQ ID NO: 2.

In some embodiments, the nucleic acid is a synthetic nucleic acid. In some embodiments, the nucleic acid is a DNA molecule. In some embodiments, the nucleic acid is an RNA molecule (*e.g.*, an mRNA molecule encoding the CAR). In some embodiments, the mRNA is capped, polyadenylated, substituted with 5-methyl cytidine, substituted with pseudouridine, or a combination thereof.

In some embodiments, the nucleic acid (*e.g.*, DNA) is operably linked to a regulatory element (*e.g.*, a promoter) in order to control the expression of the nucleic acid. In some embodiments, the promoter is a constitutive promoter. In some embodiments, the promoter is an inducible promoter. In some embodiments, the promoter is a cell-specific promoter. In some embodiments, the promoter is an organism-specific promoter.

Suitable promoters are known in the art and include, for example, a pol I promoter, a pol II promoter, a pol III promoter, a T7 promoter, a U6 promoter, a H1 promoter, retroviral Rous sarcoma virus LTR promoter, a cytomegalovirus (CMV) promoter, a SV40 promoter, a dihydrofolate reductase promoter, and a β-actin promoter.

In one aspect, the present disclosure provides nucleic acid sequences that are at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the nucleic acid sequences described herein, *i.e.*, nucleic acid sequences encoding the CAR described herein.

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In general, the length of a reference sequence aligned for comparison purposes should be at least 80% of the length of the reference sequence, and in some embodiments is at least 90%, 95%, or 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are

identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. For purposes of the present disclosure, the comparison of sequences and determination of percent identity between two sequences can be accomplished using a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

In certain embodiments, the nucleic acid molecule encoding the CAR proteins, derivatives or functional fragments thereof are codon-optimized for expression in a host cell or organism. The host cell may include established cell lines (such as T/NK cells) or isolated primary cells. The nucleic acid can be codon optimized for use in any organism of interest, in particular human immune cells. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at www.kazusa.orjp/codon/, and these tables can be adapted in a number of ways. See Nakamura *et al.*, Nucl. Acids Res. 28:292, 2000 (incorporated herein by reference). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, Pa.).

An example of a codon optimized sequence, is in this instance a CAR coding sequence optimized for expression in a eukaryote, *e.g.*, humans (*i.e.* being optimized for expression in humans), or for another eukaryote, animal or mammal as herein discussed). Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species other than human, or for codon optimization for specific organs is known. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (*e.g.* about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for

optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at http://www.kazusa.orjp/codon/ and these tables can be adapted in a number of ways. See Nakamura, Y., *et al.* "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" Nucl. Acids Res. 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or more codons (*e.g.*, 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a CAR correspond to the most frequently used codon for a particular amino acid.

In some embodiments, the polynucleotide(s) or nucleic acid(s) of the invention are present in a vector (*e.g.*, a viral vector).

The term "vector" as used herein generally refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (*e.g.*, circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art.

In certain embodiments, the vector can be a cloning vector, or an expression vector. The vectors can be plasmids, phagemids, Cosmids, *etc.* The vectors may include one or more regulatory elements that allow for the propagation of the vector in a cell of interest (*e.g.*, a mammalian cell such as a human immune cell like T/NK cell).

In certain embodiments, the vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques.

In certain embodiments, the vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (*e.g.*, retroviruses, lentiviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, HSV, and adeno-associated viruses (AAV)). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell.

In certain embodiments, the vector is a lentiviral vector. In certain embodiments, the lentiviral vector is a self-inactivating lentiviral vector. See, for example, Zufferey *et al.*, "Self-Inactivating Lentivirus Vector for Safe and Efficient *In vivo* Gene Delivery." *J Virol.* 72(12): 9873–9880, 1998 (incorporated herein by reference).

In certain embodiments, the vector is based on the Sleeping Beauty (SB) transposon, which has been used as a non-viral vector for introducing genes into genomes of vertebrate animals and for gene therapy. Because the SB system is composed solely of DNA, the costs of production and delivery are considerably reduced compared to viral vectors. SB transposons have been used to genetically modify T cell in human clinical trials.

In certain embodiments, the vector is capable of autonomous replication in a host cell into which they are introduced. In certain embodiments, the vector (*e.g.*, non-episomal mammalian vectors) is integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. In certain embodiments, the vector, referred to herein as "expression vector," is capable of directing the expression of genes to which they are operatively-linked. Vectors for and that result in expression in a eukaryotic cell are "eukaryotic expression vectors."

In certain embodiments, the vector is a recombinant expression vector that comprises a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell. The recombinant expression vector may include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Here, "operably linked" means that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell).

The term "regulatory element" include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (*e.g.*, transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (*e.g.*, liver, pancreas), or particular cell types (*e.g.*, lymphocytes such as T cells, or NK cells). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may

not also be tissue or cell-type specific.

In some embodiments, a vector comprises one or more pol III promoter (*e.g.*, 1, 2, 3, 4, 5, or more pol III promoters), one or more pol II promoters (*e.g.*, 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (*e.g.*, 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and Hl promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, *e.g.*, Boshart *et al*, Cell, 41 :521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1a promoter.

Also encompassed by the term "regulatory element" are enhancer elements, such as WPRE; CMV enhancers; the R-U5' segment in LTR of HTLV-I (Mol. Cell. Biol., Vol. 8(1), p. 466-472, 1988); SV40 enhancer; and the intron sequence between exons 2 and 3 of rabbit b-globin (Proc. Natl. Acad. Sci. USA., Vol. 78(3), p. 1527-31, 1981).

It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, *etc*. A vector can be introduced into host cells to thereby produce transcripts, proteins, or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein.

In certain embodiments, the vector is a lentiviral or AAV vector, which can be selected for targeting particular types of cells (*e.g.*, with tissue and/or cell type-specific tropism).

The vectors of the invention can be introduced into a target cell, such as a primary T/NK cell, or an "off-the-shelf" allogeneic T/NK cell, using any of many art-recognized methods, such as transfection, lipid vectors, infection, electroporation, microinjection, parenteral injections, aerosol, gene guns, or use of ballistic particles, *etc*.

In certain embodiments, transfection includes chemical transfection that introduces the vector by, *e.g.*, calcium phosphate, lipid, or protein complexes. Calcium phosphate, DEAE-dextran, liposomes, and lipoplexes (for oral delivery of gene) surfactants and perfluro chemical liquids for aerosol delivery of gene.

In certain embodiments, lipid vectors are generated by a combination of plasmid DNA and a lipid solution that result in the formation of a liposome, which can be fused with

the cell membranes of a variety of cell types, thus introducing the vector DNA into the cytoplasm and nucleus, where the encoded gene is expressed. In certain embodiments, folate is linked to DNA or DNA-lipid complexes to more efficiently introduce vectors into cells expressing high levels of folate receptor. Other targeting moieties can be similarly used to target the delivery of the vectors to specific cell types targted by the targeting moieties.

In certain embodiments, the vector DNA is internalized via receptor-mediated endocytosis.

In certain embodiments, the vector is a lentiviral vector, and the target cell infection spectrum of the vector is expanded by replacing the genes for surface glycoproteins with genes from another viral genome in the packaging cell lines packaging cell lines (PCL) of the vector.

### 6. *Immune Cells*

The CAR of the invention can be introduced into various kinds of immune cells for CAR-mediated therapy. The immune cells into which the CAR of the invention can be introduced include T cells, NK cells, monocytes (including peripheral monocytes), monocyte derived dendritic cells, macrophages, hematopoietic stem cells, and/or induced pluripotent stem cell (PSC), *etc*.

Thus in one aspect, the invention also provides a cell comprising any of the CAR of the invention, polynucleotide encoding the CAR protein, or vector of the invention comprising the polynucleotide of the invention.

In certain embodiments, the cell is a eukaryote. In certain embodiments, the cell is a human cell. In certain embodiments, the cell is an immune cell. In certain embodiments, the cell is a T cell, such as $CD4^+$ or $CD8^+$ T cell. In certain embodiments, the cell is an NK cell. In certain embodiments, the cell is a monocyte. In certain embodiments, the cell is a mactrophage. In certain embodiments, the cell is a primary cell isolated from a patient into which cell a CAR-expressing vector is to be introduced to express the CAR before the cell is reintroduced to the patient. In certain embodiments, the cell is from a healthy donor into which cell a CAR-expressing vector is to be introduced to express the CAR before the cell is reintroduced to a patient different from the healthy donor. Optionally, the HLA-type of the healthy donor matches that of the patient.

In certain embodiments, the T cells and/or NK cells and/or monocytes and/or

macrophages of the present invention can be obtained from a number of non-limiting source by various non-limiting method, comprising peripheral blood mononuclear cells (PBMCs), bone marrow, lymph node tissue, cord blood, thymus tissue, ascites, pleural effusion, spleen tissue, and tumors.

In some embodiments, the immune cells are isolated from patients in need of CAR-based therapy, *e.g.*, from patients diagnosed with cancer or inflammatory disease. In this embodiment, the T cells / NK cells / monocytes / macrophages are autologous.

As used herein, "autologous" refers to cell treatment subject, the cell line or cell population derived from the object.

In some embodiments, the immune cells are isolated from healthy donors that are not the patient in need of treatment. In this embodiment, the immune cells are derived from a heterologous host, preferably from a host that is human leukocyte antigen (HLA)-compatible.

In some embodiments, the T-cells comprise $CD4^+$ T cells. In some embodiments, the T-cells comprise $CD8^+$ T cells.

The subject CAR T cells can be prepared by any means known in the art. For example, expression constructs such as viral-based vectors (*e.g.*, lentiviral vectors) comprising and capable of expressing the CAR polynucleotides of the invention can be used to transduct the isolated immune cells to obtain the subject CAR-T, CAR-NK *etc.* cells. One of skill in the art can easily construct expression constructs such as viral vectors suitable for protein expression.

In certain embodiments, the cell (*e.g.*, immune cell) further expresses a cytokine, such as IL-2, IL-7, IL-12, IL-15, or IL-21, or combination thereof. In certain embodiments, expression of the one or more cytokine is activated upon binding of the CAR to its target antigen. In certain embodiments, expression of the cytokine is under the control of a promoter that is activated by activation of the immune cell.

In certain embodiments, the cell further comprises a safety switch for down-regulating the activity of the immune cell.

In certain embodiments, the safety switch comprises a coding sequence for an iCaspase9 (inducible caspase-9) monomer that can be activated by dimerization with, *e.g.*, FKBP, to trigger apoptosis of the immune cell.

*7. Pharmaceutical Composition and Application Thereof*

Another aspect of the present invention provides a pharmaceutical composition for the treatment of a disease or condition, such as cancer or inflammatory disease, which comprises a modified T / NK cell / monocyte / macrophage of the invention and a pharmaceutically acceptable carrier. In addition, the invention further claims modified T / NK / monocyte / macrophage of the invention in preparation for use of the medicine for treating the disease.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like physiologically compatible. In certain embodiments, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion).

In certain embodiments, the invention provides a method of treating a patient having a solid tumor or an inflammatory condition by administering a CAR-based immune cell (*e.g.*, T cell or NK cell) expressing a CAR of the invention. In another embodiment, the invention provides a method of recruiting immune cells to a solid tumor in a patient by administering a CAR-T or CAR-NK cell expressing a CAR. In some instances, the CAR-T / CAR-NK cells can be administered using lymphocyte infusion. Preferably, autologous lymphocyte infusion is used in the treatment. Autologous PBMCs are collected from a patient in need of treatment and T /NK cells are activated and expanded using the methods described herein and known in the art, and then infused back into the patient.

