

CHIMERIC ANTIGEN RECEPTOR WITH 4-1BB COSTIMULATORY DOMAIN
CROSS-REFERENCE TO RELATED APPLICATION

This application is a PCT application which claims priority to and the benefit of US Application 62/867,503 filed June 27, 2019; International Application PCT/KR2019/010244, filed August 12, 2019; US Application 16/715,462, filed December 16, 2019; US Application 62/991,493, filed March 18, 2020; US Application 63/004,827, filed April 3, 2020; US Application 63/043,237, filed June 24, 2020, the disclosure of each of which is herein incorporated by reference in its entirety.

BACKGROUND

Cancer remains one of the leading causes of death in the world. Recent statistics report that 13% of the world population dies from cancer. According to estimates from the International Agency for Research on Cancer (IARC), in 2012 there were 14.1 million new cancer cases and 8.2 million cancer deaths worldwide. By 2030, the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer deaths due to population growth and aging and exposure to risk factors such as smoking, unhealthy diet and physical inactivity. Further, pain and medical expenses for cancer treatment cause reduced quality of life for both cancer patients and their families.

T cells engineered with chimeric antigen receptors (CAR-T) have great therapeutic potential for treating diseases such as cancers. CAR-T therapeutics confer powerful target affinity and signaling function on T cell. However, the impressive efficacy of CAR-T therapies is frequently accompanied by severe side effects, such as cytokine release syndrome (CRS). Thus, there remains an unmet need to develop CAR-T therapeutics and strategies that have reduced side effects.

SUMMARY

Provided herein are immune cells comprising a chimeric antigen receptor (CAR), wherein the (CAR) comprises: (a) an extracellular domain comprising an antigen-binding domain; (b) a transmembrane domain; and (c) an intracellular domain comprising a costimulatory endodomain, wherein the costimulatory endodomain comprises an intracellular

signaling domain from 4-1BB/CD137 and five additional amino acids.

In some embodiments, the chimeric antigen receptor is a single polypeptide. In some embodiments, the chimeric antigen receptor is comprised of two polypeptides.

In some embodiments, the costimulatory endodomain comprises an intracellular signaling domain from 4-1BB/CD137 and five additional amino acids, wherein the five additional amino acids are encoded by SEQ ID NO: 1. In some embodiments, the costimulatory endodomain comprises SEQ ID NO: 2.

In some embodiments, the antigen-binding domain is humanized. In some embodiments, the antigen-binding domain is human. In some embodiments, the antigen-binding domain is an scFv. In some embodiments, the antigen-binding domain specifically binds an antigen associated with a disease. In some embodiments, the antigen-binding domain specifically binds a tumor antigen. In some embodiments, the antigen-binding domain specifically binds to an antigen selected from the group consisting of: glypican-3 (GPC3), malignancy variant receptor (MVR), and CD19.

In some embodiments, the transmembrane domain is a transmembrane domain selected from a protein selected from the group consisting of: 4-1BB/CD137, an activating NK cell receptor, an immunoglobulin protein, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD100 (SEMA4D), CD103, CD160 (BY55), CD18, CD19, CD19a, CD2, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3 delta, CD3 epsilon, CD3 gamma, CD3 zeta, CD30, CD4, CD40, CD49a, CD49D, CD49f, CD69, CD7, CD84, CD8, CD8 alpha, CD8 beta, CD96 (Tactile), CD11a, CD11b, CD11c, CD11d, CDS, CEACAM1, CRT AM, cytokine receptor, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, Ig alpha (CD79a), IL-2R beta, IL-2R gamma, IL-7R alpha, inducible T cell costimulator (ICOS), an integrin, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, LFA-1, a ligand that specifically binds with CD83, LIGHT, LTBR, Ly9 (CD229), lymphocyte function-associated antigen-1 (LFA-1), an MHC class I molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX-40, PAG/Cbp, programmed death-1 (PD-1), PSGL1, SELPLG (CD162), a Signaling Lymphocytic Activation Molecule (a SLAM protein), SLAM (SLAMF1), SLAMF4 (CD244), SLAMF6 (NTB-A), SLAMF7, SLP-76, a TNF receptor protein, TNFR2, TNFSF14, a Toll ligand receptor, TRANCE/RANKL, VLA1, and VLA-6. In some embodiments, the transmembrane domain is a transmembrane domain from

CD8 α . In some embodiments, the intracellular domain further comprises an intracellular domain from CD3 ζ .

In some embodiments, the chimeric antigen receptor further comprises a signal peptide or leader sequence. In some embodiments, the chimeric antigen receptor further comprises a hinge region. In some embodiments, the hinge region is a CD8 α hinge. In some embodiments, the chimeric antigen receptor further comprises an additional antigen-binding domain. In some embodiments, the additional antigen-binding domain is an scFv.

In some embodiments, the immune cell is a human immune cell. In some embodiments, the human immune cell is an autologous human immune cell. In some embodiments, the human immune cell is an allogenic human immune cell. In some embodiments, the immune cell is a T cell. In some embodiments, the immune cell is an NK cell.

Provided herein are nucleic acids encoding a chimeric antigen receptor (CAR), wherein the chimeric antigen receptor comprises: (a) an extracellular domain comprising an antigen-binding domain; (b) a transmembrane domain; and (c) an intracellular domain comprising a costimulatory endodomain, wherein the costimulatory endodomain comprises an intracellular signaling domain from 4-1BB/CD137 and five additional amino acids.

In some embodiments, the chimeric antigen receptor is a single polypeptide. In some embodiments, the chimeric antigen receptor is comprised of two polypeptides.

In some embodiments, the costimulatory endodomain comprises an intracellular signaling domain from 4-1BB/CD137 and five additional amino acids, wherein the five additional amino acids are encoded by a nucleotide sequence of SEQ ID NO: 1. In some embodiments, the costimulatory endodomain comprises SEQ ID NO: 2.

In some embodiments, the antigen-binding domain is humanized. In some embodiments, the antigen-binding domain is human. In some embodiments, the antigen-binding domain is an scFv. In some embodiments, the antigen-binding domain specifically binds an antigen associated with a disease. In some embodiments, the antigen-binding domain specifically binds a tumor antigen. In some embodiments, the antigen-binding domain specifically binds to an antigen selected from the group consisting of: glycan-3 (GPC3), malignancy variant receptor (MVR), and CD19.

In some embodiments, the transmembrane domain is a transmembrane domain selected from a protein selected from the group consisting of: 4-1BB/CD137, an activating NK cell

receptor, an immunoglobulin protein, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD100 (SEMA4D), CD103, CD160 (BY55), CD18, CD19, CD19a, CD2, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3 delta, CD3 epsilon, CD3 gamma, CD3 zeta, CD30, CD4, CD40, CD49a, CD49D, CD49f, CD69, CD7, CD84, CD8, CD8 alpha, CD8 beta, CD96 (Tactile),

5 CD11a, CD11b, CD11c, CD11d, CDS, CEACAM1, CRT AM, cytokine receptor, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, Ig alpha (CD79a), IL-2R beta, IL-2R gamma, IL-7R alpha, inducible T cell costimulator (ICOS), an integrin, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, LFA-1, a ligand that specifically binds with CD83, LIGHT, LTBR, Ly9

10 (CD229), lymphocyte function-associated antigen-1 (LFA-1), an MHC class 1 molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX-40, PAG/Cbp, programmed death-1 (PD-1), PSGL1, SELPLG (CD162), a Signaling Lymphocytic Activation Molecule (a SLAM protein), SLAM (SLAMF1), SLAMF4 (CD244), SLAMF6 (NTB-A), SLAMF7, SLP-76, a TNF receptor protein, TNFR2, TNFSF14, a Toll ligand receptor, TRANCE/RANKL, VLA1,

15 and VLA-6. In some embodiments, the transmembrane domain is a transmembrane domain from CD8 alpha.

In some embodiments, the intracellular domain further comprises an intracellular domain from CD3 ζ . In some embodiments, the chimeric antigen receptor further comprises a signal peptide or leader sequence. In some embodiments, the chimeric antigen receptor further

20 comprises a hinge region. In some embodiments, the hinge region is a CD8 α hinge.

Provided herein are vectors comprising any one of the nucleic acids described herein. In some embodiments, the vector further comprises a promoter operationally linked to 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 vector is a viral vector. In some

25 embodiments, the viral vector is a lentiviral vector.

Provided herein are methods of producing an engineered immune cell, the method comprising: introducing into an immune cell any one of the nucleic acids described herein or any one of the vectors described herein, thereby producing the engineered immune cell. In some

30 embodiments, the method further comprises, after the introducing step, culturing the engineered immune cell. In some embodiments, the immune cell is a T cell. In some embodiments, the immune cell is a NK cell.

In some embodiments, the method further comprises, before the introducing step, obtaining the immune cell from a subject. In some embodiments, the method further comprises administering the engineered immune cell to the subject. In some embodiments, the subject has been diagnosed or identified as having a cancer.

5 Provided herein are engineered immune cells produced by any one of the methods described herein.

 Provided herein are pharmaceutical compositions comprising any one of the engineered immune cells described herein and a pharmaceutically acceptable carrier.

 Provided herein are methods of treating a cancer in a subject, the method comprising
10 administering to the subject any one of the engineered immune cells described herein or any one of the pharmaceutical compositions described herein. In some embodiments, the cancer is an anti-glypican-3-associated cancer, an anti-CD19-associated cancer, or an anti-MVR-associated cancer. In some embodiments, the cancer is carcinoma, lymphoma (e.g., Hodgkin's and non-Hodgkin's lymphomas), blastoma, sarcoma, leukemia, squamous cell carcinoma, small cell lung
15 cancer, non-small cell lung cancer, lung adenocarcinoma, squamous cell carcinoma of the lung, peritoneal cancer, hepatocellular carcinoma, gastric cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary carcinoma, kidney cancer, prostate cancer, vulvar cancer, thyroid cancer, liver carcinoma, other lymphoproliferative disorders, and various
20 types of head and neck cancer. In some embodiments, the subject has previously been administered one or more additional anticancer therapies selected from the group consisting of: ionizing radiation, a chemotherapeutic agent, a therapeutic antibody, and a checkpoint inhibitor. In some embodiments, the subject has been identified or diagnosed as having the cancer.

25 **BRIEF DESCRIPTION OF DRAWINGS**

FIG. 1 shows a schematic of an exemplary MVR CAR construct.

FIG. 2 shows exemplary enzyme mapping results after MVRL2H2-4-1BB cloning.

FIG. 3 shows restriction enzyme digestion results of huGC33(VH-VL)-euBBz, wherein the expected size is marked on the DNA ladders and the results are shown in gel electrophoresis
30 images.

FIG. 4 shows restriction enzyme digestion results of huGC33(VH-VL)-BBz, wherein the expected size is marked on the DNA ladder and the result is shown in gel electrophoresis image.

FIG. 5A is a graph showing total fold expansion of the CAR-T cells over a 11 day period *in vitro*.

5 **FIG. 5B** is a graph comparing fold expansion of the CAR-T cells *in vitro*.

FIG. 5C is a graph showing cell viability of the CAR-T cells *in vitro*.

FIG. 6 shows analyses of T cells transduced with huGC33(VH-VL)-euBBz and huGC33(VH-VL)-BBz for CAR expression.

10 **FIG. 7A** is a graph showing LDH-based cytotoxicity assays with Target cells from Huh-7 cell line.

FIG. 7B is a graph showing LDH-based cytotoxicity assays with Target cells from PLC/PRF/5 cell line.

FIG. 8 is a set of graphs comparing *in vivo* efficacy of huGC33(VH-VL)-euBBz and huGC33(VH-VL)-BBz CAR-T cells.

15 **FIG. 9A** is a graph showing the CAR-T cell count from the mouse model, 5 weeks post injection of huGC33(VH-VL)-euBBz and huGC33(VH-VL)-BBz CAR-T cells.

FIG. 9B is a set of graphs comparing the CAR-T cell count from the mouse model, 5 weeks post injection of huGC33(VH-VL)-euBBz and huGC33(VH-VL)-BBz CAR-T cells.

20 **FIG. 10** show analyses of CAR-T cells in mouse blood, bone marrow, spleen, and liver, 5 weeks post injection of huGC33(VH-VL)-euBBz and huGC33(VH-VL)-BBz CAR-T cells using FACS staining.

FIG. 11A shows CAR expression in T cells transduced with CD19-BBz and CD19-euBBz.

FIG. 11B is a graph from Luciferase-based cytotoxicity assay showing killing activity in T cells transduced with CD19-BBz and CD19-euBBz.

25 **FIG. 12** shows results of IVIS imaging of the effects of CD19-BBz CAR-T and CD19-euBBz CAR-T cells using an animal model.

FIG. 13 are graphs showing photon values of the cancer cells in the animal model post injection of CD19-BBz CAR-T and CD19-euBBz CAR-T cells.

30 **FIG. 14A** is a set of graphs showing percentage of total CD19 CAR-T cells present in the blood using FACS after performing orbital blood collection in the mouse at 3-4 day intervals.

FIG. 14B is a set of graphs showing percentage of CD4/CD8 CAR-T cells present in the blood using FACS after performing orbital blood collection in the mouse at 3-4 day intervals.

FIG. 14C is a graph showing the number of total CD19 CAR-T present in the blood using FACS after performing orbital blood collection in the mouse at 3-4 day intervals.

5 **FIG. 15A** shows a schematic of an exemplary GPC3 CAR construct.

FIG. 15B shows a schematic of an exemplary GPC3 CAR construct.

DETAILED DESCRIPTION

This disclosure describes chimeric antigen receptors (CARs) that include a 4-1BB
10 costimulatory endodomain, as well as methods of making and using the same.

Definitions

About: The term “about”, when used herein in reference to a value, refers to a value that is similar, in context to the referenced value. In general, those skilled in the art, familiar with the
15 context, will appreciate the relevant degree of variance encompassed by “about” in that context. For example, in some embodiments, the term “about” may encompass a range of values that are within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less of the referred value.