*8. Method of Use*

Another aspect of the invention provides a method for inhibiting angiogenesis in a subject having a disease or condition treatable by angiogenesis inhibition, such as cancer or inflammatory condition, the method comprising administering to the subject a therapeutically effective amount of an immune cell expressing a chimeric antigen receptor (CAR) comprising: (1) an antigen-binding domain specific for the extra domain B (EDB) of fibronectin; (2) a transmembrane (TM) domain of a membrane protein selected from CD3, CD4, CD8, CD28, OX40 or CD137; and, (3) an intracellular ITAM (Immunoreceptor Tyrosine-based Activation Motif) domain of CD3ζ, with or without a costimulatory domain, or a pharmaceutical composition comprising the immune cell.

As used herein, a "therapeutically effective amount" or "therapeutically effective

dose" or "effective amount" means administering a sufficient amount of a substance, compound, material or cell to produce a desired therapeutic effect. Therefore, the administered amount is sufficient to prevent, cure, or ameliorate at least one symptom of, or completely or partially blocking the progression / worsening of the disease or condition. The administered amount is also below a threshold toxicity level, above which could / would cause the subject to terminate or discontinue with the therapy.

For example, the immune cells and pharmaceutical composition comprising the immune cells of the present invention, when administered in an effective amount to the subject, may results in reduced / delayed / eliminated one or more disease symptoms, reduced frequency and/or duration of the symptoms of the disease, or prevent or lessen the pain caused by injury or disability due to the disease. For example, for treatment of tumor, the immune cells and pharmaceutical composition comprising the immune cells of the present invention may inhibit cancer cell growth by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, as compared to an untreated control or control population. The ability of the subject immune cells and pharmaceutical composition comprising the immune cells of the present invention to inhibit tumor growth may be evaluated in a suitable animal model system for predicting curative effect for the human tumor. Alternatively or in addition, the ability to inhibit tumor cell growth may be measured *in vitro* using model system reasonably correlated to the disease or condition.

The amount and the dosage level of the immune cells in the pharmaceutical composition of the invention may be varied depending on specific patient need, the mode of administration, the type and/or degree of cancer in a subject, the desired therapeutic response, the tolerable toxicity to the patient, as well as other factors deemed relevant by an attending physician. That is, the selected dosage level may depend on a variety of pharmacokinetic factors including the particular composition used, the route of administration, the age of the patient, other pharmaceutical composition used in conjunction, duration and time of administration, rate of excretion or elimination, gender, weight, condition, general health condition and medical history, and like factors of the patient, as is generally known in the medical field. One of ordinary skill in the art can empirically determine the effective amount of the invention without necessitating undue experimentation. Combined with the teachings provided herein, by choosing among the various active immune cells and weighing factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects

and preferred mode of administration, an effective prophylactic or therapeutic treatment regimen can be planned which does not cause substantial toxicity in and of itself and yet is entirely effective to treat the particular subject.

Toxicity and efficacy of the protocols of the present invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio LD50/ED50. Prophylactic and/or therapeutic agents that exhibit large therapeutic indices are preferred. While prophylactic and/or therapeutic agents that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such agents to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

In certain embodiments, data obtained from the cell culture assays, animal studies and human studies can be used in formulating a range of dosage of the prophylactic and/or therapeutic agents for use in humans. The dosage of such agents lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any agent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

In certain embodiments, the CAR in the immune cells is any one of the CARs of the invention described herein.

In certain embodiments, the immune cell is an autologous or allogeneic T cell, NK cell, monocyte, or macrophage.

In certain embodiments, the disease or condition is a solid tumor, a chronic inflammatory condition, atherosclerosis, myocardial infarction, fibrosis, or a wound.

Examples of cancer or solid tumor includes: lung cancer, ovarian cancer, colon cancer, colorectal cancer, melanoma, renal cancer, bladder cancer, breast cancer, liver cancer,

lymphoma, hematologic malignancies, head and neck cancer, glioma, gastric cancer, nasopharyngeal carcinoma, laryngeal carcinoma, cervical cancer, uterine body cancer and osteosarcoma.

Examples of other cancers can using the method or pharmaceutical composition of the present invention for treating comprising: bone cancer, pancreatic cancer, skin cancer, prostate cancer, skin or intraocular malignant melanoma, uterine cancer, anal region cancer, testicular cancer, uterine cancer, endometrial cancer, vaginal cancer, vulva cancer, Hodgkin's disease, non-Hodgkin ' s lymphoma, esophageal cancer, small intestine cancer, endocrine system cancer, thyroid cancer, parathyroid cancer, adrenal cancer, soft tissue sarcoma, urethral cancer, penile cancer, chronic or acute leukemia (including acute myeloid leukemia, myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia), childhood solid tumor, lymphocytic lymphoma, bladder cancer, kidney or ureter cancer, renal cancer, cancer of the central nervous system (CNS), primary CNS lymphoma, spinal tumor, brain stem glioma, pituitary adenoma, Kaposi sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, cancer induced by environment, comprising asbestos-induced cancer, and a combination of said cancer.

In certain embodiments, the cancer is a solid tumor / cancer. In some embodiments, the cancer is lung cancer such as lung squamous cell carcinoma. In some embodiments, the cancer is ovarian cancer. In some embodiments, the cancer is colon cancer.

In certain embodiments, cancer cells from the solid tumor do not express EDB on cell surface.

In certain embodiments, the method further comprises administering an immune checkpoint inhibitor such as a PD-1 inhibitor (*e.g.* pembrolizumab, nivolumab, and cemiplimab), a PD-L1 inhibitor (*e.g.* atezolizumab, avelumab, and durvalumab), a CTLA-4 targeting agents (*e.g.* ipilimumab), or an immunomodulating agent (*e.g.* thalidomide and lenalidomide).

In some embodiments, the method further comprises administering to the subject radiotherapy and/or chemotherapy and/or surgery and/or other tumor-targeting drug (*e.g.*, targeting monoclonal antibody of other antigen or small molecule compounds).

In certain embodiments, the chemotherapy includes one or more of all-trans retinoic acid, Actinomycin D, Adriamycin, anastrozole, Azacitidine, Azathioprine, Alkeran, Ara-C, Arsenic Trioxide (Trisenox), BiCNU Bleomycin, Busulfan, CCNU, Carboplatin, Capecitabine, Cisplatin, Chlorambucil, Cyclophosphamide, Cytarabine, Cytoxan, DTIC,

Daunorubicin, Docetaxel, Doxifluridine, Doxorubicin, 5-flurouracil, Epirubicin, Epothilone, Etoposide, exemestane, Erlotinib, Fludarabine, Fluorouracil, Gemcitabine, Hydroxyurea, Herceptin, Hydrea, Ifosfamide, Irinotecan, Idarubicin, Imatinib, letrozole, Lapatinib, Leustatin, 6-MP, Mithramycin, Mitomycin, Mitoxantrone, Mechlorethamine, megestrol, Mercaptopurine, Methotrexate, Mitoxantrone, Navelbine, Nitrogen Mustard, Oxaliplatin, Paclitaxel, pamidronate disodium, Pemetrexed, Rituxan, 6-TG, Taxol, Topotecan, tamoxifen, taxotere, Teniposide, Tioguanine, toremifene, trimetrexate, trastuzumab, Valrubicin, Vinblastine, Vincristine, Vindesine, Vinorelbine, Velban, VP-16, and/or Xeloda.

In certain embodiments, the chronic inflammatory condition is psoriasis, rheumatoid arthritis, Crohn's disease, psoriatic arthritis, ulcerative colitis, osteoarthritis, asthma, pulmonary fibrosis, IBD, inflammation-induced lymphangiogenesis, obesity, diabetes, retinal neovascularization (RNV), diabetic retinopathy, choroidal neovascularization (CNV), age-related macular degeneration (AMD), metabolic syndrome-associated disorder, prolonged peritoneal dialysis, juvenile arthritis, or atherosclerosis.

In certain embodiments, the method further comprises administering a second therapeutic agent effective to inhibit angiogenesis.

In certain embodiments, the second therapeutic agent comprises axitinib, bevacizumab, cabozantinib, everolimus, lenalidomide, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, thalidomide, vandetanib, and/or ziv-aflibercept.

In certain embodiments, the immune cell is produced by introducing *in vitro* a vector of the invention into a primary immune cell isolated from the subject, and optionally culturing and/or expanding *in vitro* the primary immune cells introduced by the vector.

In certain embodiments, the method further comprises administering a reagent that suppresses cytokine release syndrome (CRS), such as an anti-IL-6 monoclonal antibody (*e.g.*, tocilizumab); and/or immunoglobulin therapy.

In certain embodiments, the subject is a human, non-human primate, cow, horse, pig, sheep, goat, dog, cat, or rodent. In certain embodiments, the subject is a human subject. In aspects of the invention pertaining to predictive therapy in cancers, the subject is a human either suspected of / at high risk of having the cancer, or having been diagnosed with cancer. Methods for identifying subjects suspected of having cancer may include physical examination, subject's family medical history, subject's medical history, biopsy, or a number of imaging technologies such as ultrasonography, computed tomography, magnetic resonance imaging, magnetic resonance spectroscopy, or positron emission tomography. Diagnostic

methods for cancer and the clinical delineation of cancer diagnoses are well known to those of skill in the medical arts.

*9. Kits*

Another aspect of the invention provides a kit, for the method of preparing the immune cells of the present invention, said kit comprising one or more of: the reagents for isolating immune cells from the patient, medium for culturing and expanding the isolated immune cells, reagents including vectors of the invention for infecting the isolated immune cells for expressing the CAR of the invention, reagents for activating the immune cells (*e.g.*, T cells), reagents for detecting / verifying the expression of the CAR induced to express the CAR of the invention, reagents for determining the presence or absence of EDB in a diseased tissue in a subject (such as reagents for immunohistochemistry or immunofluorescence or other imaging modalities such as noninvasive *in vivo* imaging modalities such as Immuno-PET/CT), *etc*.

The kit may further comprise instructions to carry out the process of the invention to produce the CAR-bearing immune cells and uses thereof.

The kit may contain any one or more of the components described herein in one or more containers. As an example, in one embodiment, the kit may include instructions for mixing one or more components of the kit and/or isolating and mixing a sample and applying to a subject. The kit may include a container housing agents described herein. The agents may be prepared sterilely, packaged in syringe and shipped refrigerated. Alternatively it may be housed in a vial or other container for storage. A second container may have other agents prepared sterilely. Alternatively the kit may include the active agents premixed and shipped in a syringe, vial, tube, or other container.

**EXAMPLES**

**Example 1: Generation of EDB-CAR T Cells**

The chimeric antigen receptor (EDB-CAR, SEQ ID NO: 1) was designed *de novo* and the coding sequence (SEQ ID NO: 2) was synthesized by Genewiz. The single chain variable domain (scFv) of the EDB-CAR was based on CAA06864.2, which recognizes the EDB antigen. Specifically, the EDB-CAR was constituted as a fusion of gene fragments, in that the EDB specific scFv was fused to CD8α extracellular and transmembrane domain, 4-1BB

intracellular domain, and CD3zeta intracellular domain. The entire EDB-CAR receptor was directed to plasma membrane by using a human interleukin-2 signal peptide. The nucleotide sequence encoding the EDB-CAR (SEQ ID NO: 2) was synthesized *de novo* by Genewiz.

To express the EDB CAR, the fusion gene DNA fragment was cloned into lentiviral vector M1, and the pseudotyped lentivirus was transduced into activated T cells.

T cells were isolated from peripheral blood mononuclear cells (PBMC) using negative magnetic selection method according to protocol as provided by manufacturer (Miltenyi, 130-096-535). T cells were activated with magnetic beads coupled with anti-human CD3 and CD28 antibodies (Thermo Fisher Scientific, 11131D) for 24 hours in complete RPMI (RPMI supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL Penicillin / streptomycin), 500 U/mL recombinant human IL-2 (SinoBiological, GMP-11848-HNAE), 10 ng/mL IL-7 (SinoiBological, GMP-11840-HNAE), and 10 ng/mL IL-15 (SinoBiological, GMP-11846-HNAE) media and then spin-fected using the lentivirus vector mixed with FuSure (Boston 3T Biotechnologies). Cells were expanded for 12 days and used for *in vitro* assays.