Administration: As used herein, the term “administration” typically refers to the
20 administration of a composition to a subject or system to achieve delivery of an agent that is, or is included in, the composition. Those of ordinary skill in the art will be aware of a variety of routes that may, in appropriate circumstances, be utilized for administration to a subject, for example a human. For example, in some embodiments, administration may be ocular, oral, parenteral, topical, etc. In some particular embodiments, administration may be bronchial (e.g.,
25 by bronchial instillation), buccal, dermal (which may be or comprise, for example, one or more of topical to the dermis, intradermal, interdermal, transdermal, etc.), enteral, intra-arterial, intradermal, intragastric, intramedullary, intramuscular, intranasal, intraperitoneal, intrathecal, intravenous, intraventricular, within a specific organ (e.g. intrahepatic), mucosal, nasal, oral, rectal, subcutaneous, sublingual, topical, tracheal (e.g., by intratracheal instillation), vaginal,
30 vitreal, etc. In some embodiments, administration may involve a single dose, multiple doses, or a fixed number of doses. In some embodiments, administration may involve dosing that is

intermittent (e.g., a plurality of doses separated in time) and/or periodic (e.g., individual doses separated by a common period of time) dosing. In some embodiments, administration may involve continuous dosing (e.g., perfusion) for at least a selected period of time.

Affinity: As is known in the art, “affinity” is a measure of the strength a particular ligand binds to its partner. Affinity can be measured in different ways. In some embodiments, affinity is measured by a quantitative assay. In some such embodiments, binding partner concentration may be fixed to be in excess of ligand concentration so as to mimic physiological conditions. Alternatively or additionally, in some embodiments, binding partner concentration and/or ligand concentration may be varied. In some such embodiments, affinity may be compared to a reference under comparable conditions (e.g., concentrations).

Antibody agent: As used herein, the term “antibody agent” can refer to an agent that specifically binds to a particular antigen. In some embodiments, the term encompasses any polypeptide or polypeptide complex that includes immunoglobulin structural elements sufficient to confer specific binding. Exemplary antibody agents include, but are not limited to monoclonal antibodies, polyclonal antibodies, and fragments thereof. In some embodiments, an antibody agent may include one or more sequence elements are humanized, primatized, chimeric, etc., as is known in the art. In many embodiments, the term “antibody agent” is used to refer to one or more of the art-known or developed constructs or formats for utilizing antibody structural and functional features in alternative presentation. For example, in some embodiments, an antibody agent utilized in accordance with the present invention is in a format selected from, but not limited to, intact IgA, IgG, IgE, or IgM antibodies; bi- or multi- specific antibodies (e.g., Zybodies®, etc.); antibody fragments such as Fab fragments, Fab’ fragments, F(ab’)2 fragments, Fd’ fragments, Fd fragments, and isolated CDRs or sets thereof; single chain Fvs; polypeptide-Fc fusions; single domain antibodies (e.g., shark single domain antibodies such as IgNAR or fragments thereof); cameloid antibodies; masked antibodies (e.g., Probodies®); Small Modular ImmunoPharmaceuticals (“SMIPsTM”); single chain or Tandem diabodies (TandAb®); VHHs; Anticalins®, Nanobodies® minibodies; BiTE®s; ankyrin repeat proteins or DARPINs®; Avimers®; DARTs; TCR-like antibodies; Adnectins®; Affilins®; Trans-bodies®; Affibodies®; TrimerX®; MicroProteins; Fynomers®, Centyrins®, and KALBITOR®s. In some embodiments, an antibody agent may lack a covalent modification (e.g., attachment of a glycan) that it would have if produced naturally. In some embodiments, an antibody agent may contain a

covalent modification (e.g., attachment of a glycan, a payload [e.g., a detectable moiety, a therapeutic moiety, a catalytic moiety, etc.], or other pendant group [e.g., poly-ethylene glycol, etc.]. In many embodiments, an antibody agent is or comprises a polypeptide whose amino acid sequence includes one or more structural elements recognized by those skilled in the art as a

5 complementarity determining region (CDR). In some embodiments an antibody agent is or comprises a polypeptide whose amino acid sequence includes at least one CDR (e.g., at least one heavy chain CDR and/or at least one light chain CDR) that is substantially identical to one found in a reference antibody. In some embodiments, an included CDR is substantially identical to a reference CDR in that it is either identical in sequence or contains between 1-5 amino acid

10 substitutions as compared with the reference CDR. In some embodiments, an included CDR is substantially identical to a reference CDR in that it shows at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference CDR. In some embodiments, an included CDR is substantially identical to a reference CDR in that it shows at least 96%, 96%, 97%, 98%, 99%, or 100% sequence identity with the

15 reference CDR. In some embodiments, an included CDR is substantially identical to a reference CDR in that at least one amino acid within the included CDR is deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical with that of the reference CDR. In some embodiments, an included CDR is substantially identical to a reference CDR in that 1-5 amino acids within the included CDR are

20 deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical to the reference CDR. In some embodiments, an included CDR is substantially identical to a reference CDR in that at least one amino acid within the included CDR is substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical with that of the reference CDR. In some

25 embodiments, an included CDR is substantially identical to a reference CDR in that 1-5 amino acids within the included CDR are deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical to the reference CDR. In some embodiments, an antibody agent is or comprises a polypeptide whose amino acid sequence includes structural elements recognized by those skilled in the art as an

30 immunoglobulin variable domain. In some embodiments, an antibody agent is a polypeptide protein having a binding domain which is homologous or largely homologous to an

immunoglobulin-binding domain. In some embodiments, an antibody agent is or comprises at least a portion of a chimeric antigen receptor (CAR).

Antigen: The term “antigen”, as used herein, can refer to an agent that binds to an antibody agent. In some embodiments, an antigen binds to an antibody agent and may or may not induce a particular physiological response in an organism. In general, an antigen may be or include any chemical entity such as, for example, a small molecule, a nucleic acid, a polypeptide, a carbohydrate, a lipid, a polymer (including biologic polymers [e.g., nucleic acid and/or amino acid polymers] and polymers other than biologic polymers [e.g., other than a nucleic acid or amino acid polymer]), etc. In some embodiments, an antigen is or comprises a polypeptide. In some embodiments, an antigen is or comprises a glycan. Those of ordinary skill in the art will appreciate that, in general, an antigen may be provided in isolated or pure form, or alternatively may be provided in crude form (e.g., together with other materials, for example in an extract such as a cellular extract or other relatively crude preparation of an antigen-containing source). In some certain embodiments, an antigen is present in a cellular context (e.g., an antigen is expressed on the surface of a cell or expressed in a cell). In some embodiments, an antigen is a recombinant antigen.

Antigen binding domain: The term “antigen binding domain”, as used herein, can refer to an antibody agent or portion thereof that specifically binds to a target moiety or entity. Typically, the interaction between an antigen binding domain and its target is non-covalent. In some embodiments, a target moiety or entity can be of any chemical class including, for example, a carbohydrate, a lipid, a nucleic acid, a metal, a polypeptide, or a small molecule. In some embodiments, an antigen binding domain may be or comprise a polypeptide (or complex thereof). In some embodiments, an antigen binding domain is part of a fusion polypeptide. In some embodiments, an antigen binding domain is part of a chimeric antigen receptor (CAR).

Associated with: Two events or entities are “associated” with one another, as that term is used herein, if the presence, level, and/or form of one is correlated with that of the other. For example, a particular entity (e.g., polypeptide, genetic signature, metabolite, microbe, etc.) is considered to be associated with a particular disease, disorder, or condition, if its presence, level and/or form correlates with incidence of and/or susceptibility to the disease, disorder, or condition (e.g., across a relevant population). In some embodiments, two or more entities are physically “associated” with one another if they interact, directly or indirectly, so that they are

and/or remain in physical proximity with one another. In some embodiments, two or more entities that are physically associated with one another are covalently linked to one another. In some embodiments, two or more entities that are physically associated with one another are not covalently linked to one another but are non-covalently associated, for example by means of
5 hydrogen bonds, van der Waals interaction, hydrophobic interactions, magnetism, and combinations thereof.

Binding: It will be understood that the term “binding”, as used herein, typically refers to a non-covalent association between or among two or more entities. “Direct” binding involves physical contact between entities or moieties. Indirect binding involves physical interaction by
10 way of physical contact with one or more intermediate entities. Binding between two or more entities can typically be assessed in any of a variety of contexts – including where interacting entities or moieties are studied in isolation or in the context of more complex systems (e.g., while covalently or otherwise associated with a carrier entity and/or in a biological system or cell).

Cancer: The terms “cancer”, “malignancy”, “neoplasm”, “tumor”, and “carcinoma”, are
15 used herein to refer to cells that exhibit relatively abnormal, uncontrolled, and/or autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. In some embodiments, a tumor may be or comprise cells that are precancerous (e.g., benign), malignant, pre-metastatic, metastatic, and/or non-metastatic. The present disclosure specifically identifies certain cancers to which its teachings may be
20 particularly relevant. In some embodiments, a relevant cancer may be characterized by a solid tumor. In some embodiments, a relevant cancer may be characterized by a hematologic tumor. In general, examples of different types of cancers known in the art include, for example, hematopoietic cancers including leukemias, lymphomas (Hodgkin’s and non-Hodgkin’s), myelomas and myeloproliferative disorders; sarcomas, melanomas, adenomas, carcinomas of
25 solid tissue, squamous cell carcinomas of the mouth, throat, larynx, and lung, liver cancer, genitourinary cancers such as prostate, cervical, bladder, uterine, and endometrial cancer and renal cell carcinomas, bone cancer, pancreatic cancer, skin cancer, cutaneous or intraocular melanoma, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, head and neck cancers, breast cancer, gastro-intestinal cancers and nervous system
30 cancers, benign lesions such as papillomas, and the like.

CDR: As used herein, “CDR”, can refer to a complementarity determining region within a variable region of an antibody agent. There are three CDRs in each of the variable regions of the heavy chain and the light chain, which are designated CDR1, CDR2 and CDR3, for each of the variable regions. A “set of CDRs” or “CDR set” refers to a group of three or six CDRs that occur in either a single variable region capable of binding the antigen or the CDRs of cognate heavy and light chain variable regions capable of binding the antigen. Certain systems have been established in the art for defining CDR boundaries (e.g., Kabat, Chothia, etc.); those skilled in the art appreciate the differences between and among these systems and are capable of understanding CDR boundaries to the extent required to understand and to practice the claimed invention.

Chemotherapeutic Agent: The term “chemotherapeutic agent”, has used herein has its art-understood meaning referring to one or more pro-apoptotic, cytostatic and/or cytotoxic agents, for example specifically including agents utilized and/or recommended for use in treating one or more diseases, disorders or conditions associated with undesirable cell proliferation. In many embodiments, chemotherapeutic agents are useful in the treatment of cancer. In some embodiments, a chemotherapeutic agent may be or comprise one or more alkylating agents, one or more anthracyclines, one or more cytoskeletal disruptors (e.g. microtubule targeting agents such as taxanes, maytansine and analogs thereof), one or more epothilones, one or more histone deacetylase inhibitors HDACs), one or more topoisomerase inhibitors (e.g., inhibitors of topoisomerase I and/or topoisomerase II), one or more kinase inhibitors, one or more nucleotide analogs or nucleotide precursor analogs, one or more peptide antibiotics, one or more platinum-based agents, one or more retinoids, one or more vinca alkaloids, and/or one or more analogs of one or more of the following (i.e., that share a relevant anti-proliferative activity). In some particular embodiments, a chemotherapeutic agent may be or comprise one or more of Actinomycin, All-trans retinoic acid, an Auiristatin, Azacitidine, Azathioprine, Bleomycin, Bortezomib, Carboplatin, Capecitabine, Cisplatin, Chlorambucil, Cyclophosphamide, Curcumin, Cytarabine, Daunorubicin, Docetaxel, Doxifluridine, Doxorubicin, Epirubicin, Epothilone, Etoposide, Fluorouracil, Gemcitabine, Hydroxyurea, Idarubicin, Imatinib, Irinotecan, Maytansine and/or analogs thereof (e.g. DM1) Mechlorethamine, Mercaptopurine, Methotrexate, Mitoxantrone, a Maytansinoid, Oxaliplatin, Paclitaxel, Pemetrexed, Teniposide, Tioguanine, Topotecan, Valrubicin, Vinblastine, Vincristine, Vindesine, Vinorelbine, and combinations

thereof. In some embodiments, a chemotherapeutic agent may be utilized in the context of an antibody-drug conjugate. In some embodiments, a chemotherapeutic agent is one found in an antibody-drug conjugate selected from the group consisting of: hLL1-doxorubicin, hRS7-SN-38, hMN-14-SN-38, hLL2-SN-38, hA20-SN-38, hPAM4-SN-38, hLL1-SN-38, hRS7-Pro-2-P-Dox, hMN-14-Pro-2-P-Dox, hLL2-Pro-2-P-Dox, hA20-Pro-2-P-Dox, hPAM4-Pro-2-P-Dox, hLL1-Pro-2-P-Dox, P4/D10-doxorubicin, gemtuzumab ozogamicin, brentuximab vedotin, trastuzumab emtansine, inotuzumab ozogamicin, glembatumomab vedotin, SAR3419, SAR566658, BIIB015, BT062, SGN-75, SGN-CD19A, AMG-172, AMG-595, BAY-94-9343, ASG-5ME, ASG-22ME, ASG-16M8F, MDX-1203, MLN-0264, anti-PSMA ADC, RG-7450, RG-7458, RG-7593, RG-7596, RG-7598, RG-7599, RG-7600, RG-7636, ABT-414, IMGN-853, IMGN-529, vorsetuzumab mafodotin, and lorvotuzumab mertansine.

Engineered: In general, the term “engineered” can refer to the aspect of having been manipulated by the hand of man. For example, a polypeptide is considered to be “engineered” when the polypeptide sequence manipulated by the hand of man. For example, in some embodiments of the present invention, an engineered polypeptide comprises a sequence that includes one or more amino acid mutations, deletions and/or insertions that have been introduced by the hand of man into a reference polypeptide sequence. In some embodiments, an engineered polypeptide includes a polypeptide that has been fused (i.e., covalently linked) to one or more additional polypeptides by the hand of man, to form a fusion polypeptide that would not naturally occur *in vivo*. Comparably, a cell or organism is considered to be “engineered” if it has been manipulated so that its genetic information is altered (e.g., new genetic material not previously present has been introduced, for example by transformation, mating, somatic hybridization, transfection, transduction, or other mechanism, or previously present genetic material is altered or removed, for example by substitution or deletion mutation, or by mating protocols). As is common practice and is understood by those in the art, derivatives and/or progeny of an engineered polypeptide or cell are typically still referred to as “engineered” even though the actual manipulation was performed on a prior entity.

Host cell: The term “host cell”, as used herein, can refer to a cell of an organism that is selected, modified, transformed, grown, use or manipulated in any way, for the production of material by the cell, for example the expression by the cell of a gene, a DNA or RNA sequence, a

protein or an enzyme. Host cells can include immune cells, including but not limited to lymphocytes (e.g., T cells, B cells, and NK cells), neutrophils, and monocytes/macrophages.

***In vitro*:** The term “*in vitro*”, as used herein, refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, etc., rather than within a multi-cellular organism.

***In vivo*:** The term “*in vivo*” as used herein refers to events that occur within a multi-cellular organism, such as a human and a non-human animal. In the context of cell-based systems, the term may be used to refer to events that occur within a living cell (as opposed to, for example, *in vitro* systems).

***Isolated*:** The term “isolated” as used herein, can refer to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) designed, produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is “pure” if it is substantially free of other components. In some embodiments, as will be understood by those skilled in the art, a substance may still be considered “*isolated*” or even “*pure*”, after having been combined with certain other components such as, for example, one or more carriers or excipients (e.g., buffer, solvent, water, etc.); in such embodiments, percent isolation or purity of the substance is calculated without including such carriers or excipients. To give but one example, in some embodiments, a biological polymer such as a polypeptide or polynucleotide that occurs in nature is considered to be “*isolated*” when, a) by virtue of its origin or source of derivation is not associated with some or all of the components that accompany it in its native state in nature; b) it is substantially free of other polypeptides or nucleic acids of the same species from the species that produces it in nature; c) is expressed by or is otherwise in association with components from a cell or other expression system that is not of the species that produces it in nature. Thus, for instance, in some embodiments, a polypeptide that is chemically

synthesized or is synthesized in a cellular system different from that which produces it in nature is considered to be an “*isolated*” polypeptide. Alternatively or additionally, in some embodiments, a polypeptide that has been subjected to one or more purification techniques may be considered to be an “*isolated*” polypeptide to the extent that it has been separated from other components a) with which it is associated in nature; and/or b) with which it was associated when initially produced.

Operably linked: The term “operably linked” as used herein, can refer to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control element “*operably linked*” to a functional element is associated in such a way that expression and/or activity of the functional element is achieved under conditions compatible with the control element. In some embodiments, “*operably linked*” control elements are contiguous (e.g., covalently linked) with the coding elements of interest. In some embodiments, control elements act in *trans* to or otherwise at a distance from the functional element of interest.

Pharmaceutical composition: As used herein, the term “pharmaceutical composition” refers to a composition in which an active agent is formulated together with one or more pharmaceutically acceptable carriers. In some embodiments, the composition is suitable for administration to a human or animal subject. In some embodiments, the active agent is present in unit dose amount appropriate for administration in a therapeutic regimen that shows a statistically significant probability of achieving a predetermined therapeutic effect when administered to a relevant population.

Polypeptide: The term “polypeptide”, as used herein, generally refers to its art-recognized meaning of a polymer of at least three amino acids. Those of ordinary skill in the art will appreciate that the term “polypeptide” is intended to be sufficiently general as to encompass not only polypeptides having a complete sequence recited herein, but also to encompass polypeptides that represent functional fragments (i.e., fragments retaining at least one activity) of such complete polypeptides. Moreover, those of ordinary skill in the art understand that protein sequences generally tolerate some substitution without destroying activity. Thus, any polypeptide that retains activity and shares at least about 30-40% overall sequence identity, often greater than about 50%, 60%, 70%, or 80%, and further usually including at least one region of much higher identity, often greater than 90% or even 95%, 96%, 97%, 98%, or 99% in one or

more highly conserved regions, usually encompassing at least 3-4 and often up to 20 or more amino acids, with another polypeptide of the same class, is encompassed within the relevant term “polypeptide” as used herein. Polypeptides may contain L-amino acids, D-amino acids, or both and may contain any of a variety of amino acid modifications or analogs known in the art. Useful modifications include, e.g., terminal acetylation, amidation, methylation, etc. In some embodiments, proteins may comprise natural amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof. The term “peptide” is generally used to refer to a polypeptide having a length of less than about 100 amino acids, less than about 50 amino acids, less than 20 amino acids, or less than 10 amino acids. In some embodiments, proteins are antibody agents, antibody fragments, biologically active portions thereof, and/or characteristic portions thereof.

Prevent or prevention: The terms “prevent” or “prevention”, as used herein, when used in connection with the occurrence of a disease, disorder, and/or condition, can refer to reducing the risk of developing the disease, disorder and/or condition and/or to delaying onset and/or severity of one or more characteristics or symptoms of the disease, disorder or condition. In some embodiments, prevention is assessed on a population basis such that an agent is considered to “prevent” a particular disease, disorder or condition if a statistically significant decrease in the development, frequency, and/or intensity of one or more symptoms of the disease, disorder, or condition is observed in a population susceptible to the disease, disorder, or condition.

Recombinant: The term “recombinant”, as used herein, can refer to polypeptides that are designed, engineered, prepared, expressed, created, manufactured, and/or isolated by recombinant means, such as polypeptides expressed using a recombinant expression vector transfected into a host cell; polypeptides isolated from a recombinant, combinatorial human polypeptide library; polypeptides isolated from an animal (e.g., a mouse, rabbit, sheep, fish, etc.) that are transgenic for or otherwise has been manipulated to express a gene or genes, or gene components that encode and/or direct expression of the polypeptide or one or more component(s), portion(s), element(s), or domain(s) thereof; and/or polypeptides prepared, expressed, created or isolated by any other means that involves splicing or ligating selected nucleic acid sequence elements to one another, chemically synthesizing selected sequence elements, and/or otherwise generating a nucleic acid that encodes and/or directs expression of the polypeptide or one or more component(s), portion(s), element(s), or domain(s) thereof. In some

embodiments, one or more of such selected sequence elements is found in nature. In some embodiments, one or more of such selected sequence elements is designed *in silico*. In some embodiments, one or more such selected sequence elements results from mutagenesis (e.g., *in vivo* or *in vitro*) of a known sequence element, e.g., from a natural or synthetic source such as, for example, in the germline of a source organism of interest (e.g., of a human, a mouse, etc.).

Specific binding: As used herein, the term “specific binding” can refer to an ability to discriminate between possible binding partners in the environment in which binding can occur. A binding agent that interacts with one particular target when other potential targets are present is said to “*bind specifically*” to the target with which it interacts. In some embodiments, specific binding is assessed by detecting or determining degree of association between the binding agent and its partner. In some embodiments, specific binding is assessed by detecting or determining degree of dissociation of a binding agent-partner complex; in some embodiments, specific binding is assessed by detecting or determining ability of the binding agent to compete an alternative interaction between its partner and another entity. In some embodiments, specific binding is assessed by performing such detections or determinations across a range of concentrations.

Subject: As used herein, the term “subject” refers an organism, typically a mammal (e.g., a human, in some embodiments including prenatal human forms). In some embodiments, a subject is suffering from a relevant disease, disorder or condition. In some embodiments, a subject is susceptible to a disease, disorder, or condition. In some embodiments, a subject displays one or more symptoms or characteristics of a disease, disorder or condition. In some embodiments, a subject does not display any symptom or characteristic of a disease, disorder, or condition. In some embodiments, a subject is someone with one or more features characteristic of susceptibility to or risk of a disease, disorder, or condition. In some embodiments, a subject is a patient. In some embodiments, a subject is an individual to whom diagnosis and/or therapy is and/or has been administered.

Therapeutic agent: As used herein, the phrase “therapeutic agent” in general refers to any agent that elicits a desired pharmacological effect when administered to an organism. In some embodiments, an agent is considered to be a therapeutic agent if it demonstrates a statistically significant effect across an appropriate population. In some embodiments, the appropriate population may be a population of model organisms. In some embodiments, an

appropriate population may be defined by various criteria, such as a certain age group, gender, genetic background, preexisting clinical conditions, etc. In some embodiments, a therapeutic agent is a substance that can be used to alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more symptoms or features of a disease, disorder, and/or condition. In some embodiments, a “therapeutic agent” is an agent that has been or is required to be approved by a government agency before it can be marketed for administration to humans. In some embodiments, a “therapeutic agent” is an agent for which a medical prescription is required for administration to humans.

Therapeutically Effective Amount: As used herein, the term “therapeutically effective amount” means an amount that is sufficient, when administered to a population suffering from or susceptible to a disease, disorder, and/or condition in accordance with a therapeutic dosing regimen, to treat the disease, disorder, and/or condition. In some embodiments, a therapeutically effective amount is one that reduces the incidence and/or severity of, stabilizes one or more characteristics of, and/or delays onset of, one or more symptoms of the disease, disorder, and/or condition. Those of ordinary skill in the art will appreciate that the term “therapeutically effective amount” does not in fact require successful treatment be achieved in a particular individual. Rather, a therapeutically effective amount may be that amount that provides a particular desired pharmacological response in a significant number of subjects when administered to patients in need of such treatment. For example, in some embodiments, term “therapeutically effective amount”, refers to an amount which, when administered to an individual in need thereof in the context of inventive therapy, will block, stabilize, attenuate, or reverse a cancer-supportive process occurring in said individual, or will enhance or increase a cancer-suppressive process in said individual. In the context of cancer treatment, a “therapeutically effective amount” is an amount which, when administered to an individual diagnosed with a cancer, will prevent, stabilize, inhibit, or reduce the further development of cancer in the individual. A particularly preferred “therapeutically effective amount” of a composition described herein reverses (in a therapeutic treatment) the development of a malignancy, such as a pancreatic carcinoma, or helps achieve or prolong remission of a malignancy. A therapeutically effective amount administered to an individual to treat a cancer in that individual may be the same or different from a therapeutically effective amount administered to promote remission or inhibit metastasis. As with most cancer therapies, the

therapeutic methods described herein are not to be interpreted as, restricted to, or otherwise limited to a “cure” for cancer; rather the methods of treatment are directed to the use of the described compositions to “treat” a cancer, i.e., to effect a desirable or beneficial change in the health of an individual who has cancer. Such benefits are recognized by skilled healthcare providers in the field of oncology and include, but are not limited to, a stabilization of patient condition, a decrease in tumor size (tumor regression), an improvement in vital functions (e.g., improved function of cancerous tissues or organs), a decrease or inhibition of further metastasis, a decrease in opportunistic infections, an increased survivability, a decrease in pain, improved motor function, improved cognitive function, improved feeling of energy (vitality, decreased malaise), improved feeling of well-being, restoration of normal appetite, restoration of healthy weight gain, and combinations thereof. In addition, regression of a particular tumor in an individual (e.g., as the result of treatments described herein) may also be assessed by taking samples of cancer cells from the site of a tumor such as a pancreatic adenocarcinoma (e.g., over the course of treatment) and testing the cancer cells for the level of metabolic and signaling markers to monitor the status of the cancer cells to verify at the molecular level the regression of the cancer cells to a less malignant phenotype. For example, tumor regression induced by employing the methods of this invention would be indicated by finding a decrease in any of the pro-angiogenic markers discussed above, an increase in anti-angiogenic markers described herein, the normalization (i.e., alteration toward a state found in normal individuals not suffering from cancer) of metabolic pathways, intercellular signaling pathways, or intracellular signaling pathways that exhibit abnormal activity in individuals diagnosed with cancer. Those of ordinary skill in the art will appreciate that, in some embodiments, a therapeutically effective amount may be formulated and/or administered in a single dose. In some embodiments, a therapeutically effective amount may be formulated and/or administered in a plurality of doses, for example, as part of a dosing regimen.

Transfection: The term “transfection”, as used herein, can refer to the introduction of a foreign nucleic acid into a cell using recombinant DNA technology. The term “transformation” as used herein can refer to the introduction of a foreign gene, DNA, or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce the encoded protein or enzyme.

Transduction: The term “transduction”, as used herein, can refer to the introduction of a foreign nucleic acid into a cell using a viral vector.

Variant: As used herein in the context of molecules, e.g., nucleic acids, proteins, or small molecules, the term “variant” can refer to a molecule that shows significant structural identity with a reference molecule but differs structurally from the reference molecule, e.g., in the presence or absence or in the level of one or more chemical moieties as compared to the reference entity. In some embodiments, a variant also differs functionally from its reference molecule. In general, whether a particular molecule is properly considered to be a “variant” of a reference molecule is based on its degree of structural identity with the reference molecule. As will be appreciated by those skilled in the art, any biological or chemical reference molecule has certain characteristic structural elements. A variant, by definition, is a distinct molecule that shares one or more such characteristic structural elements but differs in at least one aspect from the reference molecule. To give but a few examples, a polypeptide may have a characteristic sequence element comprised of a plurality of amino acids having designated positions relative to one another in linear or three-dimensional space and/or contributing to a particular structural motif and/or biological function; a nucleic acid may have a characteristic sequence element comprised of a plurality of nucleotide residues having designated positions relative to one another in linear or three-dimensional space. In some embodiments, a variant polypeptide or nucleic acid may differ from a reference polypeptide or nucleic acid as a result of one or more differences in amino acid or nucleotide sequence. In some embodiments, a variant polypeptide or nucleic acid shows an overall sequence identity with a reference polypeptide or nucleic acid that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99%. In some embodiments, a variant polypeptide or nucleic acid does not share at least one characteristic sequence element with a reference polypeptide or nucleic acid. In some embodiments, a reference polypeptide or nucleic acid has one or more biological activities. In some embodiments, a variant polypeptide or nucleic acid shares one or more of the biological activities of the reference polypeptide or nucleic acid.

Vector: The term “vector”, as used herein, can refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors can encompass both non-viral and viral carriers for introducing nucleic acids into cells *in vitro*, *ex vivo*, or *in vivo*.