The expression of the EDB-CAR on the surface of the T cells was detected by flow cytometry using an Fab fragment recognizing the human variable chain framework sequences. Specifically, $10^6$ transfected cells were incubated with 8 μg/mL reconstituted biotin-labeled polyclonal goat anti-human-IgG $F(ab')_2$ fragment antibodies (Jackson Immunoresearch, Cat # 109-066-097) in FACS buffer (PBS plus 0.4% FBS) for 25 min at 4 °C. Cells were washed with FACS buffer, and incubated with 5.5 μL Phycoerythrin (R-Phycoerythrin Streptavidin, Jackson Immunoresearch, 016-110-084) in FACS buffer for 20 min on ice in dark. Cells were washed 3 times with ice-cold FACS buffer and analyzed by ACEA Novocyte Flow Cytometer. The Fab fragment only recognized T-cells transduced with the EDB chimeric receptor (FIG. 1).

Furthermore, it was discovered that the EDB-CAR transduced T cells recognize soluble EDB antigens and produced IFG-γ, suggesting that the EDB-CAR in this design was activated by soluble antigens. Notably, EDB-specific antibody partially inhibited IFN-γ induction (FIG. 2A). Further, the EDB-CAR T cells were capable of killing cells bearing EDB antigens on surface of U87-MG cells (FIG. 2B).

Thus, the present design of the EDB-CAR is unique in that both soluble and membrane-bound antigens can stimulate the receptors, leading to activation of T cells.

**Example 2: EDB-CAR T Cells are Cytotoxic to EDB-Expressing Cancer Cells**

To determine the presence of the EDB domain containing fibronectin in target cells, standard Western blot analysis were performed with anti-EDB monoclonal antibody BC-1 (Abcam, ab154210) followed by secondary antibody detection. EDB was expressed in several representative human cancer cell lines, including Caco-2 for colon adenocarcinoma, MCF-7 breast cancer, HS578T for carcinosarcoma, U87-MG, for human glioblastoma, and MDA-MB-468 for metastatic adenocarcinoma. Murine colorectal cancer cell line CT26 and HUVEC cells also expressed EDB (FIG. 3A).

To substantiate the Western blot analysis, EDB-specific mRNA levels in various cells were confirmed by qPCR using probes specific for the EDB domain. Total RNA from target cells were extracted (19221,YEASEN) and reverse transcribed using total RNA as template (11121ES60, YEASEN). Using the cDNA as template, qPCR reactions were carried out using primers: GADPH F-primer 5′-ACCCAGAAGACTGTGGATGG-3′ and R-primer 5′-TCTAGACGGCAGGTCAGGTC-3′, and EDB F-primer 5′-AAC TCA CTG ACC TAA GCT TT-3′ and R-primer 5′-CGT TTG TGT CAG TGT AG-3′, and SYBR Green dye (11199ES03, YEASEN). The data showed that EDB-specific mRNA was present at varying levels in multiple cancer cell lines and HUVEC, but not detectable in MCF-7 and MDA-MB-468 ( FIG. 3B).

Cytotoxicity of EDB-CAR T cells were tested on a panel of cells that showed variable levels of EDB. Ten thousand target cells were mixed with transduced T cells at effector to target ratios of 1:1, 5:1 and 10:1 in 96-well U-bottom plate. After 24 h culture, target cells lysis was detected by LDH detection kit (Yeasen, 40209ES76). Consistent with the expression analysis (FIGS. 3A-3B), HUVEC, U87-MG, Hs578, A549, F9 were susceptible to EDB-CAR T induced cell lysis, while MCF-7 and MDA-MB468 were not affected (FIGS. 4A-4B). For Caco-2 cell line, the low level of cell killing was probably due to the tendency of the cells forming aggregates *in vitro* (data not shown).

Interestingly, there were no apparent correlation between EDB expression and the levels of cell lysis. While not wishing to be bound by any particular theory, it is possible that the accessibility or specific conformation of the EDB domain in these cell lines varied, and might affect the susceptibility of the cells to the lysis.

Overall, the data supports cancer treatment using the EDB-CAR T cells.

**Example 3: EDB-CAR T Cells are Cytotoxic to HUVEC Endothelial Cells**

Angiogenesis is prerequisite for tumor growths and metastasis. Targeting angiogenesis for therapeutic development has resulted in successful demonstrations of therapeutic efficacies for bevacizumab and aflibercept (Keating 2014, Syed 2015).

This example demonstrates that the subject CAR T cells can be used for therapeutic targeting of angiogenesis, based on cytotoxicity of EDB-CAR T cells towards HUVEC cells. HUVEC cells are endothelial cells capable of forming tubular structures. The levels of cytotoxicity of EDB-CAR T cells increased with higher effector to target ratio (FIG. 4A, bottom row, middle).

This suggest that EBD-CAR T cells may be an effective angiogenesis inhibitor for treatment of diseases where neovascular generation play a critical role. Due to the involvement of the $EDB^+$ fibronectin during the neovascular structure formation, EDB-CAR T cells may be used alone, or as combination therapies with current available angiogenesis inhibitors such as bevacizumab or aflibercept.

**Example 4: EDB CAR-T Cells are Activated by EDB-Expressing Cancer Cells**

IFN-γ is a hallmark for T cell activation. To assess whether the EDB-CAR T cells were activated during the cytotoxic reactions, IFN-γ expression were determined by the EDB-CAR T cells by ELISA. Indeed, IFN-γ was found in the culture supernatant in the presence of the target cells (**FIG. 5**). IFN-γ induction was not observed for MCF-7 and MDA-MB468 cells, consistent with the low or undetectable expression levels of EDB in these cells. These findings strongly suggest that the cell lysis induced by EDB-CAR T cells was due to the binding to the EDB-containing fibronectins.

Interestingly, Caco-2 cells induced high levels of IFN-γ, although the cytotoxicity was low (**FIG. 2**). This finding is surprising, and is inconsistent with the common observations of cytotoxic activities, and suggests that the IFN-γ induction pathway maybe independent of the cytotoxicity pathway for the EDB-CAR T cells.

Indeed, preliminary data suggests that the subject EDB-CAR is likely not proliferative upon cytotoxic reactions, which is quite surprising. On the other hand, this data suggests that it is safer to use the subject EDB-CAR for treatment.

It has also been observed that CAR T cells can produce TNF-α upon cytotoxic killings (Jiang *et al* 2018). TNF-α induction upon the incubation of the target cells with EDB-CAR T cells was tested. Consistent with IFN-γ expression, incubation with Caco-2 and HS578T cells induced production of TNF-α, which is enhanced at higher effector to target ratios, while no significant amount of TNF-α was produced upon incubation with MCF-7 and MDA-MB-468 cells (FIG. 6).

**Example 5: EDB-CAR Natural Killer Cells are also Cytotoxic to Cancer Cells**

This experiment demonstrates the potential application of natural killer (NK) cell – based treatment options using the subject EDB-CAR.

NK-92 cell line is an immortal cell line derived from a patient and had been used in clinical studies. To demonstrate the applicability of the EDB-CAR for NK cell-based therapies, we transduced the NK-92 cell line with the subject EDB-CAR-expressing lentiviral vector, in a way similar to that used in the generation of the CAR T cells. EDB-CAR expression was analyzed by flow cytometry using the Fab fragment as previously described. Over 55% of the NK-92 cells showed expression of EDB-CAR on the cell surface (FIG. 7).

Cell lysis was induced in U87-MG, a glioblastoma cell line, by co-incubation with EDB-CAR NK-92 cells (FIG. 8A). Consistent with the cytotoxicity assay, IFN-γ was produced by the EDB-CAR NK-92 cells upon activation by target cells.

These results clearly demonstrate the potential utility of the natural killer cells for cancer therapies when transduced with EDB-CAR.

MYRMQLLSCIALSLALVTNSEVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPG
KGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFD
YWGQGTLVTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQ
QKPGQAPRLLIYYASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFG
QGTKVEIKAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAP
LAGTCGVLLLSLVITLYKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVK
FSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQK
DKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:
1)

ATGTACAGAATGCAGCTGCTGTCCTGCATCGCCCTGAGCCTGGCCCTGGTGACCAATAGCGA
GGTGCAACTCCTGGAGTCCGGCGGAGGCCTGGTCCAACCTGGAGGAAGCCTGAGGCTGAGCT
GTGCCGCCAGCGGCTTCACCTTTTCCAGCTTCTCCATGAGCTGGGTGAGACAGGCCCCCGGC
AAAGGCCTGGAGTGGGTGTCCAGCATCTCCGGCAGCTCCGGCACCACCTACTATGCTGATTC
CGTGAAGGGCAGGTTCACCATCTCCAGGGACAACAGCAAGAACACACTGTACCTCCAAATGA
ACTCCCTGAGGGCCGAAGACACCGCCGTGTACTACTGCGCCAAGCCCTTTCCCTATTTCGAC
TATTGGGGCCAGGGCACACTGGTCACCGTGAGCTCCGGCGATGGAAGCAGCGGAGGAAGCGG
AGGCGCTAGCGAAATCGTGCTGACCCAGAGCCCTGGCACACTGTCCCTGAGCCCTGGAGAAA
GAGCCACCCTGAGCTGTAGGGCCTCCCAGAGCGTGAGCAGCAGCTTCCTGGCCTGGTACCAA
CAGAAGCCCGGACAGGCCCCCAGGCTGCTGATCTACTATGCCTCCTCCAGGGCCACAGGCAT
CCCCGACAGGTTCTCCGGCTCCGGTTCTGGCACCGATTTTACCCTGACCATCTCCAGGCTGG
AGCCCGAAGACTTCGCCGTGTATTACTGCCAGCAGACCGGACGTATTCCTCCTACCTTTGGC
CAGGGCACCAAGGTGGAGATCAAAGCCAAGCCCACCACCACACCTGCCCCTAGACCCCCTAC
ACCTGCCCCCACAATCGCTTCCCAGCCTCTGTCCCTGAGGCCTGAGGCTTGTAGGCCTGCCG
CTGGAGGAGCTGTGCACACCAGAGGCCTCGACTTCGCCTGCGACATCTATATCTGGGCTCCT
CTGGCCGGCACCTGTGGAGTCCTCCTGCTGAGCCTGGTGATCACACTGTACAAGAGAGGCAG
GAAGAAGCTGCTGTACATCTTCAAGCAACCCTTCATGAGGCCTGTGCAGACCACCCAGGAAG
AAGATGGCTGCAGCTGCAGGTTCCCTGAGGAAGAAGAGGGCGGATGCGAGCTGAGAGTGAAG
TTCAGCAGGTCCGCCGATGCCCCTGCCTATCAGCAGGGCCAGAACCAGCTGTACAACGAACT
CAACCTGGGCAGGAGGGAGGAGTACGACGTCCTCGACAAGAGGAGAGGCAGGGACCCCGAGA
TGGGAGGCAAGCCTCAGAGGAGGAAGAACCCTCAAGAGGGACTGTACAACGAGCTGCAGAAG
GACAAGATGGCCGAGGCCTACTCCGAGATCGGCATGAAGGGCGAGAGAAGAAGAGGCAAGGG
CCATGATGGCCTCTACCAGGGCCTGAGCACCGCCACCAAGGACACATACGATGCCCTGCATA
TGCAGGCCCTCCCCCCTAGGTGA (SEQ ID NO: 2)

**Example 6: EDB-CAR T Cells Injected *In vivo***

Since the CAR-T cells have been shown to be cytotoxic to HUVEC cells *in vitro*, there is a concern that such EDB-specific CAR-T cells may carry unacceptable toxicity *in vivo* against normal blood vessels in a patient. This experiment demonstrated that the subject CAR-T cells are safe to use *in vivo*, despite the *in vitro* cytotoxicity against HUVEC cells.