A vector may be a replicon with another DNA fragment attached to amplify the attached fragment. The term “replicon” refers to any genetic element (e.g., a plasmid, phage, cosmid, chromosome, or virus) that is able to act as an autonomous unit of *in vivo* DNA replication. Many vectors known in the art may be used to engineer nucleic acids, incorporate response elements and promoters into genes, and the like. Preferred vectors include, but are not limited to, plasmids (e.g., PBR322 or pUC plasmid derivatives), modified viruses (e.g., adenoviruses, retroviruses, adeno-associated viruses, or herpes viruses), or Bluescript vectors. For example, DNA fragments corresponding to reaction elements and promoters may be inserted into appropriate vectors by combining the appropriate DNA fragments with selected vectors having complementary cohesive termini. In some embodiments, the termini of the DNA molecules may be enzymatically modified, or any site may be generated by binding the nucleotide sequence with the DNA ends via a linker. In some embodiments, vectors may be manipulated to contain a selection marker gene for screening cells that have incorporated the marker into the cell genome. Such markers make it possible to identify and/or screen host cells expressing the protein encoded by the marker.

One type of vector is a “plasmid”, which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as “expression vectors.” Non-limiting examples of expression vectors and packaging constructs that can be used to deliver the chimeric antigen receptors described herein include retroviral vectors (e.g., SFG, pMX, pSAMEN, pMP71, pLXSN, pMSCV, pMSGV), lentiviral vectors (e.g., epHIV7, pLenO, pSIN, pSIEW, pELPS, pELNS, pHR), packaging constructs (psPAX2, pRDF, pEQ-PAM3(-E), pVSVg, pCL, pMEVSVg, pMD2G, pMDLg/p.RRE, pRSV.REV, pTSV.rev, pCHGP, pCMV-g, pCMV-Rev2, pCMVdR8.91, pGALV).

In some embodiments, a vector provides the necessary regulatory sequences (e.g., transcriptional and translational elements) to regulate the expression of the fusion protein in the appropriate host cell. Regulatory sequences may comprise promoter regions, enhancer regions, transcription termination sites, ribosomal binding sites, initiation codons, splice signals, introns, polyadenylation signals, Shine/Dalgarno translation sequences and Kozak consensus sequences. A regulatory sequence is selected in view of the host cell in which the fusion protein will be produced. In some embodiments, suitable bacterial promoters include, but are not limited to, bacteriophage λ pL or pR, T6, T7, T7/lacO, lac, recA, gal, trp, ara, hut and trp-lac. In some embodiments, suitable eukaryotic promoters include, but are not limited to, PRBI, GAPDH, metallothionein, thymidine kinase, viral LTR, cytomegalovirus, SV40, or tissue-specific or tumor-specific promoters such as α -fetal protein, amylase, cathepsin E, M1 muscarinic receptor or γ -glutamyl transferase.

In some embodiments, additional vectors include lipoplexes (cationic liposome-DNA complexes), polyplexes (cationic polymer-DNA complexes) and protein-DNA complexes. In addition to nucleic acids, the vector may also comprise one or more regulatory regions and/or selectable markers useful for selecting, measuring, and monitoring the outcomes of nucleic acid delivery (e.g., delivery to a certain tissue, or duration of expression).

Vectors may be introduced into a desired host cell by methods known in the art, such as injection, transfection, electroporation, microinjection, transduction, cell fusion, lipofection, calcium phosphate precipitation (Graham, F.L. et al., *Virology*, 52: 456 (1973), Chen and Okayama, *Mol. Cell. Biol.* 7: 2745-2752 (1987)), liposome-mediated textured salt method (Wong, T.K. et al., *Gene*, 10:87 (1980), Nicolau and Sene, *Biochim. Biophys. Acta*, 721: 185-190 (1982), Nicolau et al., *Methods Enzymol.*, 149: 157-176 (1987)), DEAE-dextran treatment (Gopal, *Mol. Cell Biol.*, 5: 1188-1190 (1985)), gene bombardment (Yang et al., *Proc. Natl. Acad. Sci.*, 87: 9568-9572 (1990)) using gene species or DNA vector transporters (see Wu et al., *J. Biol. Chem.* 267: 963 (1992), Wu et al., *J. Biol. Chem.* 263: 14621 (1988), Hartmut et al., Canadian Patent Application No. 2,012,311).

In some embodiments, viral vectors have been used in a wide range of gene transfer applications in cells as well as in live animal subjects. Viral vectors that may be used include, but are not limited to, adenovirus, retrovirus, vaccinia virus, poxvirus, adeno-associated virus, herpes simplex virus, lentivirus, baculovirus, sendai virus, measles virus, simian virus 40, and Epstein-

Barr virus vectors. Non-viral vectors include plasmids, lipoplexes (cationic liposome-DNA complexes), polyplexes (cationic polymer-DNA complexes) and protein-DNA complexes. In addition to nucleic acids, the vector may comprise one or more regulatory regions and/or selection markers useful for screening, measuring, and monitoring nucleic acid delivery outcomes (e.g., delivery to tissue, or persistence of expression).

In some embodiments, polynucleotides may be introduced in vivo by lipofection. The use of liposomes for encapsulating and transfecting nucleic acids in vitro has increased. In some embodiments, synthetic cationic lipids, designed to limit the difficulties and risks encountered by liposome-mediated transfection may be used to prepare liposomes for in vivo transfection of genes (Feigner et al., Proc. Natl. Acad. Sci. USA. 84:7413 (1987), Mackey et al., Proc. Natl. Acad. Sci. USA 85:8027 (1988), Ulmer et al., Science 259:1745 (1993)). In some embodiments, the use of cationic lipids may promote encapsulation of negatively charged nucleic acids and may also promote fusion with negatively charged cell membranes (Feigner et al., Science 337:387 (1989)). Particularly useful lipid compounds and compositions for the delivery of nucleic acids are described in WO95/18863, WO96/17823, and U.S. 5,459,127, which are herein incorporated by reference in their entirety. In some embodiments, direct transfection to specific cell types will clearly be particularly desirable for tissues with cellular heterogeneity such as the pancreas, liver, kidney and brain. In some embodiments, lipids may chemically bind to other molecules for targeting (Mackey et al., 1988). In some embodiments, targeted peptides such as hormones or neurotransmitters, and proteins such as antibodies, or non-peptidic molecules may be chemically bound to liposomes.

Standard techniques may be used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures may be generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)), which is incorporated herein by reference for any purpose.

Engineered Immune Cells

As used herein, “immune cells” refer to cells of the immune system which can be categorized as lymphocytes (e.g., T cells, B cells, and NK cells), neutrophils, and monocytes/macrophages. In some embodiments, the immune cell is a T cell. In some
5 embodiments, the immune cell is an NK cell. In some embodiments, the immune cell is a Macrophage. In some embodiments, an immune cell is an engineered immune cell, meaning the immune cell has been genetically modified to express a non-naturally occurring protein (e.g., a chimeric antigen receptor) or to include an exogenous nucleic acid.

The immune cells (e.g., T cells) may be modified in one or more than one manner.

- 10 Immune cells (e.g., T cells) may express at least one non-natural molecule that is a receptor for an antigen that is present on the surface of one or more types of cells. In some embodiments, immune cells, include immune cells (e.g., T cells) that are not found in nature because they are engineered to comprise or express at least one synthetic molecule that is not found in nature. In specific embodiments, the immune cells (e.g., T cells) are engineered to express at least one
15 chimeric antigen receptor (CAR), including a CAR that targets a specific tumor antigen, such as glypican-3 (GPC3), malignancy variant receptor (MVR), HLA-DR (Human Leukocyte Antigen-D Related), or CD19. In specific embodiments, the immune cell can be a T cell, e.g., a CD4⁺ T cell, a CD8⁺ T cell, a Treg cell, a Th1 T cell, a Th2 T cell, a Th17 T cell, an unspecific T cell, or a population of T cells that comprises a combination of any of the foregoing. Immune cells (e.g.,
20 T cells) engineered with chimeric antigen receptors (CAR T cells) have great therapeutic potential for treating cancers. With a CAR, a receptor can be programmed to recognize an antigen, which when bound, activate immune cells to kill the cell expressing that antigen. Therefore, immune cells expressing CAR(s) for an antigen expressed on a tumor cell can target and kill the tumor cell. For example, recent clinical trials of a CD19-targeted CAR-transduced T
25 cell (CD19-CAR T cell) against hematologic malignancies showed a strong effect of CAR T technology. (Kochenderfer, J. N. et al. (2010) *Blood* 116: 4099-4102; Porter, D. L., et al. (2011) *N. Engl. J. Med.* 365: 725-733; Grupp, S. A. et al. (2013) *N. Engl. J. Med.* 368: 1509-1518; Kochenderfer, J. N. et al. (2015) *J. Clin. Oncol.* 33: 540-549; Brown, C. E. et al. (2016) *N. Engl. J. Med.* 375: 2561-2569). The clinical success of CAR T is attributed, at least in part, to the
30 fusion structure of the CAR, which is made by artificially combining a high-affinity antigen-

binding domain with multiple signaling domains (Maus, M. V. et al. (2014) *Blood* 123: 2625-2635; van der Stegen, S. J. et al. (2015) *Nat. Rev. Drug Discov.* 14: 499-509).

CARs comprise an extracellular antigen-binding domain, a transmembrane domain, and an intracellular signaling domain. In some embodiments, the extracellular antigen-binding domain comprises a single chain variable fragment (scFv) that is capable of recognizing a tumor-associated antigen, the transmembrane domain employs the transmembrane domain from molecules such as CD8 and CD28, and the intracellular signaling domain employs an immunoreceptor tyrosine-based activation motif (e.g., CD3 ζ) and the intracellular signaling domain of co-stimulatory signaling molecule (e.g., CD28 and CD137 (4-1BB)).

As used herein, “single chain variable fragment, scFv” refers to a fragment of antibody defined as a recombinant protein comprising a heavy chain variable domain (VH) and a light chain variable domain (VL) connected by a linker, which brings the two domains together into association such that an antigen-binding site is formed.

In some embodiments, the transmembrane domain is a transmembrane domain from a protein selected from 4-1BB/CD137, an activating NK cell receptor, an immunoglobulin protein, B7-H3, BAFTR, BLAME (SLAMF8), BTLA, CD100 (SEMA4D), CD103, CD160 (BY55), CD18, CD19, CD19a, CD2, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3 delta, CD3 epsilon, CD3 gamma, CD3 zeta, CD30, CD4, CD40, CD49a, CD49D, CD49f, CD69, CD7, CD84, CD8, CD8 alpha, CD8 beta, CD96 (Tactile), CD11a, CD11b, CD11c, CD11d, CDS, CEACAM1, CRT AM, cytokine receptor, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, Ig alpha (CD79a), IL-2R beta, IL-2R gamma, IL-7R alpha, inducible T cell co-stimulator (ICOS), an integrin, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, LFA-1, a ligand that specifically binds with CD83, LIGHT, LTBR, Ly9 (CD229), lymphocyte function-associated antigen-1 (LFA-1), an MHC class 1 molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLR1), OX-40, PAG/Cbp, programmed death-1 (PD-1), PSGL1, SELPLG (CD162), a Signaling Lymphocytic Activation Molecule (a SLAM protein), SLAM (SLAMF1), SLAMF4 (CD244), SLAMF6 (NTB-A), SLAMF7, SLP-76, a TNF receptor protein, TNFR2, TNFSF14, a Toll ligand receptor, TRANCE/RANKL, VLA1, and VLA-6.

In some embodiments, the intracellular signaling domain comprises an intracellular signaling domain from a protein selected from 4-1BB/CD137, an activating NK cell receptor, an

immunoglobulin protein, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD100 (SEMA4D), CD103, CD160 (BY55), CD18, CD19, CD19a, CD2, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3delta, CD3epsilon, CD3gamma, CD3zeta, CD30, CD4, CD40, CD49a, CD49D, CD49f, CD69, CD7, CD84, CD8, CD8alpha, CD8beta, CD96 (Tactile), CD11a, CD11b, CD11c, CD11d, CDS, CEACAM1, CRTAM, a cytokine receptor, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, Ig alpha (CD79a), IL-2Rbeta, IL-2R gamma, IL-7R alpha, inducible T cell co-stimulator (ICOS), an integrin, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, ligand that specifically binds with CD83, LIGHT, LTBR, Ly9 (CD229), Lyl08, lymphocyte function-associated antigen-1 (LFA-1), a MHC class 1 molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX-40, PAG/Cbp, programmed death-1 (PD-1), PSGL1, SELPLG (CD162), a Signaling Lymphocytic Activation Molecules (SLAM protein), SLAM (SLAMF1), SLAMF4 (CD244), SLAMF6 (NTB-A), SLAMF7, SLP-76, a TNF receptor protein, TNFR2, TNFSF14, a Toll ligand receptor, TRANCE/RANKL, VLA1, and VLA-6, or any combination thereof.

In some embodiments, the chimeric antigen receptor further comprises an additional antigen-binding domain. In some embodiments, the chimeric antigen receptor is a bi-specific CAR (i.e., targeting two antigen binding domains). In some embodiments, the chimeric antigen receptor is multivalent (i.e., targeting multiple antigen binding domains). In some embodiments, the additional antigen-binding domain is a scFv.

The immune cells, (e.g., T cells) can come from any source known in the art. For example, immune (e.g., T) cells can be differentiated in vitro from a hematopoietic stem cell population, or immune (e.g., T) cells can be obtained from a subject. T cells can be obtained from peripheral blood mononuclear cells (PBMCs), bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, or tumors. In addition, immune (e.g., T) cells can be derived from one or more immune cell lines available in the art. In some embodiments, T cells can be obtained from blood collected from a subject using any number of techniques known to the skilled artisan, such as FICOLL™ separation and/or apheresis. Additional methods of isolating T cells for a T cell therapy are disclosed in U.S. Patent Publication No. 2013/0287748. Other non-limiting examples can be found in International Application No. PCT/US2015/014520 (published as WO2015/120096).

and in International Application No. PCT/US2016/057983 (published as WO2017/070395), each of which is herein incorporated by reference in its entirety.

In some embodiments, the immune cells are autologous T cells. In some embodiments, the immune cells are obtained from a subject that is not the patient. In some embodiments, T cells for using in a therapeutic method are syngeneic (the donor and the recipients are different but are identical twins). In some embodiments, T cells for using in a therapeutic method are allogenic (from the same species but different donor) as the recipient subject. In some embodiments, the T cells are autologous stem cells (for autologous stem cell therapy or ASCT). In some embodiments, the immune cells are non-autologous T-cells. In some embodiments, the immune cells are obtained from a healthy donor. In some embodiments, the immune cells are obtained from a patient afflicted with a cancer or a tumor.