In this experiment, mice were injected either with $1 \times 10^7$ T cells, $1 \times 10^7$ EDB CAR-T cells, or $2 \times 10^7$ EDB CAR-T cells. Mice were sacrificed on days 21 following T-cell infusion. Different tissues were harvested, formalin-fixed, paraffin-embedded, and stained with H&E. Representative photomicrographs are shown in FIG. 9. The images were taken through Leica Aperio VERSA 8 slice scanner under magnification × 20. Each scale bar represents 100 μm.

The results showed that there was no obvious pathological changes in all tissues examined, and there were no significant differences among all groups. See FIG. 9.

In this experiment, 20 million EDB-specific CAR-T cells were at the very high end of injectable CAR-T cells for a 20 gram or so mouse, which amount of CAR-T is equivalent to about 60 billion cells in a typical 60 kg human, a very high dose that probably cannot be reached in practice.

**Example 7: EDB-CAR T Cells Injected *In vivo***

Although CAR-mediated immune therapy has gained wider therapeutic usage in recent years, the understanding of the precise mechanisms through which such immune therapy kills cancer cells remains incomplete. It has recently been reported that long-term killing capability, but not secretion of conventional cytokines or standard 4-hr cytotoxicity, correlates positively with the quality of the CAR-mediated immunological synapse (IS) in two different CAR T cells that share identical antigen specificity, and thus the quality of the IS has been proposed as being predictive of the effectiveness of CAR-modified immune cells. Xenograft model data also confirmed that the quality of the IS *in vitro* correlates positively with performance of CAR-modified immune cells *in vivo*. See Xiong *et al.*, *Molecular Therapy* 26(4):963-975, 2018.

This exmple, however, demonstrates that EDB-CAR-transduced macrophages produce TNF alpha, suggesting that the EDB-CAR-mediated killing of cancer cells may be partially through the secretion of certain anti-tumor cytokines, and may be less or not dependent on a mechanism that involves immunological synapse, as is typical in CAR-T-mediated killing of cancer cells.

In this experiment, monocytes were isolated from PBMC using positive magnetic selection method according to protocol provided by the manufacturer (130-050-201, Miltenyi). Selected $CD14^+$ monocytes were seeded in non-treated cell culture flasks in RPMI with 10% FBS and 10 ng/mL recombinant human GM-CSF (300-03-20, PeproTech) for 8 days, before spin-fecting using the SEQ ID NO: 1 EDB-CAR-expressing lentiviral vector mixed with FuSure (Boston 3T Biotechnologies) at day 6. Monocytes / macrophages were harvested at day 7 and tested for expression of EDB-CAR (SEQ ID NO: 1). See FIGs. 10A-10B.

For cytokine induction of EDB-CAR-transduced monocytes/macrophages, target cells or EDB protein were mixed in effector-to-target ratios of 10, 20 and 40 in a 96-well U-bottom plate. After 24-hr culture, IFN-γ, TNF-α, and IL-12 expression were measured by ELISA (DAKEWE). See FIGs. 11A-11J.

A summary of the experimental procedure is provided below:

| 0 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|
| 8.31 | 9.4 | 9.5 | 9.6 | 9.7 | 9.8 | 9.9 |

Day 1: Monocytes were isolated from peripheral blood and the ratio of CD14 + cells was detected by flow cytometry
10 ng / ml GM-CSF was added for 5 days.
Day 5:The adherent cells were digested and stained with trypan blue to calculate the cell viability.
Day 6: Virus transduction.
Day 7: Cell activity was observed.
Day 8: The positive rate of car was detected by flow cytometry.
Day 9: Incubated with target cells or EDB antigen.
Day 10:The expression of cytokines was detected by ELISA.

Earlier observation on the cytotoxicity of EDB-CAR T cells seemed to suggest that killing was not proportional to the expression levels of the EDB-fibronectin on the target cells. See Example Example 4. Further, EDB-CAR T cells secreted TNFα when co-cultured with target cells.

TNFα possesses cytotoxicity function. This example shows that the EDB-CAR-expressing monocytes / macrophages also secrete TNFα. See FIGs. 11C and 11J. It is known that interferon-γ and TNFα are both strong stimulators of immune cells and inhibitors of cancer cells. Thus it is believed that the subject EDB-CAR modified immune cells can be used to deliver cytokines such as IFN-γ and/or TNFα to cancer tissues to effect EDB-CAR-mediated killing.

WE CLAIM:

1. A chimeric antigen receptor (CAR) comprising:
   (1) an antigen-binding domain specific for the extra domain B (EDB) of fibronectin;
   (2) a transmembrane (TM) domain of a membrane protein selected from CD3, CD4, CD8, CD28, OX40 or CD137; and,
   (3) an intracellular ITAM (Immunoreceptor Tyrosine-based Activation Motif) domain of CD3ζ, with or without a costimulatory domain;

   wherein the CAR, when expressed on the surface of a T cell, is capable of activating the T cell (a) upon binding to a soluble EDB, (b) upon binding to a membrane-bound EDB, and/or (c) upon binding to EDB in extracellular matrices (*e.g.*, those that are part of fibronectin mesh functioning as scaffold for cell attachment).

2. The CAR of claim 1, wherein the antigen-binding domain is an scFv, a single chain antibody, a nanobody (*e.g.*, a derivative of VHH (camelid Ig)), a domain antibody (dAb, a derivative of VH or VL domain), a Bispecific T cell Engager (BiTE, a bispecific diabody); a Dual Affinity ReTargeting (DART, a bispecific diabody); an anticalin (a derivative of Lipocalins); an adnectin (10th FN3 (Fibronectin)); a Designed Ankyrin Repeat Proteins (DARPins); or an avimer.

3. The CAR of claim 1, wherein the antigen-binding domain is a human scFv or a humanized scFv.

4. The CAR of any one of claims 1-3, further comprising a hinge / spacer domain between the antigen-binding domain and the TM domain.

5. The CAR of claim 4, wherein the hinge / spacer domain and the TM domain originate from the same protein.

6. The CAR of claim 5, wherein the same protein is CD8α, and wherein the hinge / spacer domain is the extracellular domain of CD8α.

7. The CAR of any one of claims 1-6, wherein (3) comprises the costimulatory domain.

8. The CAR of claim 7, wherein the costimulatory domain is from CD28.

9. The CAR of any one of claims 1-8, wherein (3) comprises two costimulatory domains.

10. The CAR of claim 9, wherein said two costimulatory domains comprise a costimulatory domain from CD28, and/or a costimulatory domain from CD27, 4-1BB, or OX-40.

11. The CAR of claim 1, comprising as the scFv of residues 21-236 of SEQ ID NO: 1, a CD8α extracellular and transmembrane domain, a 4-1BB intracellular domain, and a CD3zeta intracellular domain.

12. The CAR of claim 11, further comprising an N-terminal signal peptide sequence (such as the hIL-2 signal peptide sequence, or residues 1-20 of SEQ ID NO: 1).

13. The CAR of claim 12, which comprises a polypeptide of SEQ ID NO: 1.

14. A polynucleotide encoding the CAR of any one of claims 1-13, such as SEQ ID NO: 2.

15. The polynucleotide of claim 14, which is codon-optimized for expression in a human cell.

16. A vector comprising the polynucleotide of claim 14 or 15.

17. The vector of claim 16, which is a viral vector capable of infecting and/or expressing said CAR in T cells, macrophages, and/or NK cells, such as primary human T cells, macrophages, or NK cells.

18. The vector of claim 17, which is a lentiviral vector.

19. The vector of claim 18, wherein the lentiviral vector is a self-inactivating lentiviral vector.

20. A cell expressing the CAR of any one of claims 1-13, comprising the polynucleotide of claim 14 or 15, or the vector of any one of claims 16-19.

21. The cell of claim 20, wherein the cell is an immune cell.

22. The cell of claim 20, wherein the cell is a T cell.

23. The cell of claim 20, wherein the cell is an NK cell.

24. The cell of claim 20, wherein the cell is a macrophage.

25. The cell of any one of claims 20-24, wherein the cell is a primary cell isolated from a patient.

26. The cell of any one of claims 20-24, wherein the cell is from an established cell line, such as an allogeneic cell line with respect to a patient to whom the cell is to be administered.

27. The cell of any one of claims 20-26, wherein the cell expresses a cytokine.

28. The cell of claim 27, wherein the cytokine comprises IL-2, IL-7, IL-12, IL-15, or IL-21.

29. The cell of claim 27 or 28, wherein expression of the cytokine is under the control of a promoter that is activated by activation of the immune cell.

30. The cell of any one of claims 20-30, further comprising a safety switch for down-regulating the activity of the immune cell.

31. The cell of claim 30, wherein the safety switch comprises a coding sequence for an iCaspase9 (inducible caspase-9) monomer that can be activated by dimerization with, *e.g.*, FKBP, to trigger apoptosis of the immune cell.

32. A method of inhibiting angiogenesis in a subject having a disease or condition treatable by angiogenesis inhibition, the method comprising administering to the subject a therapeutically effective amount of an immune cell expressing a chimeric antigen receptor (CAR) comprising:
    (1) an antigen-binding domain specific for the extra domain B (EDB) of fibronectin;
    (2) a transmembrane (TM) domain of a membrane protein selected from CD3, CD4, CD8, CD28, OX40 or CD137; and,
    (3) an intracellular ITAM (Immunoreceptor Tyrosine-based Activation Motif) domain of CD3ζ, with or without a costimulatory domain.

33. The method of claim 32, wherein the CAR is any one of claims 1-13.

34. The method of claim 32 or 33, wherein the disease or condition is a solid tumor or a chronic inflammatory condition.

35. The method of claim 34, wherein cancer cells from the solid tumor do not express EDB on cell surface.

36. The method of claim 34 or 35, wherein the disease or condition is a solid tumor, and wherein the method further comprises administering an immune checkpoint inhibitor such as a PD-1 inhibitor (*e.g.* pembrolizumab, nivolumab, and cemiplimab), a PD-L1

inhibitor (*e.g.* atezolizumab, avelumab, and durvalumab), a CTLA-4 targeting agents (*e.g.* ipilimumab), or an immunomodulating agent (*e.g.* thalidomide and lenalidomide).

37. The method of claim 34, wherein the chronic inflammatory condition is psoriasis, rheumatoid arthritis, Crohn's disease, psoriatic arthritis, ulcerative colitis, osteoarthritis, asthma, pulmonary fibrosis, IBD, inflammation-induced lymphangiogenesis, obesity, diabetes, retinal neovascularization (RNV), diabetic retinopathy, choroidal neovascularization (CNV), age-related macular degeneration (AMD), metabolic syndrome-associated disorder, prolonged peritoneal dialysis, juvenile arthritis, or atherosclerosis.

38. The method of any one of claims 32-37 further comprising administering a second therapeutic agent effective to inhibit angiogenesis.

39. The method of claim 38, wherein the second therapeutic agent comprises axitinib, bevacizumab, cabozantinib, everolimus, lenalidomide, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, thalidomide, vandetanib, and/or ziv-aflibercept.

40. The method of any one of claims 32-39, wherein the immune cell is produced by introducing *in vitro* a vector of any one of claims 16-19 into a primary immune cell isolated from the subject, and optionally culturing and/or expanding *in vitro* the primary immune cells introduced by the vector.

41. The method of any one of claims 32-40, further comprising administering a reagent that suppresses cytokine release syndrome (CRS), such as an anti-IL-6 monoclonal antibody (*e.g.*, tocilizumab); and/or immunoglobulin therapy.

FIG. 1

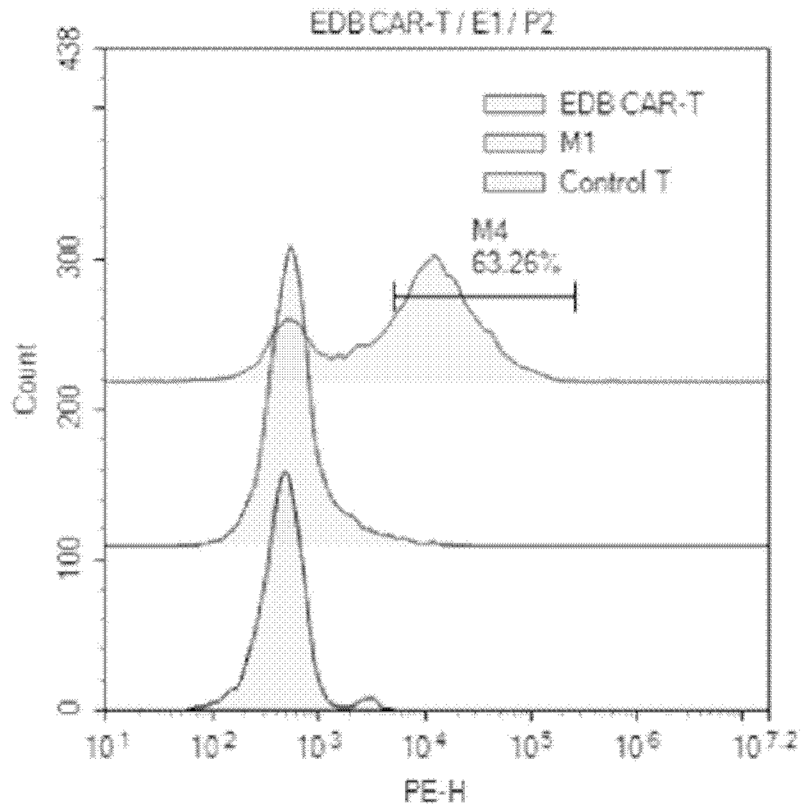


FIG. 2A

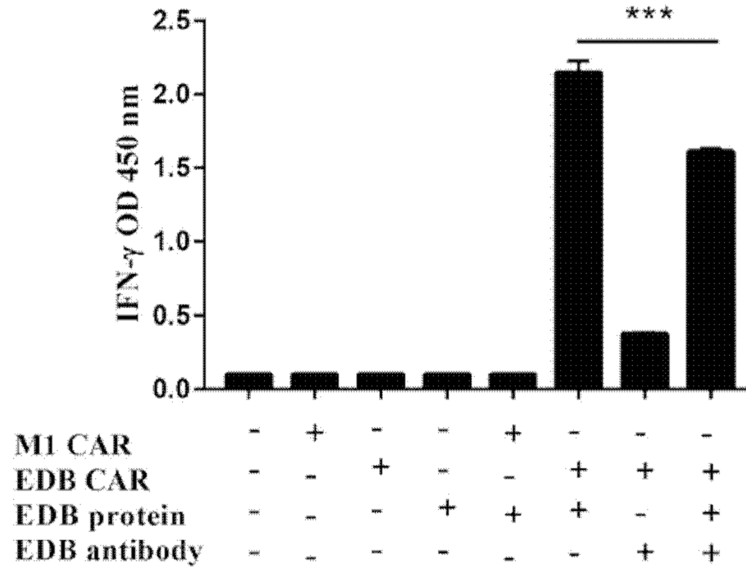



FIG. 2B

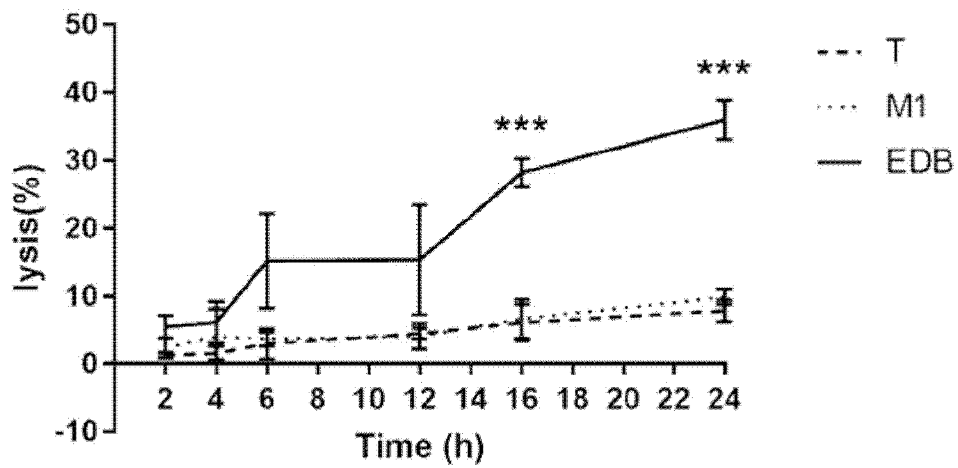


FIG. 3A

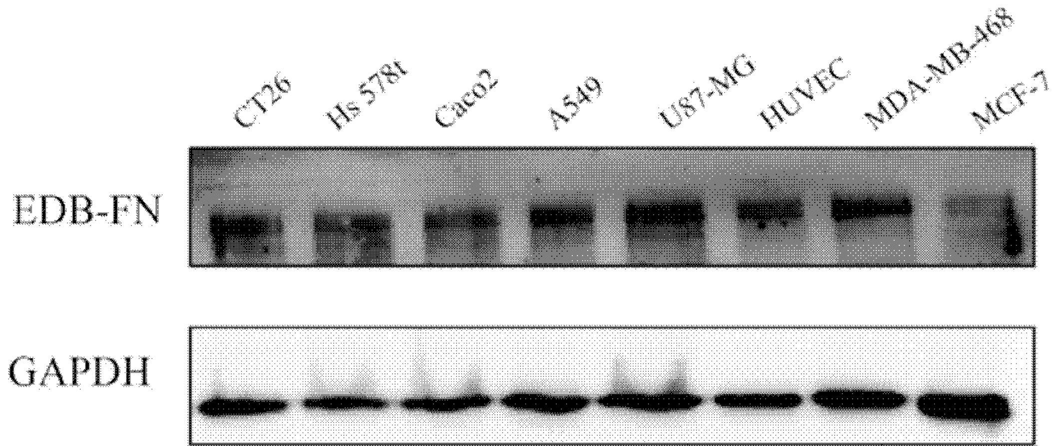



FIG. 3B

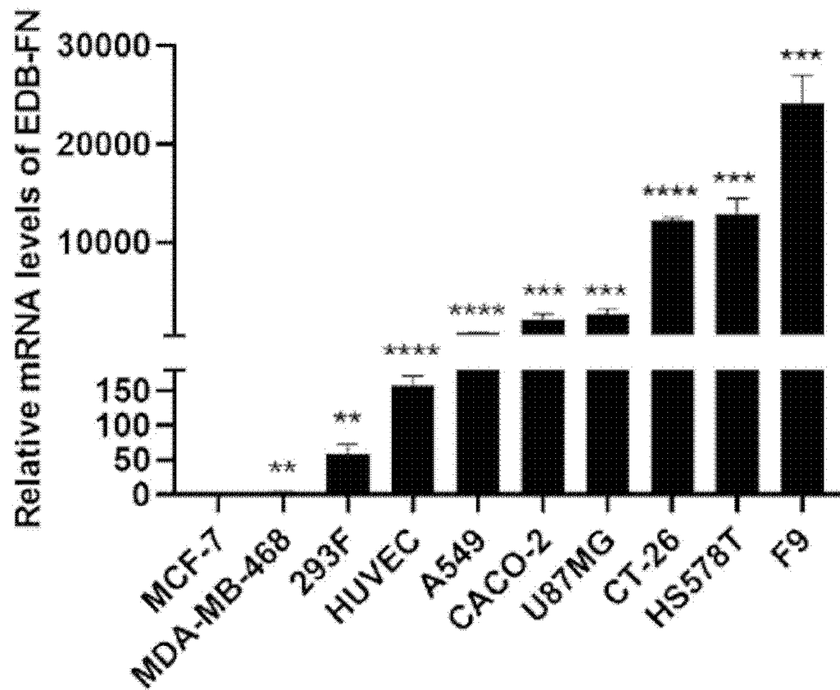
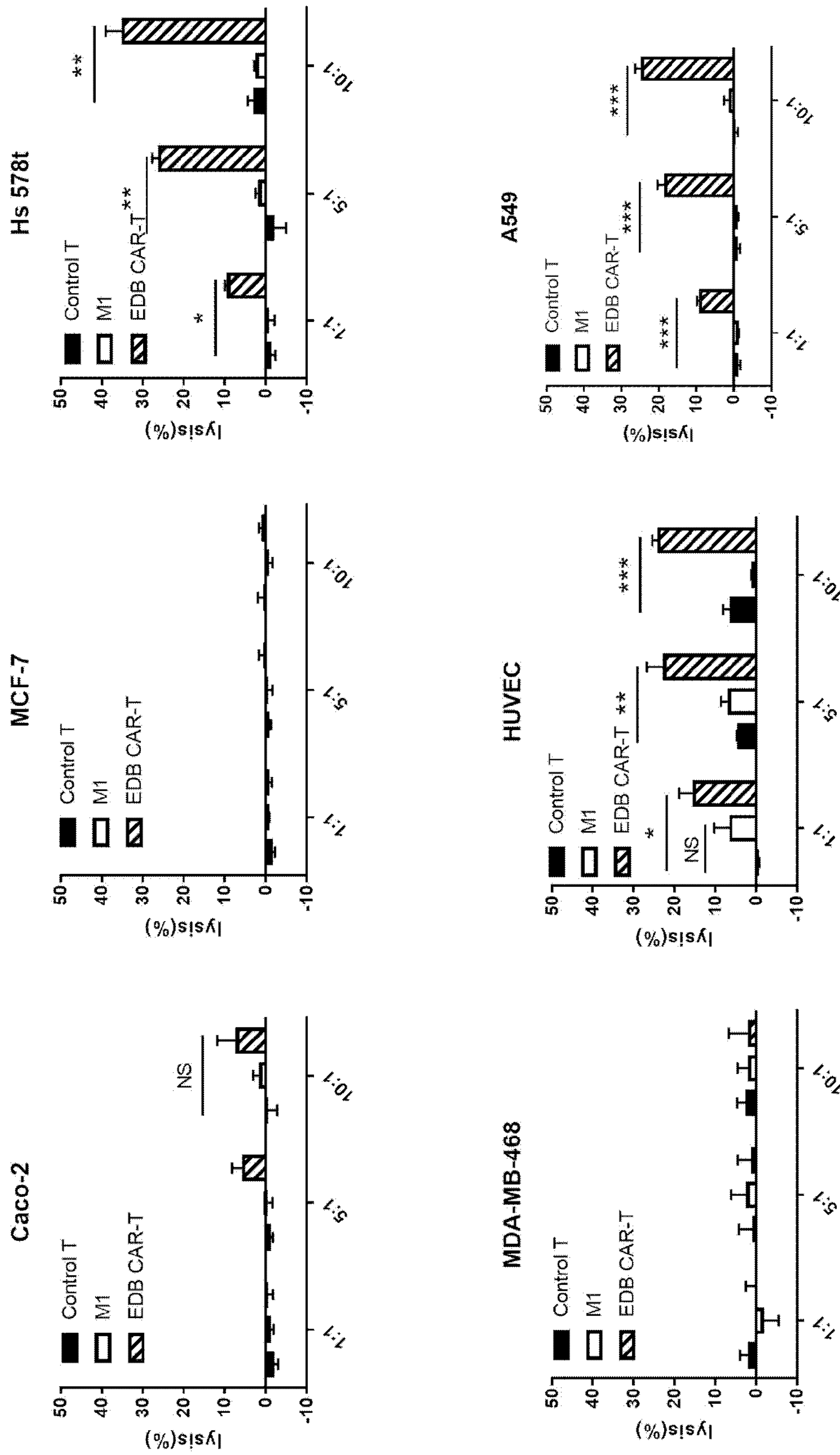