T cells can be engineered to express, for example, chimeric antigen receptors (CARs). In some embodiments, CAR-T cells can be engineered to express an extracellular single chain variable fragment (scFv). In some embodiments, the CAR is engineered such that the costimulatory domain is expressed as a separate polypeptide chain. Exemplary CAR-T cell therapies and constructs are described in U.S. Patent Publication Nos. 2013/0287748, 2014/0227237, 2014/0099309, and 2014/0050708, which are herein incorporated by reference in their entirety.

CAR construct

The present disclosure provides, at least in part, chimeric antigen receptor (CAR) polypeptides. As used herein, “chimeric antigen receptor (CAR)” refers to a receptor not present in nature and is capable of providing an immune effector cell with a specificity to a particular antigen. In some embodiments, a CAR refers to a receptor used for delivering the specificity of a monoclonal antibody agent to a T cell. Generally, a CAR comprises an extracellular binding domain (ectodomain), a transmembrane domain, and an intracellular signaling domain (endodomain).

In some embodiments, in order to achieve robust immune (e.g., CAR-T) cell expansion, function, persistence and antitumor activity, costimulatory signals can be provided by incorporating intracellular signaling domains from immune (e.g., T cell) cell costimulatory molecules into the CAR construct. In some embodiments, the selection and positioning of co-

stimulatory domains within a CAR construct can influence immune (e.g., CAR-T) cell function and fate, and have differential impacts on immune (e.g., CAR-T) cell kinetics, cytotoxic function and potentially safety profile. Non-limiting examples of costimulatory molecules include CD28, ICOS, CD27, 4-1BB/CD137, OX40, and CD40L.

5 As used herein, 4-1BB/CD137 is an activation-induced T cell costimulatory molecule, expressed on a subset of resting CD8⁺ T cells and is upregulated on both CD4⁺ and CD8⁺ T cells following activation. In some embodiments, T cells expressing CARs that incorporate 4-1BB/CD137 domains can express granzyme B, IFN- γ , TNF- α , GM-CSF and the anti-apoptotic protein Bcl-XL (Zhong et al., Mol. Ther. 2010; 18: 413-420), wherein CARs incorporating a 4-1BB/CD137 costimulatory domain can show longer CAR-T cell persistence (Zhao et al., Cancer Cell 2015; 28: 415-428). In some embodiments, intracellular domain of the chimeric receptors described herein include a 4-1BB signaling domain followed by a five amino acid sequence, which may be further combined with any other desired extracellular, transmembrane, and/or intracellular domain(s) useful in the context of the chimeric receptor.

15 In some embodiments, a CAR includes: (a) extracellular domain comprising an antigen-binding domain; (b) a transmembrane domain; and (c) an intracellular domain comprising a costimulatory endodomain, wherein the costimulatory endodomain comprises an intracellular signaling domain from 4-1BB/CD137 and five additional amino acids. As contemplated herein, the CAR construct can include an extracellular domain directed to any desired antigen-binding domain. In some embodiments, the costimulatory endodomain includes an intracellular signaling domain from 4-1BB/CD137 and five additional amino acids, wherein the five additional amino acids are encoded by SEQ ID NO: 1. In some embodiments, the costimulatory endodomain comprises SEQ ID NO: 2. In some embodiments, the co-stimulatory endodomain includes a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 25 98%, 99%, or 100% identical to SEQ ID NO: 2 or 4.

SEQ ID NO: 1 – five additional amino acids (DNA sequence)

CGTTTCTCTGTTGTT

30 SEQ ID NO: 2 – 4-1BB costimulatory domain with five additional amino acids (DNA sequence)

CGTTTCTCTGTTGTTAAACGGGGCAGAAAGAACTCCTGTATATATTCAAACA
 ACCATTTATGAGACCAGTACAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGAT
 TTCCAGAAGAAGAAGAAGGAGGATGTGAACTG

5 SEQ ID NO: 3 – five additional amino acids (Amino acid sequence)
 RFSVV

SEQ ID NO: 4 – 4-1BB costimulatory domain with five additional amino acids (Amino
 acid sequence)
 10 RFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEEGGCEL

In some embodiments, an extracellular binding domain of a CAR comprises an antigen-
 binding domain. In some embodiments, the antigen-binding domain specifically binds an
 antigen associated with a disease. In some embodiments, the antigen-binding domain specifically
 15 binds a tumor antigen. In some embodiments, the antigen-binding domain specifically binds to
 any number of targets, including surface antigens, cytoplasmic, or nuclear antigens. For
 example, the antigen binding domain can bind to BCMA, CD2, CD3, CD4, CD8, CD10, CD19,
 CD20, CD22, CD23, CD33, CD38, CD44, CD52, CD70, CD99, CD138, CD123, CD274, TIM-
 3, members of the epidermal growth factor receptor family (erb1, erb2, erb3, erb4 and mutants
 20 thereof), members of the ephrin receptor family (EphA1-10, EphB1-6), prostate specific antigens
 (e.g., prostate stem cell antigen PSCA, prostate specific membrane antigen PSMA), embryonic
 antigens (e.g. carcinoembryonic antigen CEA, fetal acetylcholine receptor), members of the
 vascular endothelia growth factor family (VEGFR 1-3), epithelia cell adhesion molecule
 EpCAM, alphafetoprotein AFP, members of the mucin protein family (e.g. MUC1, MUC16),
 25 follicle stimulating hormone receptor (FSHR), the human high molecular weight-melanoma-
 associated antigen (HMW-MAA), folate binding protein FBP, a-Folate receptor, ligands of the
 NKG2D receptor, members of the epithelia glycoprotein family (e.g. EGP-2, EGP-4),
 diasialogangliosides (e.g. GD2, GD3), members of the carbonic anhydrase family (e.g. CAIX),
 and members of the carbohydrate antigen family (e.g. Ley), including mutants of the named
 30 proteins and protein families. In some embodiments, the antigen-binding domain can bind to
 antibodies or fragments thereof that binds to cytoplasmic or nuclear antigens like the La/SSB

- antigen, members of the Rho family of GTPases, members of the high mobility group proteins and others. Likewise, the antigen binding domain can bind to the alpha and beta or the gamma and delta chains of a T cell receptor (TCR) or fragments thereof. In some embodiments, the antigen binding domain can bind to peptides presented by human leukocyte antigen class (HLA)
- 5 I and II protein complexes. Examples are, but are not limited to, TCRs specific for peptides derived from proteins like the EGFR family, survivin, srylike high motility group box (SOX) protein family, melanoma-associated antigens (e.g. autoimmunogenic cancer/testis antigen NY-ESO-1, members of the melanoma antigen family A MAGEA, the preferentially expressed antigen in melanoma PRAME, gp100, MART-1), and leukemia-associated antigens (e.g.,
- 10 AML1-ETO, DEK-CAN, PML-RARalpha, Flt3-ITD, NPM1, AurA, Bcl-2, B1-1, BMI1, BRAP, CML28, CML66, Cyclin A, Cyclin B1, Cyclin E, CYP1B1, ETO/MTG8, G250/CAIX, HOXA9, hTERT, Mcl-1, MAGE, Mesothelin, mHAg, Myeloperoxidase, MPP11, MUC1, NuSAP1, OFA/iLRP, PASD1, PRAME, Proteinase 3, RAGE-1, RGS5, RHAMM, SSX2IP, Syrvivin, Wilms tumor gene 1 (WT1)). The antigen binding domain can bind to cytokine receptors (e.g.,
- 15 IL-13 receptor, IL-22 receptor), NKG2D receptors (e.g., ULBP1, ULBP2), EGFR family members, or auto-reactive TCRs. In some embodiments, the antigen-binding domain specifically binds to tumor antigens. Examples are, but are not limited to, glypican-3 (GPC), malignancy variant receptor (MVR), HLA-DR (Human Leukocyte Antigen-D Related), AFP, CEA, CA-125, MUC-1, ETA, Tyrosinase, MAGE, Immature laminin receptor, TAG-72, HPV
- 20 E6, HPV E7, BING-4, Calcium-activated chloride channel 2, Cyclin-B1, 9D7, Ep-CAM, EphA3, Her2/neu, Telomerase, Mesothelin, SAP-1, Survivin, NY-ESO-1/LAGE-1, PRAME, SSX-2, BRCA1/2, CDK4, CML66, or CD19. In some embodiments, an antigen-binding domain is or comprises an antibody agent. In some embodiments, an antigen-binding domain is or comprises an antibody agent that specifically binds to GPC3, MVR, HLA-DR, or CD19.
- 25 In some embodiments, the transmembrane domain is a transmembrane domain from a protein selected from 4-1BB/CD137, an activating NK cell receptor, an immunoglobulin protein, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD100 (SEMA4D), CD103, CD160 (BY55), CD18, CD19, CD19a, CD2, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3delta, CD3 epsilon, CD3 gamma, CD3 zeta, CD30, CD4, CD40, CD49a, CD49D, CD49f, CD69, CD7,
- 30 CD84, CD8, CD8alpha, CD8beta, CD96 (Tactile), CD11a, CD11b, CD11c, CD11d, CDS, CEACAM1, CRT AM, cytokine receptor, DAP-10, DNAM1 (CD226), Fc gamma receptor,

GADS, GTR, HVEM (LIGHTR), IA4, ICAM-1, Ig alpha (CD79a), IL-2R beta, IL-2R gamma, IL-7R alpha, inducible T cell co-stimulator (ICOS), an integrin, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, LFA-1, a ligand that specifically binds with CD83, LIGHT, LTBR, Ly9 (CD229), lymphocyte function-associated antigen-1 (LFA-1), an MHC class 1 molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX-40, PAG/Cbp, programmed death-1 (PD-1), PSGL1, SELPLG (CD162), a Signaling Lymphocytic Activation Molecule (a SLAM protein), SLAM (SLAMF1), SLAMF4 (CD244), SLAMF6 (NTB-A), SLAMF7, SLP-76, a TNF receptor protein, TNFR2, TNFSF14, a Toll ligand receptor, TRANCE/RANKL, VLA1, and VLA-6. In some embodiments, the transmembrane domain is a transmembrane domain from CD8 α . In some embodiments, the transmembrane domain comprises SEQ ID NO: 5. In some embodiments, the transmembrane domain includes a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 5.

SEQ ID NO: 5 – CD8/Hinge/Transmembrane

ACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGCTAGCC
AGCCCCTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCGGCGGGGGGCGCAGTGCAC
ACGAGGGGGCTGGACTTCGCCTGTGATATCTACATCTGGGCGCCCTTGGCCGGGACT
TGTGGGGTCCTTCTCCTGTCACTGGTTATCACCTTTACTGC

20

In some embodiments, the intracellular domain further comprises an intracellular domain from CD3 ζ . In some embodiments, the intracellular domain comprises SEQ ID NO: 6. In some embodiments, the intracellular domain includes a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 6.

25

SEQ ID NO: 6 – CD3 ζ

AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCGCGTACAAGCAGGGCCAG
AACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGA
CAAGAGACGTGGCCGGGACCCTGAGATGGGGGAAAGCCGAGAAGGAAGAACCCT
CAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGA
GATTGGGATGAAAGGCGAGCGCCGAGGGGCAAGGGGCACGATGGCCTTTACCAG

30

GGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCCC
CCTCGCTAA

In some embodiments, the CAR further comprises a T2A self-cleaving peptide. In some
5 embodiments, the CAR further comprises a signal peptide or leader sequence. In some
embodiments, the CAR further comprises a CD8 α leader sequence. In some embodiments, the
CAR further comprises a flag-tag sequence. In some embodiments, the CAR further comprises a
hinge region. In some embodiments, the hinge region is a CD8 α hinge. In some embodiments,
the CAR further comprises SEQ ID NO: 7. In some embodiments, the CAR further comprises
10 SEQ ID NO: 8. In some embodiments, the extracellular domain includes a sequence that is at
least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to
SEQ ID NO: 7 or 8.

SEQ ID NO: 7 – CD8 α leader sequence

15 GGATCCATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCT
CCACGCCGCCAGGCCG

SEQ ID NO: 8 – Flag-tag sequence

GACTACAAGGACGACGATGACAAG

20

GPC3 CAR

Glypican-3 (GPC3) is a cell surface protein encoded by the GPC3 gene in humans and an
oncofetal antigen re-expressed in a high frequency of neoplastic hepatocytes (Vidali, et al., 2008,
J hepatol 48:399-406). GPC3 is highly expressed in fetal liver and not expressed in normal adult
25 liver tissue, but its expression is reactivated in hepatocellular carcinoma, and has close
association with the development of liver cancer, where the detection rate of GPC3 expression is
relatively high during early stage of liver cancer and increases along with the development of
liver cancer. Further, GPC3 is also expressed in tumors such as melanoma, ovarian clear cell
carcinoma, yolk sac tumor, neuroblastoma and other tumors. Considering its specifically high
30 expression in hepatocellular carcinoma, melanoma and other tumors, GPC3 has emerged as a

useful immunohistochemical diagnostic test (Anatelli, et al., 2008, Am J Clin Path 130:219-223) and potential biomarker (Aburatani, 2005, J Gastroenterol 40. S16:1-6).

GPC3 is a member of the proteoglycan family that functions as extracellular matrix in cell adhesion in organogenesis or as a receptor of a cell growth factor. The protein core of GPC3 comprises two subunits, and N-terminal subunit and a C-terminal subunit. A glycosyl phosphatidylinositol (GPI) anchor is added to serine at position 560 located on the carboxyl (C)-terminal side of GPC3. The GPI anchor plays a role in localizing GPC3 on cell surface through covalent binding to cell membrane lipid. Also, serine at position 495 and serine at position 509 of GPC3 are modified with a heparan sulfate chain (HS chain) wherein the HS chain is known to regulate a plurality of growth signal transduction pathways such as Wnt signal, FGF signal, and BMP signal transduction pathways. A growth signal transduction pathway involved is known to differ among the types of cancers. For example, in hepatocellular carcinoma (HCC), cells grow by the stimulation of the Wnt signal pathway.

The present disclosure provides, at least in part, GPC3 CAR polypeptides. In some embodiments, an extracellular binding domain of a GPC3 CAR comprises an antigen binding domain. In some embodiments, an antigen binding domain is or comprises an antibody agent. In some embodiments, an antigen binding domain is or comprises an antibody agent that specifically binds to GPC3.