FIG. 4A



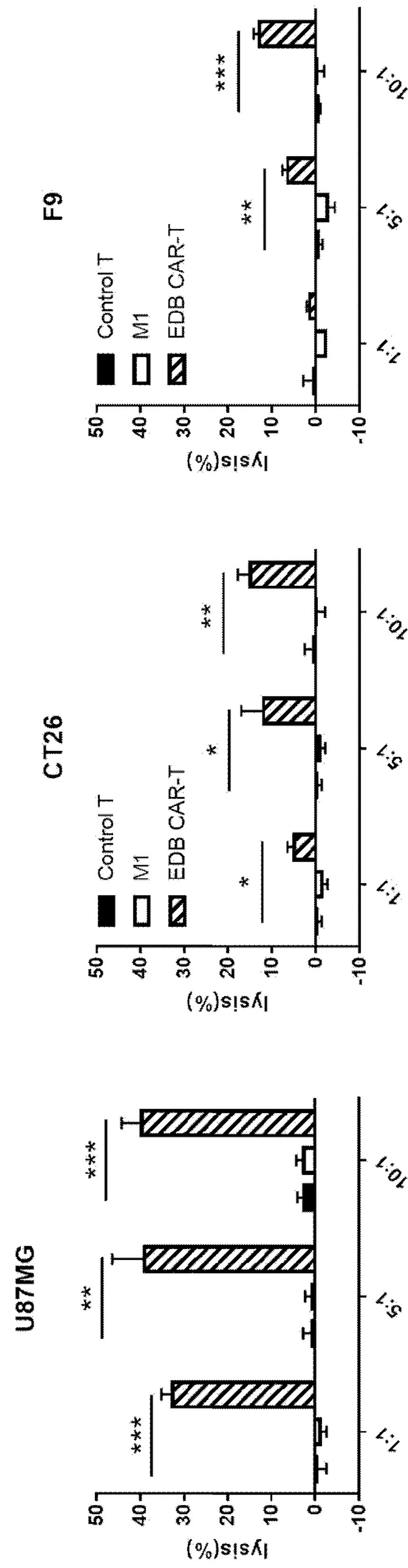


FIG. 4B
U87MG
CT26
F9
lysis(%)
50
40
30
20
10
0
-10
1:1
5:1
10:1
*
**
***
Control T
M1
EDB CAR-T

FIG. 5

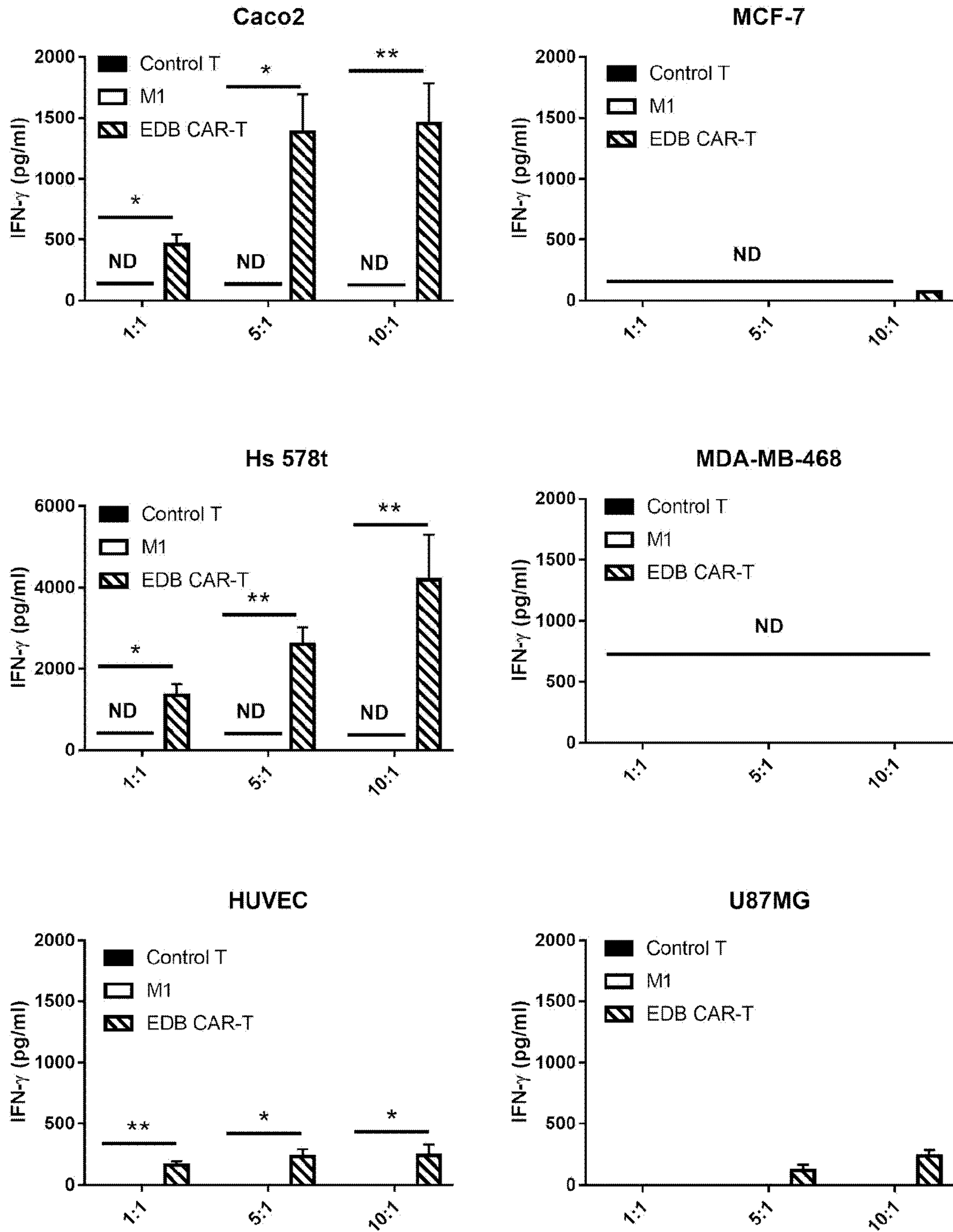


FIG. 6

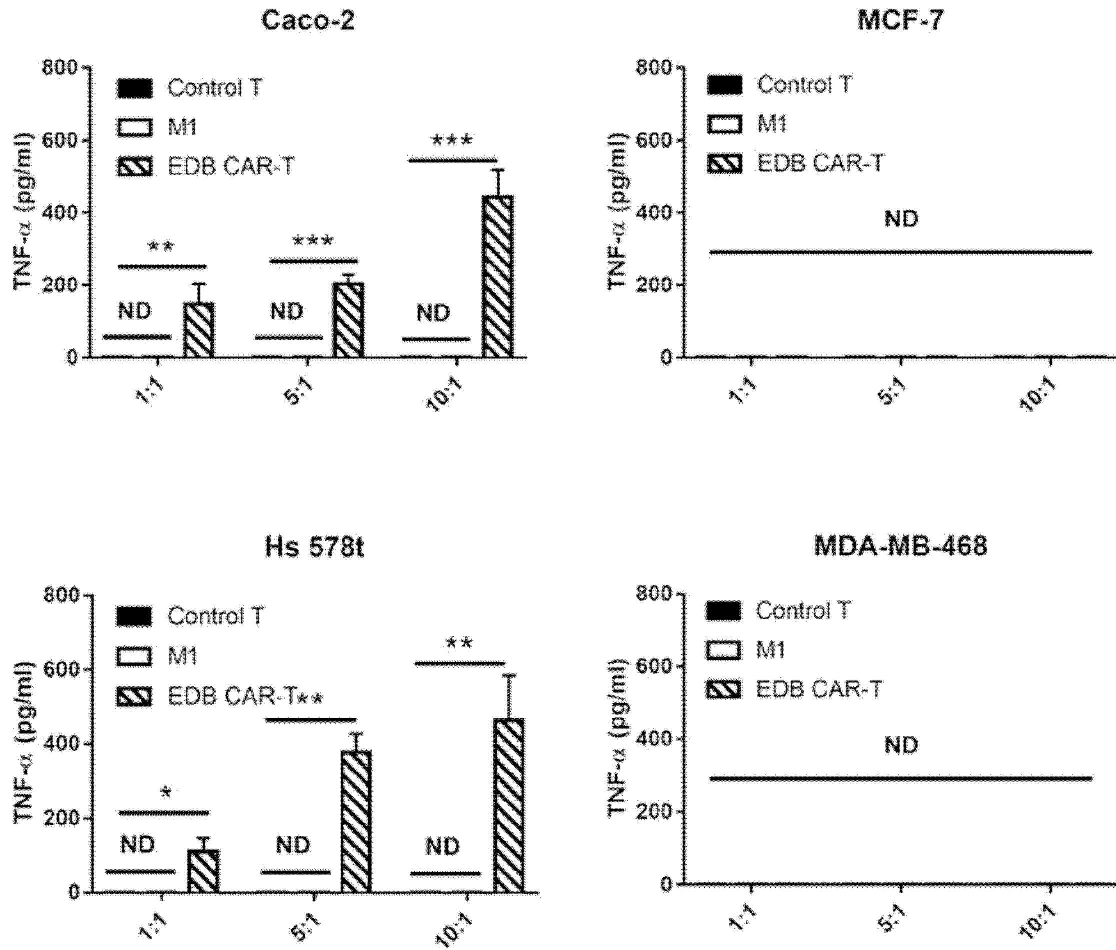


**FIG. 7**

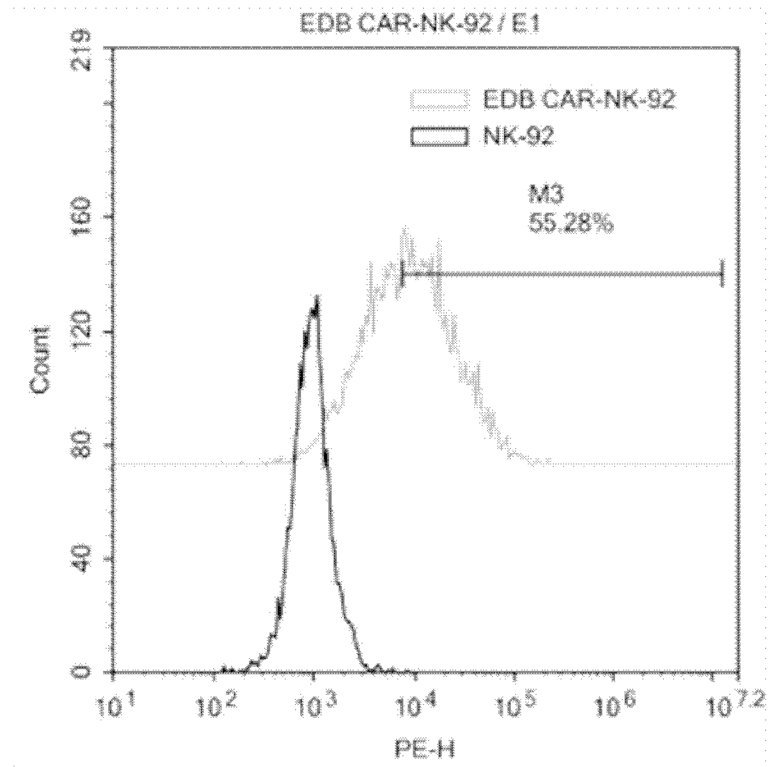


FIG. 8A

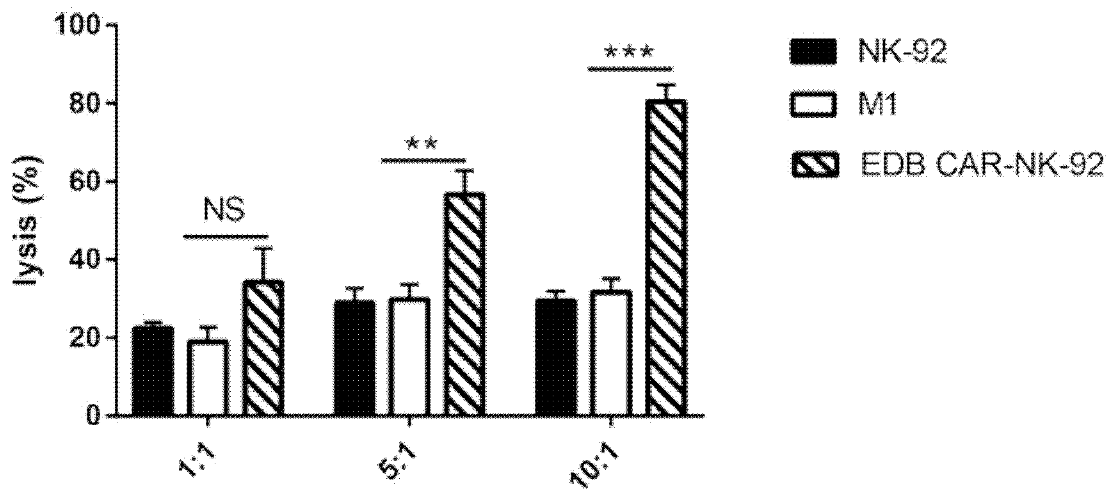



FIG. 8B

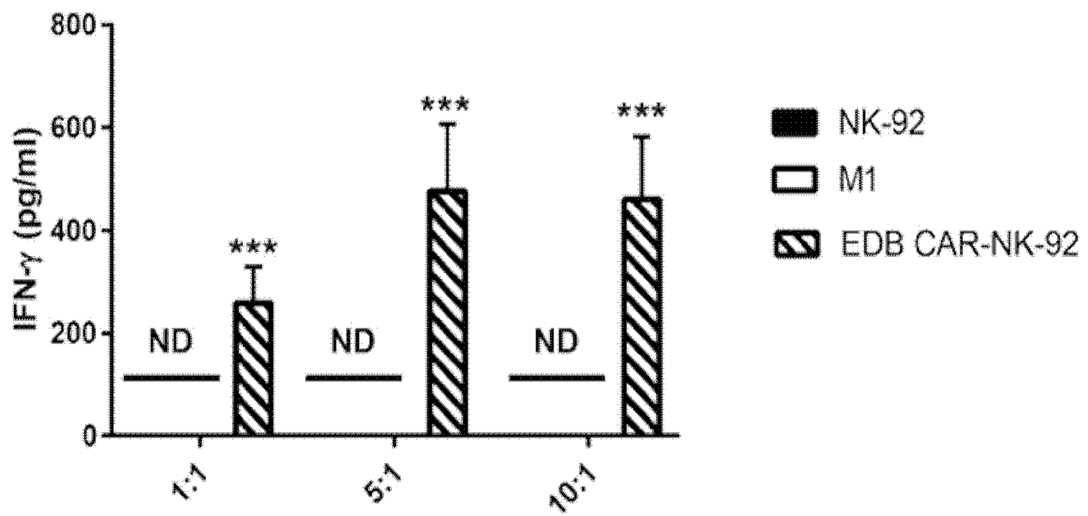


FIG. 9

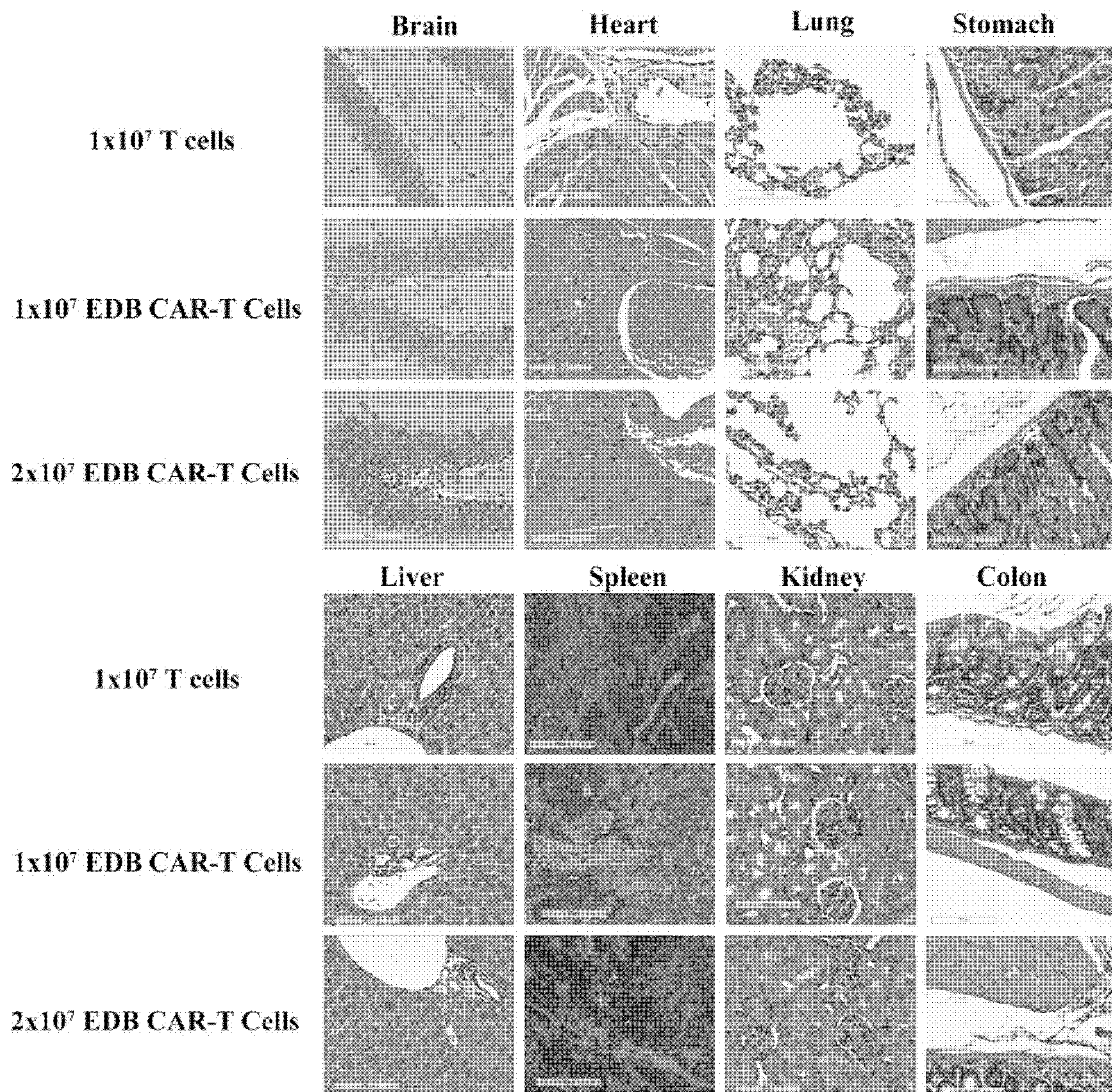


FIG. 10

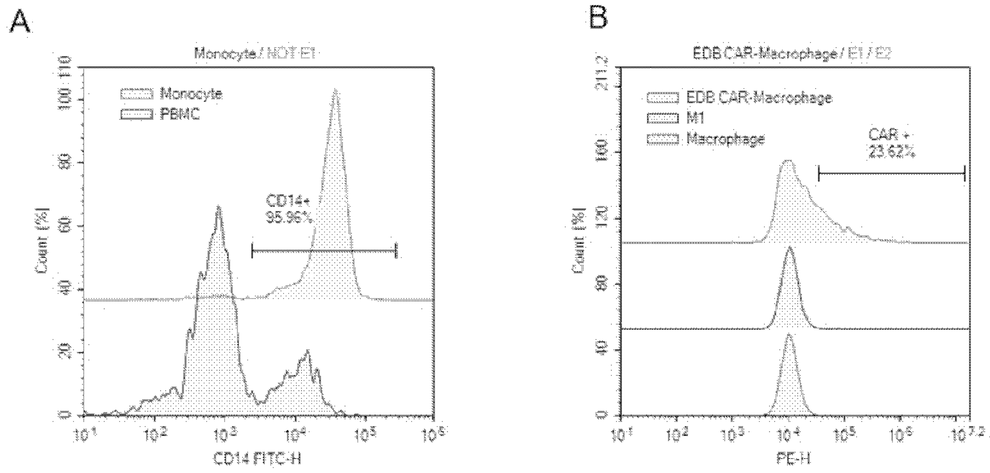



FIG. 11A

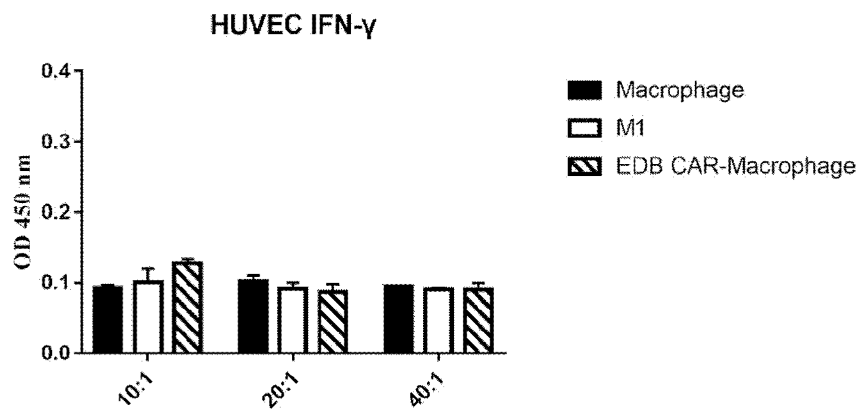



FIG. 11B

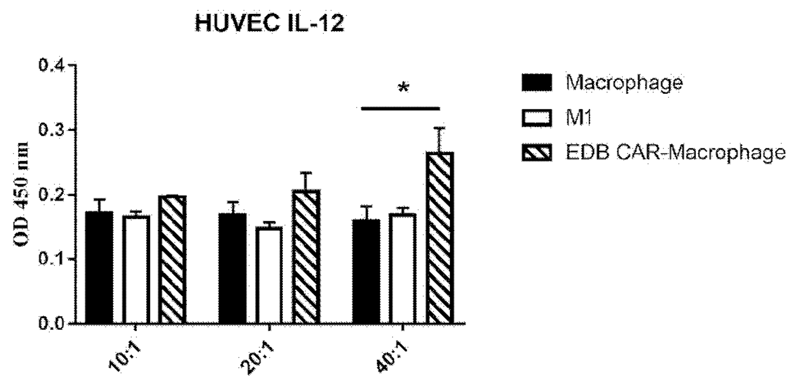


FIG. 11C

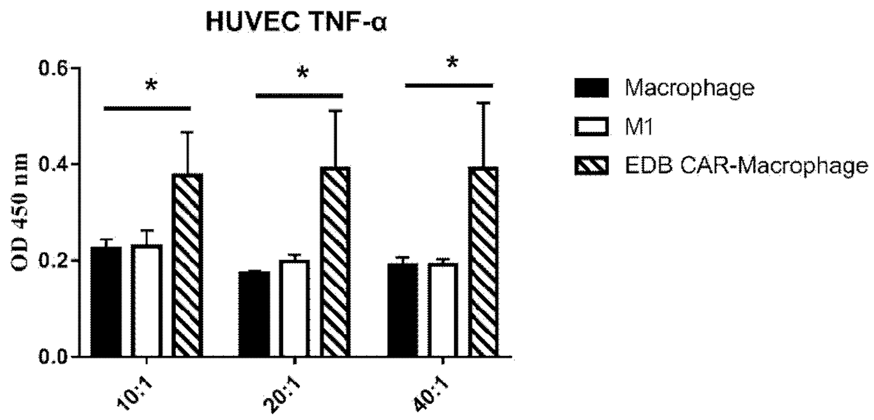



FIG. 11D

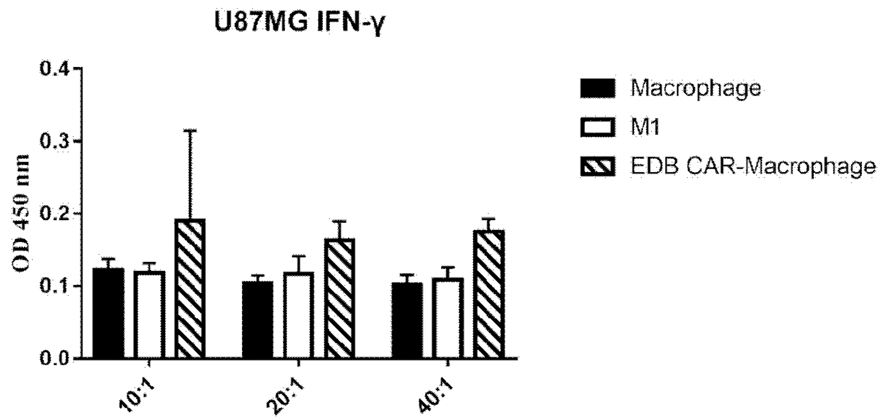



FIG. 11E

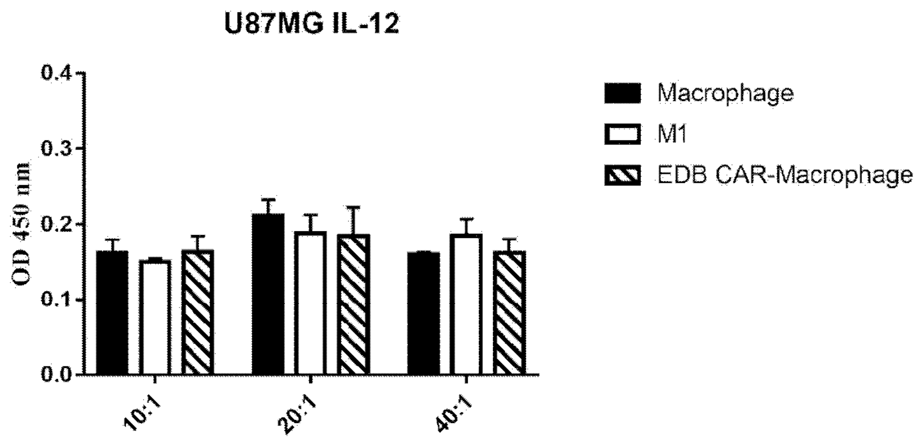


FIG. 11F

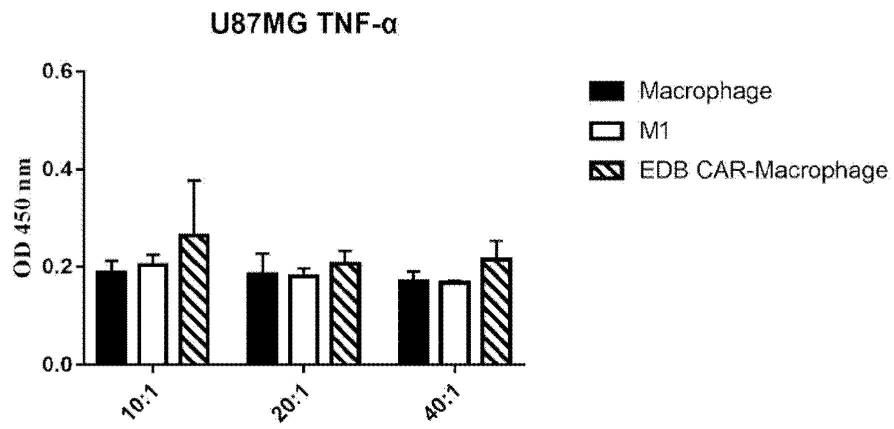



FIG. 11G

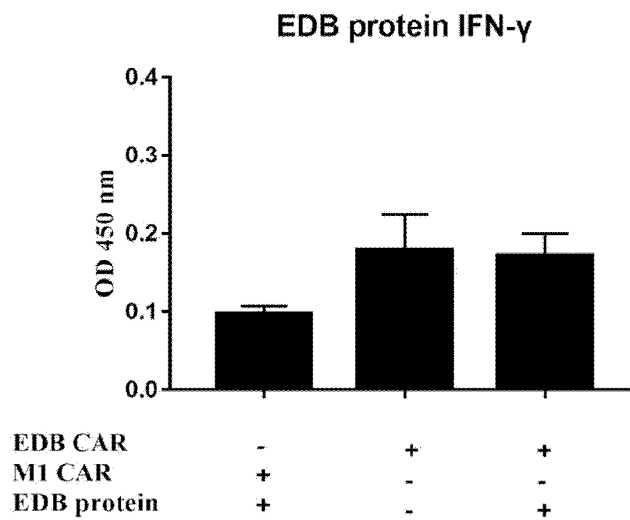


FIG. 11H

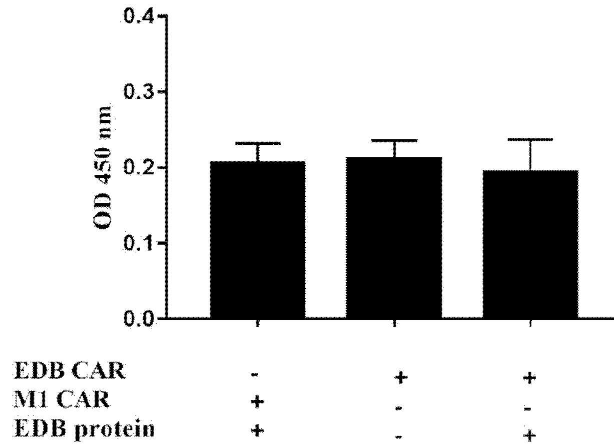
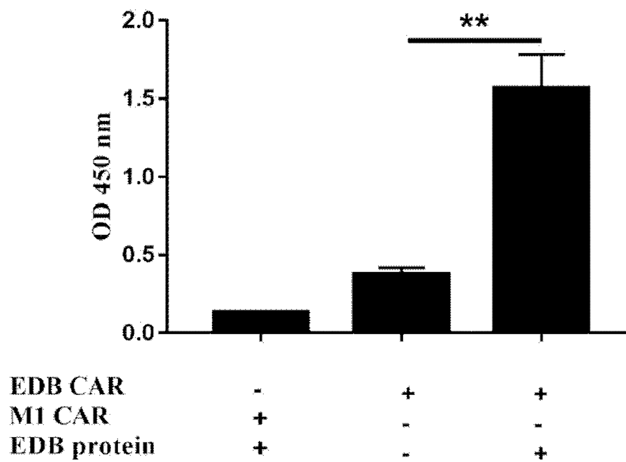



FIG. 11J



## INTERNATIONAL SEARCH REPORT

International application No.

**PCT/CN2020/118184**

### A. CLASSIFICATION OF SUBJECT MATTER

A61K 48/00(2006.01)i; A61K 39/395(2006.01)i; C07K 16/00(2006.01)i; A61P 35/04(2006.01)i

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

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K; C07K; A61P

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

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

VEN,CNABS,PubMed,ISI Web of Science,CNTXT,WOTXT,EPTXT,USTXT,CNKI,WANFANG,BAIDUXUESHU, DUXIU, STN,GenBank:CAR-T,Cancer,fibronectin,ED-B,EDB,SEQ ID NO:1-2.

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | XIE, Y.S. et al. "Nanobody-based CAR T cells that target the tumor microenvironment inhibit the growth of solid tumors in immunocompetent mice" *PNAS*, Vol. 116, No. 16, 01 April 2019 (2019-04-01), Abstract, Page 7628, paragraph 2-page7629 paragraph 2, figures 1, 6, 7 | 1-10, 14-41 |
| Y | CN 110904045 A (INSTITUTE OF ZOOLOGY CHINESE ACADEMY OF SCIENCE) 24 March 2020 (2020-03-24) Claims 1-22 | 1-10, 14-41 |
| Y | CN 110872577 A (INSTITUTE OF ZOOLOGY CHINESE ACADEMY OF SCIENCE) 10 March 2020 (2020-03-10) Claims 1-17 | 1-10, 14-41 |
| A | WO 2020020210 A1 (CARSGEN THERAPEUTICS CO., LTD.) 30 January 2020 (2020-01-30) the whole document | 1-41 |
| A | CN 109152824 A (CARTHERICS PTY. LTD.) 04 January 2019 (2019-01-04) the whole document | 1-41 |

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

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| **04 June 2021** | **24 June 2021** |

| Name and mailing address of the ISA/CN | Authorized officer |
|---|---|