In some embodiments, the chimeric antigen receptor (CAR) polypeptide includes: i) an extracellular antigen-binding domain comprising a light chain variable domain comprising a light chain CDR1 comprising SEQ ID NO: 9; a light chain CDR2 comprising SEQ ID NO: 10; and a light chain CDR3 comprising SEQ ID NO: 11; and a heavy chain variable domain comprising a heavy chain CDR1 comprising SEQ ID NO: 12; a heavy chain CDR2 comprising SEQ ID NO: 13; and a heavy chain CDR3 comprising SEQ ID NO: 14; ii) a transmembrane domain; and iii) an intracellular signaling domain, which leads to T cell activation when an antigen binds to the antibody agent.

	SEQUENCE	SEQ ID NO:
Light chain CDR1	RSSQSLVHSNGNTYLH	9
Light chain CDR2	KVSNRFS	10
Light chain CDR3	SQNTHPPT	11

Heavy chain CDR1	DYEMH	12
Heavy chain CDR2	ALDPKTGDTAYSQKFKG	13
Heavy chain CDR3	FYSYTY	14

In some embodiments, the CAR polypeptide includes: i) an extracellular antigen-binding domain comprising a light chain variable domain comprising a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 15 and a heavy chain variable domain comprising a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 16; ii) a transmembrane domain; and iii) an intracellular signaling domain, which leads to T cell activation when an antigen binds to the antibody agent. In some embodiments, the CAR polypeptide includes: i) an extracellular antigen-binding domain comprising a light chain variable domain comprising a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 17 and a heavy chain variable domain comprising a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 18; ii) a transmembrane domain; and iii) an intracellular signaling domain, which leads to T cell activation when an antigen binds to the antibody agent.

In some embodiments, the CAR polypeptide includes: i) an extracellular antigen-binding domain comprising a light chain variable domain comprising SEQ ID NO: 15 and a heavy chain variable domain comprising SEQ ID NO: 16; ii) a transmembrane domain; and iii) an intracellular signaling domain, which leads to T cell activation when an antigen binds to the antibody agent.

SEQ ID NO: 15 – human GC33 light chain variable region (Amino acid sequence)
DVVMTQSPLSLPVTLGQPASISCRSSQSLVHSNGNTYLHWYQQRPGQSPRLLIYK
VSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCSQNTHPPTFGSGTKLEIK

SEQ ID NO: 16 – human GC33 heavy chain variable region (Amino acid sequence)

QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYEMHWVRQAPGQGLEWIGALD
PKTGD TAYSQKFKGRATLTADKSTSTAYMELSSLRSEDTAVYYCTRFYSYTYWGQGT
LTVSS

5 SEQ ID NO: 17 – human GC33 light chain variable region (DNA sequence)

GACGTCGTTATGACACAGAGTCCCCTCTCCTTGCCGGTGACCCTGGGTCAGCC
TGCCTCCATCTCTTGCAGATCCTCCCAGTCTCTGGTAACTCCAACGGCAACACATA
CTTGCACTGGTACCAACAAAGACCTGGTCAGTCACCGCGACTTCTCATATATAAAGT
TTCCAATAGGTTCACTGGAGTGCCAGACAGGTTCACTGGTTCAGGATCAGGCACTGA
10 TTTCACGCTTAAATCAGTCGGGTTGAGGCGGAGGACGTAGGAGTTTACTATTGCAG
CCAGAATACGCACGTGCCGCCTACTTTTGGCTCTGGAACCAAGTTGGAAATAAAG

SEQ ID NO: 18 – human GC33 heavy chain variable region (DNA sequence)

CAAGTGCAACTCGTACAATCAGGTGCTGAAGTCAAAAAGCCGGGAGCCTCTG
15 TTAAAGTGTCTGTAAAGCCAGCGGCTACACCTTTACCGATTATGAGATGCACTGGG
TTCGGCAGGCTCCGGGCCAAGGTCTGGAGTGGATCGGGGCTCTTGACCCAAAGACG
GGCGACACGGCTTATTCACAAAAATTCAAAGGTAGGGCTACTCTGACTGCCGATAA
GTCCACCAGCACCGCGTATATGGAGCTCTCTAGCTTGCGAAGCGAGGACACGGCGG
TGTA CTATTGCACACGCTTCTATAGTTACACATATTGGGGTCAAGGCACGCTTGTGA
20 CCGTGTCTAGC

MVR CAR

Human Leukocyte Antigen-DR (HLA-DR) is a classic major histocompatibility complex II molecule (Shackelford, D. A. et al., 1982 *Immunol. Rev.* 66: 133-187). HLA-DR and its
25 ligand, a peptide of 9 amino acids in length or longer, constitutes a ligand for the T cell receptor (TCR). HLA-DR molecules are upregulated in response to signaling. In the instance of an infection, the peptide (such as the staphylococcal enterotoxin I peptide) is bound into a DR molecule and presented to T-cell receptors found on T-helper cells. These cells then bind to antigens on the surface of B-cells stimulating B-cell proliferation.

30 The primary function of HLA-DR is to present peptide antigens, potentially foreign in origin, to the immune system for the purpose of eliciting or suppressing T-(helper)-cell responses

that eventually lead to the production of antibodies against the same peptide antigen. HLA-DR is an $\alpha\beta$ heterodimer, cell surface receptor, each subunit of which contains two extracellular domains, a membrane-spanning domain and a cytoplasmic tail. Both α and β chains are anchored in the membrane. The N-terminal domain of the mature protein forms an alpha-helix that constitutes the exposed part of the binding groove, the C-terminal cytoplasmic region interact with the other chain forming a beta-sheet under the binding groove spanning to the cell membrane. The majority of the peptide contact positions are in the first 80 residues of each chain.

HLA-DR has restricted expression on antigen presenting cells, e.g., dendritic cells, macrophages, monocytes, and B cells. Increased abundance of HLA-DR 'antigen' on the cell surface is often in response to stimulation, and, therefore, HLA-DR is also a marker for immune stimulation. Due to the high expression level of HLA-DR in B cell malignancies and the limited expression spectrum on normal cells, antibodies against HLA-DR have been developed and tested for B cell malignancies in preclinical and clinical studies. (Nagy, Z.A., et al. (2002) *Nat. Med.* 8: 801-807; DeNardo, G.L., et al. (2005) *Clin. Cancer Res.* 11: 7075s-7079s; Ivanov, A., et al. (2009) *J. Clin. Invest.* 119: 2143-2159; Lin, T.S., et al. (2009) *Leuk. Lymphoma* 50: 1958-1963). In a phase I/II trial, although the toxicity was not serious, further study was discontinued due to limited efficacy (Lin, T.S., et al. (2009) *Leuk. Lymphoma* 50: 1958-1963).

As used herein, the malignancy variant receptor (MVR) antibody agent recognizes a polymorphic region of HLA-DR (described in U.S. Patent Application Publication No. US 2016-0257762, which is herein incorporated by reference in its entirety). The present disclosure provides, at least in part, MVR CAR polypeptides. A schematic of exemplary MVR CAR constructs in accordance with the present disclosure is shown in **FIG. 1**. In some embodiments, an extracellular domain of a CAR comprises an antigen-binding domain. In some embodiments, an antigen-binding domain is or comprises an antibody agent. In some embodiments, an antigen-binding domain is or comprises an antibody agent that specifically binds to HLA-DR.

In some embodiments, the CAR polypeptide includes a single-chain variable fragment (scFv) form of an anti-MVR antibody agent. In some embodiments, the CAR polypeptide comprises SEQ ID NO: 19. In some embodiments, the CAR includes a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 19.

In some embodiments, the CAR polypeptide includes a single-chain variable fragment (scFv) form of an anti-MVR antibody agent. In some embodiments, the CAR polypeptide comprises SEQ ID NO: 20. In some embodiments, the CAR includes a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 20.

SEQ ID NO: 19 –MVRL2H2 (Amino acid sequence)

DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKPGKAPKLLISGATSLE
TGVPSRFSGSGSGKDYTLTISSLQPEDFATYYCQYWSTPFTFGQGTKVEIKGGGSGGGG
10 GSGGGGSQVQLQESGPGLVKPSSETLSLTCTVSGFSLSRYSVHWIRQPPGKGLEWLGMIW
GGGSTDYNSALKSRLTISKDNSKNQVSLKLSSVTAADTAVYYCARNEGDTTAGTWFAFAY
WGQGTLLVTVSS

SEQ ID NO: 20 – MVRL2H2 (DNA sequence)

15 GATATTCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCCTCTGTGGGCGATAG
GGTCACCATCACCTGCAAGGCCAGTGACCACATCAACAACCTGGCTGGCCTGGTATC
AACAGAAACCAGGAAAAGCTCCGAACTACTGATCAGCGGCGCCACCTCTCTGGAA
ACCGGAGTCCCTTCTCGCTTCTCTGGTTCCGGATCTGGGAAGGATTACACTCTGACC
ATCAGCAGTCTGCAGCCGGAAGACTTCGCAACTTATTACTGTCAGCAGTACTGGTCC
20 ACCCCCTTCACCTTCGGACAGGGTACCAAGGTGGAGATCAAAGGCGGAGGCGGATC
TGGCGGCGGAGGAAGTGGCGGAGGGGGATCTCAGGTGCAGCTGCAGGAGTCGGGC
CCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTTTC
TCCCTGAGTCGGTACTCTGTGCATTGGATCCGGCAGCCCCCAGGGAAGGGACTGGA
GTGGCTGGGGATGATCTGGGGAGGCGGCAGCACCGACTACAACAGCGCCCTGAAGT
25 CCCGACTGACCATATCAAAGGACAACCTCCAAGAACCAGGTGTCCTTGAAGCTGAGC
TCTGTGACCGCTGCGGACACGGCCGTGTATTACTGTGCGAGAAATGAGGGCGATAC
CACCGCCGGCACTTGGTTTGCCTATTGGGGCCAGGGAACCCTGGTCACCGTCTCCTC
A

CD19 CAR

CD19 is a biomarker for normal and neoplastic B cells, as well as follicular dendritic cells. CD19 is critically involved in establishing intrinsic B cell signaling thresholds through modulating both B cell receptor-dependent and independent signaling. Furthermore, CD19 functions as the dominant signaling component of a multimolecular complex on the surface of mature B cells, alongside complement receptor CD21, and the tetraspanin membrane protein CD81 (TAPA-1), as well as CD225, wherein CD19 also plays a critical role in maintaining the balance between humoral, antigen-induced response and tolerance induction.

The present disclosure provides, at least in part, CD19 CAR polypeptides. In some embodiments, an extracellular domain of a CAR comprises an antigen-binding domain. In some embodiments, an antigen-binding domain is or comprises an antibody agent. In some embodiments, an antigen-binding domain is or comprises an antibody agent that specifically binds to CD19.

In some embodiments, the CAR polypeptide includes a single-chain variable fragment (scFv) form of an anti-CD19 antibody agent. In some embodiments, the CAR polypeptide comprises SEQ ID NO: 21. In some embodiments, the CAR includes a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 21.

In some embodiments, the CAR polypeptide includes a single-chain variable fragment (scFv) form of an anti-CD19 antibody agent. In some embodiments, the CAR polypeptide comprises SEQ ID NO: 22. In some embodiments, the CAR includes a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 22.

SEQ ID NO: 21 – CD19 (Amino acid sequence)

DIQMTQTTSSLSASLGDRVITISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLH
SGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGGGGSGGG
GSGGGGSEVKLQESGPGVLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIW
GSETTYYN SALKSRLTIKD NSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYW
GQGTSVTVSS

SEQ ID NO: 22 – CD19 (DNA sequence)

GACATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACA
GAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGTAAATATTTAAATTGGTATC
AGCAGAAACCAGATGGAAGTGTAACTCCTGATCTACCATACATCAAGATTACACT
5 CAGGAGTCCCATCAAGGTTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCA
TTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAGGGTAATACGC
TTCCGTACACGTTCCGAGGGGGGACCAAGCTGGAGATCACAGGTGGCGGTGGCTCG
GGCGGTGGTGGGTCTGGGTGGCGGCGGATCTGAGGTGAACTGCAGGAGTCAGGACC
TGGCCTGGTGGCGCCCTCACAGAGCCTGTCCGTACATGCACTGTCTCAGGGGTCTC
10 ATTACCCGACTATGGTGTAAGCTGGATTTCGCCAGCCTCCACGAAAGGGTCTGGAGTG
GCTGGGAGTAATATGGGGTAGTGAAACCACATACTATAATTCAGCTCTCAAATCCAG
ACTGACCATCATCAAGGACAACCTCCAAGAGCCAAGTTTCTTAAAAATGAACAGTCT
GCAAAGTATGACACAGCCATTTACTACTGTGCCAAACATTATTACTACGGTGGTAG
CTATGCTATGGACTACTGGGGCCAAGGAACCTCAGTCACCGTCTCCTCA

15 Nucleic Acids

As used herein, “nucleic acid” is used to include any compound and/or substance that comprises polynucleotides. Exemplary nucleic acids or polynucleotides can include, but are not limited to, ribonucleic acids (RNAs) and/or deoxyribonucleic acids (DNAs).

In some embodiments, nucleic acid constructs include regions that encode a CAR,
20 wherein the CAR comprises: (a) an extracellular domain comprising an antigen-binding domain;
(b) a transmembrane domain; and (c) an intracellular domain comprising an intracellular
signaling domain from 4-1BB/CD137 and five additional amino acids. In some embodiments,
nucleic acid constructs may be inserted into an expression vector or viral vector by methods
known to the art, and nucleic acid molecules may be operably linked to an expression control
25 sequence. Non-limiting examples of expression vectors include plasmid vectors, transposon
vectors, cosmid vectors, and viral derived vectors (e.g., any adenoviral derived vectors (AV),
cytomegaloviral derived (CMV) vectors, simian viral derived (SV40) vectors, adeno-associated
virus (AAV) vectors, lentivirus vectors, and retroviral vectors). In some embodiments, the
expression vector is a viral vector. In some embodiments, the viral vector is a lentiviral vector.

In some embodiments, the expression vector further comprises a promoter operationally linked to 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 expression vector comprises SEQ ID NO: 23, 24, 25, 26, 27, and/or 28. In some embodiments, the expression vector includes a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 23, 24, 25, 26, 27, and/or 28.

SEQ ID NO: 23 – EF1 α -promoter

```
CGTGAGGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCACAGTCCC
10 CGAGAAGTTGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGC
GGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGG
GGAGAACCGTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTTCGCAACGGGTTT
GCCGCCAGAACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACG
GGTTATGGCCCTTGCCTTGAATTACTTCCACCTGGCTGCAGTACGTGATTCTTG
15 ATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCTTGCGCTTAAGGA
GCCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTG
CGAATCTGGTGGCACCTTCGCGCCTGTCTCGCTGCTTTTCGATAAGTCTCTAGCCATTT
AAAATTTTTGATGACCTGCTGCGACGCTTTTTTTTCTGGCAAGATAGTCTTGTAATGC
GGGCCAAGATCTGCACACTGGTATTTTCGGTTTTTGGGGCCGCGGGCGGCGACGGGG
20 CCCGTGCGTCCCAGCGCACATGTTCCGGCGAGGCGGGGCTGCGAGCGCGGCCACCG
AGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGCCTCGC
GCCGCCGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGGCCCCGGTCGGCACCAAGTTG
CGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAATGGAGG
ACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCAACACACAAAGGAAAAGGGCCT
25 TTCCGTCTCAGCCGTCGCTTCATGTGACTCCACTGAGTACCGGGCGCCGTCCAGGC
ACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGGAGGGGT
TTTATGCGATGGAGTTTCCCCACACTGAGTGGGTGGAGACTGAAGTTAGGCCAGCTT
GGCACTTGATGTAATTCTCCTTGGAATTTGCCCTTTTTGAGTTTGGATCTTGTTTCAT
TCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCATTCAGGTGTCGTGA
```

30

SEQ ID NO: 24 – U5 repeat

AGTAGTGTGTGCCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCTCAGAC
CCTTTTAGTCAGTGTGGAAAATCTCTAGCAG

SEQ ID NO: 25– Gag/Pol

5 CGAACAGGGACTTGAAAGCGAAAGGGAAACCAGAGGAGCTCTCTCGACGCA
GGACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGAGT
ACGCCAAAAATTTGACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGT
CAGTATTAAGCGGGGAGAATTAGATCGCGATGGGAAAAAATTCGGTTAAGGCCAG
GGGGAAAGAAAAAATATAAATTAACATATAGTATGGGCAAGCAGGGAGCTAGA
10 ACGATTTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAGGCTGTAGACAAATACT
GGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAAGCTTAGATCATTATATA
ATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAG
GAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGACCACCGCACAGC
AAGCGGCCGCTGATCTTCAGACCTGGAGGAGGAGATATGAGGGACAATTGGAGAAG
15 TGAATTATATAAATATAAAGTAGTAAAAATTGAACCATTAGGAGTAGCACCCACCA
AGGCAAAGAGAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAATAGGAGCTTT
GTTCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGACGCT
GACGGTACAGGCCAGACAATTATTGTCTGGTATAGTGCAGCAGCAGAACAATTTGCT
GAGGGCTATTGAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGCATCAAGC
20 AGCTCCAGGCAAGAATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTCCTG
GGGATTTGGGGTTGCTCTGGAAACTCATTTGCACCACTGCTGTGCCTTGGAATGCT
AGTTGGAGTAATAAATCTCTGGAACAGATTTGGAATCACACGACCTGGATGGAGTG
GGACAGAGAAATTAACAATTACACAAGCTTAATACACTCCTTAATTGAAGAATCGC
AAAACCAGCAAGAAAAGAATGAACAAGAATTATTGGAATTAGATAAATGGGCAAG
25 TTTGTGGAATTGGTTTAACATAACAAATTGGCTGTGGTATATAAAATTATTCATAAT
GATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTTTCTATAGTGAA
TAGAGTTAGGCAGGGATATTCACCATTATCGTTTCAGACCCACCTCCCAACCCGAG
GGGACCCGACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGAC
AGATCCATTTCGATTAGTGAACGGATCTCGACGGTAT

30

SEQ ID NO: 26 – cPPT

TAGACTGTAGCCCAGGAATATGGCAGCTAGATTGTACACATTTAGAAGGAAA
AGTTATCTTGGTAGCAGTTCATGTAGCCAGTGGATATATAGAAGCAGAAGTAATTCC
AGCAGAGACAGGGCAAGAAACAGCATACTTCCTCTTAAAATTAGCAGGAAGATGGC
CAGTAAAAACAGTACATACAGACAATGGCAGCAATTTACCCAGTACTACAGTTAAG
5 GCCGCCTGTTGGTGGGCGGGGATCAAGCAGGAATTTGGCATTCCCTACAATCCCCAA
AGTCAAGGAGTAATAGAATCTATGAATAAAGAATTAAGAAAATTATAGGACAGGT
AAGAGATCAGGCTGAACATCTTAAGACAGCAGTACAAATGGCAGTATTCATCCACA
ATTTTAAAAGAAAAGGGGGGATTGGGGGGTACAGTGCAGGGGAAAGAATAGTAGA
CATAATAGCAACAGACATACAACTAAAGAATTACAAAAACAAATTACAAAAATTC
10 AAAATTTTCGGGTTTATTACAGGGACAGCAGAGATCCAGTTTGGCT

SEQ ID NO: 27 – Woodchuck/PRE

ATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTTA ACTAT
GTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTATTG
15 CTTCCCGTATGGCTTTCATTTTCTCCTCCTTGTATAAATCCTGGTTGCTGTCTCTTTAT
GAGGAGTTGTGGCCCGTTGT CAGGCAACGTGGCGTGGTGTGCACTGTGTTTGCTGAC
GCAACCCCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTC
GCTTTCCCCCTCCCTATTGCCACGGCGGA ACTCATCGCCGCCTGCCTTGCCCGCTGCT
GGACAGGGGCTCGGCTGTTGGGCACTGACAATTC CGTGTTGTTCGGGGAAGCTG
20 ACGTCCTTTCCATGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCTT
TCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCC
GGCTCTGCGGCCTCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGGATCTCCCTT
TGGGCCGCCTCCCCGCCTG

25 SEQ ID NO: 28 – R/region

GGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGG
GAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCA

30 A lentiviral vector is derived from a lentivirus. Lentiviral vectors are based on the single-
stranded RNA lentiviruses, which are a subclass of retrovirus. They combine the advantages of
midrange cloning capacity with stable gene expression, wherein they are able to transduce

dividing and non-dividing cells, including neurons. Upon infection, the lentiviral genome integrates transgenes into the host genome and promotes long-term gene expression. Lentiviral vectors, such as HIV-based vectors, are exemplary of retroviral vectors used for gene delivery. Unlike other retroviruses, HIV-based vectors are known to incorporate their passenger genes into
5 non-dividing cells and, therefore, can be of use in treating persistent forms of disease.

Additional sequences can be added to such cloning and/or expression sequences to optimize their function in cloning and/or expression, to aid in isolation of the polynucleotide, or to improve the introduction of the polynucleotide into a cell. Use of cloning vectors, expression vectors, adapters, and linkers is well known in the art.

10 In some embodiments, nucleic acid molecules are inserted into a vector that is able to express a CAR of the present disclosure when introduced into an engineered immune cell. In some embodiments, an engineered immune cell is a T cell.

Production of CAR-T cells

15 Provided herein are methods for producing immune cells comprising a CAR. In some embodiments, the immune cell where a CAR is introduced therein is a human immune cell. In some embodiments, the immune cell is an autologous human immune cell. In some embodiments, the immune cell is an allogeneic human immune cell. In some embodiments, the immune cell is a CD4⁺ T cell (helper T cell, T_H cell), a CD8⁺ T cell (cytotoxic T cell, CTL), a
20 memory T cell, a regulatory T cell (Treg cell), an apoptotic T cell, but is not limited thereto. In some embodiments, the immune cell is an NK cell.

In some embodiments, viral infection of an immune cell can comprise transfecting a host cell (e.g., 293T cell, PBMC, Plat-GP cell, or PA317) with the CAR expression vector and a packaging plasmid to prepare recombinant viruses and infecting the immune cell with the
25 recombinant viruses. The viral infection method may be performed by any method known in the art. In some embodiments, transfer of the CAR expression vector to the immune cell can be confirmed by examining the expression of the CAR by flow cytometry, Northern blotting, Southern blotting, PCR (e.g., RT-PCR), ELISA, or Western blotting, or examining the expression of a marker gene inserted in the vector.

30 In some embodiments, the present disclosure provides methods of producing an engineered immune cell, comprising: introducing into an immune cell (i) a nucleic acid encoding

a CAR, wherein the CAR comprises (a) an extracellular domain comprising an antigen-binding domain; (b) a transmembrane domain; and (c) an intracellular domain comprising a costimulatory endodomain, wherein the costimulatory endodomain comprises an intracellular signaling domain from 4-1BB/CD137 and five additional amino acids, or (ii) a vector comprising the nucleic acid encoding a CAR, wherein the CAR comprises (a) an extracellular domain comprising an antigen-binding domain; (b) a transmembrane domain; and (c) an intracellular domain comprising a costimulatory endodomain, wherein the costimulatory endodomain comprises an intracellular signaling domain from 4-1BB/CD137 and five additional amino acids.

In some embodiments, to increase immunological efficacy in the cytoplasmic signal domain 4-1BB, 5 amino acids are added in the 4-1BB cytoplasmic domain used in producing a CAR as a costimulatory signal factor. In some embodiments, the completed construct comprises an antigen-binding domain, which is an scFv; an EF1- α promoter; a hinge region and a transmembrane domain of human CD8; and an intracellular signaling domain. In particular, the intracellular signaling domain comprises a stimulatory domain and a costimulatory signaling domain. In some embodiments, the transmembrane domain can include the alpha, beta or zeta chain of the T cell receptor, or one or more of CD28, CD45, CD4, CD5, CD8, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 or CD154, but it is not limited thereto. In some embodiments, the transmembrane domain includes CD8. In some embodiments, intracellular signaling domains include the costimulatory signaling domains in the CD3zeta primary signaling domain, selected from among CD28, OX40, CD27, ICAM-1, ICOS (CD278), and 4-1BB/CD137. In some embodiments, the costimulatory domain comprises 4-1BB to which 5 consecutive amino acids were added. In some embodiments, the costimulatory domain is linked to CD3zeta.

In some embodiments, a method of producing an engineered immune cell of the present disclosure further comprises culturing the engineered immune cell *in vitro* for at least 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, or 12 days.

In some embodiments, the method of producing an engineered immune cell further comprises, after the introducing step, culturing the engineered immune cell. In some embodiments, the method of producing an engineered immune cell further comprises, before the introducing step, obtaining the immune cell from a subject.

Any method known in the art for expressing a CAR in immune cells can be used in the context of the present disclosure. For example, there are various nucleic acid vectors for expression known in the art, such as linear polynucleotides, polynucleotides to which an ionic or amphiphilic compound is bound, plasmids, or viral vectors, though the present disclosure is not limited thereto. In some embodiments, a vector for expression of a CAR in immune cells may be or include an autonomously replicating plasmid or virus or derivative thereof. Viral vectors can include, but are not limited to adenovirus vector, adeno-associated viral vector, retrovirus vector, etc. In some embodiments a lentivirus vector, which is a retroviral vector, can be used. In some embodiments, a vector is a non-plasmid and a non-viral compound, such as, for example, a liposome.

The present disclosure encompasses the recognition that CAR-T cells, generated by the methods described herein may be therapeutically useful (e.g., for the treatment of cancer).

Therapeutic Applications

Provided herein are methods of treating a subject having a cancer or other malignancy, wherein the method comprises administering to a subject a composition that comprises or delivers an immune cell comprising a CAR. In some embodiments, the cancer is an anti-glypican-3-associated cancer. In some embodiments, the cancer is an anti-CD19-associated cancer. In some embodiments, the cancer is an anti-MVR-associated cancer.

Cancer can refer to a broad group of diseases characterized by the uncontrolled growth of abnormal cells in the body. Unregulated cell division and growth results in the formation of malignant tumors that invade neighboring tissues and may also metastasize to distant parts of the body through the lymphatic system or bloodstream. Cancer or cancer tissue may include a tumor.

An “anti-glypican-3-associated cancer” is a cancer that is characterized by a cancer cell having glypican-3 present on its surface. GPC3, a membrane-bound heparan sulfate proteoglycan, is overexpressed in approximately 70% to 80% of hepatocellular carcinomas, but is not expressed commonly in healthy tissues. In addition, GPC3 overexpression is found in several tumors, such as but not limited to hepatocellular carcinomas, hepatoblastoma, germ cell tumors (e.g., yolk sac tumors, choriocarcinomas), Wilms tumor, gastric carcinoma, non-small lung cancer, and thyroid cancer.

An “anti-CD19-associated cancer” is a cancer that is characterized by CD19 expression, wherein CD19 shows an essential role in B cell development and maturation. CD19 expression is highly conserved on most B cell tumors. It is expressed in most acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and B cell lymphomas.

5 An “anti-MVR-associated cancer” is characterized by cancer cells with increased expression of HLA-DR antigen relative to a non-cancer cell from a subject. In some embodiments, a cancer with higher expression of HLA-DR antigen can include, but are not limited to, bladder cancer, breast cancer, cervical cancer, colon cancer, endometrial cancer, esophageal cancer, fallopian tube cancer, gall bladder cancer, gastrointestinal cancer, head and
10 neck cancer, hematological cancer, laryngeal cancer, liver cancer, lung cancer, lymphoma, melanoma, mesothelioma, ovarian cancer, primary peritoneal cancer, salivary gland cancer, sarcoma, stomach cancer, thyroid cancer, pancreatic cancer, and prostate cancer. In some embodiments, diseases associated with HLA-DR expression include, but are not limited to, atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative
15 diseases expressing HLA-DR, or any combination thereof.

In some embodiments, a cancer for treatment by a method of the present disclosure can include, but is not limited to, carcinoma, lymphoma (e.g., Hodgkin’s and non-Hodgkin’s lymphomas), blastoma, sarcoma, and leukemia. In some embodiments, cancer may include squamous cell carcinoma, small cell lung cancer, non-small cell lung cancer, lung
20 adenocarcinoma, squamous cell carcinoma of the lung, peritoneal cancer, hepatocellular carcinoma, gastric cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatocellular carcinoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary carcinoma, kidney cancer, prostate cancer, vulvar cancer, thyroid cancer, liver carcinoma, leukemia and other lymphoproliferative disorders, and
25 various types of head and neck cancer.