| **National Intellectual Property Administration, PRC**<br>**6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088**<br>**China** | **LI,Youzhao** |
| Facsimile No. **(86-10)62019451** | Telephone No. **86-(10)-53961930** |

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | HAN，Z. et al. "Targeting Fibronectin for Cancer Imaging and Therapy" *J Mater Chem B Mater Biol Med.,* Vol. 5, No. 4, 28 January 2017 (2017-01-28), pages 639-654 | 1-41 |
| T | WAGNER，J. et al. "Antitumor Effects of CAR T Cells Redirected to the EDB Splice Variant of Fibronectin" *Cancer Immunol Res.,* Vol. 9, No. 3, 31 March 2021 (2021-03-31), Abstract | 1-41 |

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

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

1. ☑ Claims Nos.: **32-41**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] Claims 32-41 are directed to methods of inhibiting angiogenesis in a subject having a disease or condition treatable by angiogenesis inhibition, and therefore do not warrant an international search according to the criteria set out in Rule 39.1(iv). However, the search has been carried out and based on the use of the cells expressing the CAR according to claim 32, for manufacturing of a medicament for the treatment of diseases.

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

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

## INTERNATIONAL SEARCH REPORT
**Information on patent family members**

International application No.
**PCT/CN2020/118184**

| Patent document cited in search report | Publication date (day/month/year) | Patent family member(s) | Publication date (day/month/year) |
|---|---|---|---|
| CN 110904045 A | 24 March 2020 | WO 2020057486 A1 | 26 March 2020 |
| CN 110872577 A | 10 March 2020 | CN 110872577 B | 08 May 2020 |
| WO 2020020210 A1 | 30 January 2020 | AU 2019310855 A1 | 11 March 2021 |
| | | CA 3107515 A1 | 30 January 2020 |
| CN 109152824 A | 04 January 2019 | IL 259586 D0 | 31 July 2018 |
| | | EP 3380117 B1 | 06 January 2021 |
| | | AU 2016361451 A9 | 03 January 2019 |
| | | JP 2018535701 A | 06 December 2018 |
| | | CA 3004120 A1 | 01 June 2017 |
| | | AU 2016361451 A1 | 07 June 2018 |
| | | EP 3380117 A4 | 03 April 2019 |
| | | SG 10201912825X A | 27 February 2020 |
| | | WO 2017088012 A1 | 01 June 2017 |
| | | EP 3380117 A1 | 03 October 2018 |
| | | US 2018353588 A1 | 13 December 2018 |
| | | SG 11201803493U A | 30 May 2018 |
| | | EP 3708588 A1 | 16 September 2020 |
| | | EP 3708587 A1 | 16 September 2020 |
| | | WO 2017088012 A9 | 07 June 2018 |