In some embodiments, a cancer suitable for treatment by methods of the present disclosure is a hematologic cancer. In some embodiments, a hematologic cancer is a leukemia. In some embodiments, a cancer is selected from the group consisting of one or more acute leukemias including but not limited to B-cell acute lymphoid leukemia (“BALL”), T-cell acute
30 lymphoid leukemia (“TALL”), acute lymphoid leukemia (ALL); one or more chronic leukemias including but not limited to chronic myelogenous leukemia (CML), chronic lymphocytic

leukemia (CLL); additional hematologic cancers or hematologic conditions including, but not limited to B cell prolymphocytic leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma, follicular lymphoma, hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions, MALT lymphoma, mantle cell lymphoma, Marginal zone lymphoma, multiple myeloma, myelodysplasia and myelodysplastic syndrome, non-Hodgkin's lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, and "preleukemia" which are a diverse collection of hematological conditions united by ineffective production (or dysplasia) of myeloid blood cells.

10 In some embodiments, a cancer for treatment by methods of the present disclosure is a B cell lymphoma (i.e., a malignant lymphoma of B cell origin). B cell lymphomas include Hodgkin's lymphoma and non-Hodgkin's lymphoma, diffuse large B cell lymphoma (DLBCL), follicular lymphoma, mucosa-associated lymphatic tissue lymphoma (MALT), chronic lymphocytic leukemia, mantle cell lymphoma (MCL), burkitt lymphoma, mediastinal large B
15 cell lymphoma, waldenstrom macroglobulinemia, nodal marginal zone B cell lymphoma (NMZL), splenic marginal zone lymphoma (SMZL), intravascular large B-cell lymphoma, primary effusion lymphoma, lymphomatoid granulomatosis, and AIDS-related lymphoma, but is not particularly limited thereto as long as it is lymphoma of B cell origin.

The immune cells (e.g., CAR-T cells) may be administered at a therapeutically effective
20 amount to a patient in need thereof. For example, a therapeutically effective amount of the immune cells (e.g. CAR-T cells) may be at least about 10^4 cells, at least about 10^5 cells, at least about 10^6 cells, at least about 10^7 cells, at least about 10^8 cells, at least about 10^9 , or at least about 10^{10} . In some embodiments, a therapeutically effective amount of T cells is about 10^4 cells, about 10^5 cells, about 10^6 cells, about 10^7 cells, or about 10^8 cells. In some embodiments, the
25 therapeutically effective amount of the T cells is between about 0.4×10^8 and about 2×10^8 T cells. In some embodiments, the therapeutically effective amount of the T cells is about 0.4×10^8 , about 0.5×10^8 , about 0.6×10^8 , about 0.7×10^8 , about 0.8×10^8 , about 0.9×10^8 , about 1.0×10^8 , about 1.1×10^8 , about 1.2×10^8 , about 1.3×10^8 , about 1.4×10^8 , about 1.5×10^8 , about 1.6×10^8 , about 1.7×10^8 , about 1.8×10^8 , about 1.9×10^8 , or about 2.0×10^8 T cells.

30 In some embodiments, a therapeutically effective amount of the CAR T cells is about 2×10^6 cells/kg, about 3×10^6 cells/kg, about 4×10^6 cells/kg, about 5×10^6 cells/kg, about

6×10⁶ cells/kg, about 7×10⁶ cells/kg, about 8×10⁶ cells/kg, about 9×10⁶ cells/kg, about 1×10⁷ cells/kg, about 2×10⁷ cells/kg, about 3×10⁷ cells/kg, about 4×10⁷ cells/kg, about 5×10⁷ cells/kg, about 6×10⁷ cells/kg, about 7×10⁷ cells/kg, about 8×10⁷ cells/kg, or about 9×10⁷ cells/kg. In some embodiments, a therapeutically effective amount of immune cells (e.g., CAR-T cells) is between about 1×10⁶ and about 2×10⁶ T cells per kg body weight up to a maximum dose of about 1×10⁸ T cells. In some embodiments, the therapeutically effective amount of the T cells is about 1×10⁶ or about 2×10⁶ T cells per kg body weight up to a maximum dose of about 1×10⁸ T cells.

The number of cells will depend upon the ultimate use for which the composition is intended as will the type of cells included therein. For example, in some embodiments, a population of T cells comprising a CAR will contain greater than 10%, greater than 15%, greater than 20%, greater than 25%, greater than 30%, or greater than 35% of such cells. In some embodiments, a population of T cells comprising a CAR will contain 10% to 50%, 15% to 45%, 20% to 40%, 25% to 35%, or 20% to 30% of such T cells. In some embodiments, a population of T cells for administration is in a volume of a liter or less. In some embodiments, T cells for administration are in a volume of less than 500 ml, less than 250 ml, or 100 ml or less. In some embodiments, a density of the desired T cells is typically greater than 10⁶ cells/ml and generally is greater than 10⁷ cells/ml, generally 10⁸ cells/ml or greater. A clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed 10⁷ cells, 10⁸ cells, 10⁹ cells, 10¹⁰ cells, 10¹¹ cells, or 10¹² cells.

In some embodiments, a composition may be administered to a patient parenterally. In some embodiments, a composition that comprises or delivers a T cell comprising a CAR may be parenterally administered to a patient in one or multiple administrations. In some embodiments, a composition that comprises or delivers a T cell comprising a CAR may be parenterally administered to a patient once every day, once every 2 to 7 days, once every week, once every two weeks, once every month, once every three months, or once every 6 months.

In some embodiments, the present disclosure provides methods of treating a cancer in a subject in need thereof, the method comprising administering to the subject an engineered immune cell comprising a CAR, wherein the CAR comprises (a) an extracellular domain comprising an antigen-binding domain; (b) a transmembrane domain; and (c) an intracellular

domain comprising a costimulatory endodomain, wherein the costimulatory endodomain comprises an intracellular signaling domain from 4-1BB/CD137 and five additional amino acids.

In some embodiments, the subject has previously been administered one or more additional anticancer therapies selected from the group consisting of: ionizing radiation, a
5 chemotherapeutic agent, a therapeutic antibody, and a checkpoint inhibitor. In some embodiments, the subject has been identified or diagnosed as having a cancer.

Pharmaceutical Compositions

In some embodiments, the present disclosure provides pharmaceutical compositions that
10 include a T cell comprising a CAR, wherein the CAR comprises (a) an extracellular domain comprising an antigen-binding domain; (b) a transmembrane domain; and (c) an intracellular domain comprising a costimulatory endodomain, wherein the costimulatory endodomain comprises an intracellular signaling domain from 4-1BB/CD137 and five additional amino acids, and a pharmaceutically acceptable carrier. In some embodiments, a T cell comprising the CAR
15 is an autologous T cell. In some embodiments, a pharmaceutical composition can include a buffer, a diluent, solubilizer, emulsifier, preservative, adjuvant, an excipient, or any combination thereof. In some embodiments, a composition, if desired, can also contain one or more additional therapeutically active substances.

In some embodiments, T cells of the present disclosure are formulated by first harvesting
20 them from their culture medium, and then washing and concentrating the cells in a medium and container system suitable for administration (a “pharmaceutically acceptable” carrier) in a treatment-effective amount. Suitable infusion medium can be any isotonic medium formulation, typically normal saline, Normosol R (Abbott) or Plasma-Lyte A (Baxter), but also 5% dextrose in water or Ringer’s lactate can be utilized. The infusion medium can be supplemented with
25 human serum albumin.

In some embodiments, compositions are formulated for parenteral administration. For example, a pharmaceutical composition provided herein may be provided in a sterile injectable form (e.g., a form that is suitable for subcutaneous injection or intravenous infusion). For example, in some embodiments, a pharmaceutical composition is provided in a liquid dosage
30 form that is suitable for injection. In some embodiments, a pharmaceutical composition is provided as powders (e.g., lyophilized and/or sterilized), optionally under vacuum, which can be

reconstituted with an aqueous diluent (e.g., water, buffer, salt solution, etc.) prior to injection. In some embodiments, a pharmaceutical composition is diluted and/or reconstituted in water, sodium chloride solution, sodium acetate solution, benzyl alcohol solution, phosphate buffered saline, etc. In some embodiments, a powder should be mixed gently with the aqueous diluent (e.g., not shaken).

In some embodiments, a T cell comprising the CAR and/or a nucleic acid encoding the CAR of the present disclosure is formulated with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 1-10% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils can also be used. A vehicle or lyophilized powder can contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). In some embodiments, a formulation is sterilized by known or suitable techniques. A pharmaceutical composition may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro (Lippincott, Williams & Wilkins, Baltimore, MD, 2006) discloses various excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this disclosure.

In some embodiments, a composition including a population of T cells comprising the CAR and/or a nucleic acid encoding the CAR of the present disclosure is stably formulated. In some embodiments, a stable formulation of a population of T cells comprising the CAR and/or a nucleic acid encoding the CAR of the present disclosure may comprise a phosphate buffer with saline or a chosen salt, as well as preserved solutions and formulations containing a preservative as well as multi-use preserved formulations suitable for pharmaceutical or veterinary use.

Preserved formulations contain at least one known preservative or optionally selected from the group consisting of at least one phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol,

phenylmercuric nitrite, phenoxyethanol, formaldehyde, chlorobutanol, magnesium chloride (e.g., hexahydrate), alkylparaben (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof in an aqueous diluent. Any suitable concentration or mixture can be used as known in the art, such as

5 0.001-5%, or any range or value therein, such as, but not limited to 0.001, 0.003, 0.005, 0.009, 0.01, 0.02, 0.03, 0.05, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.3, 4.5, 4.6, 4.7, 4.8, 4.9, or any range or value therein. Non-limiting examples include, no preservative, 0.1-2% m-cresol (e.g., 0.2, 0.3, 0.4, 0.5, 0.9, 1.0%), 0.1-3% benzyl alcohol (e.g.,

10 0.5, 0.9, 1.1, 1.5, 1.9, 2.0, 2.5%), 0.001-0.5% thimerosal (e.g., 0.005, 0.01), 0.001-2.0% phenol (e.g., 0.05, 0.25, 0.28, 0.5, 0.9, 1.0%), 0.0005-1.0% alkylparaben(s) (e.g., 0.00075, 0.0009, 0.001, 0.002, 0.005, 0.0075, 0.009, 0.01, 0.02, 0.05, 0.075, 0.09, 0.1, 0.2, 0.3, 0.5, 0.75, 0.9, 1.0%), and the like.

In some embodiments, a pharmaceutical composition is provided in a form that can be

15 refrigerated and/or frozen. In some embodiments, a pharmaceutical composition is provided in a form that cannot be refrigerated and/or frozen. In some embodiments, reconstituted solutions and/or liquid dosage forms may be stored for a certain period of time after reconstitution (e.g., 2 hours, 12 hours, 24 hours, 2 days, 5 days, 7 days, 10 days, 2 weeks, a month, two months, or longer). In some embodiments, storage of compositions including an antibody agent for longer

20 than the specified time results in degradation of the antibody agent. Liquid dosage forms and/or reconstituted solutions may comprise particulate matter and/or discoloration prior to administration. In some embodiments, a solution should not be used if discolored or cloudy and/or if particulate matter remains after filtration. General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in Remington: The

25 Science and Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005.

In some embodiments, a pharmaceutical composition including a T cell comprising the CAR and/or a nucleic acid encoding the CAR of the present disclosure can be included in a container for storage or administration, for example, an vial, a syringe (e.g., an IV syringe), or a bag (e.g., an IV bag). A pharmaceutical composition in accordance with the present disclosure

30 may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a “unit dose” is discrete amount of the pharmaceutical

composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient that would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

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Kits

The present disclosure further provides a kit comprising one or more containers filled with at least one CAR and/or a nucleic acid encoding a CAR as described herein. Kits may be used in any applicable method, including, for example, therapeutic methods, diagnostic methods, cell proliferation and/or isolation methods, etc. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects (a) approval by the agency of manufacture, use or sale for human administration, (b) directions for use, or both.

In some embodiments, a kit may include one or more reagents for detection (e.g., detection of a CAR and/or a nucleic acid encoding a CAR. In some embodiments, a kit may include a CAR and/or a nucleic acid encoding a CAR in a detectable form (e.g., covalently associated with detectable moiety or entity). In some embodiments, one or more CARs and/or nucleic acids encoding a CAR as provided herein may be included in a kit used for treatment of subjects. In some embodiments, a CAR and/or a nucleic acid encoding a CAR as provided herein may be included in a kit used for preparing an autologous T cell expressing the CAR.

In some embodiments, a kit may provide one, two, three, four or more antigen specific antibody agents, where each is suitable for cloning into a CAR construct. In some embodiments, a kit may provide other reagents for assaying binding affinity of an antibody agent and/or CAR and/or a CAR T cell for a T cell identified or isolated from a subject. In some embodiments, a kit may provide other reagents for assaying functional avidity of an antibody agent and/or CAR and/or a CAR T cell for a T cell of a subject.

EXAMPLES

The disclosure is further described in the following examples, which do not limit the scope of the disclosure described in the claims.

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Example 1 – GPC3 Lentiviral transfer plasmid

A DNA construct encoding a single-chain variable fragment (scFv) form (**FIG. 2**) of a humanized anti-GC33 antibody agent was generated by connecting the VH and VL regions, using standard DNA cloning techniques known to the art. The lentiviral transfer plasmid used herein is shown in Table 1.

Table 1.

Lentiviral Transfer Vector			
scFv	Intracellular Domain	Selectable marker	Transfer vector
huGC33 VH-VL	euBBz	Flag	pELPS4
huGC33 VH-VL	BBz	Flag	pELPS3

The huGC33 VH-VL-scFv was cloned into a lentiviral vector, pELPS4-MVRL2H2-euBBz, wherein pELPS4-MVRL2H2-euBBz is a lentiviral vector including a costimulatory domain 4-1BB with five additional amino acids. The lentivirus vector construct, pELPS4-huGC33 VH-VL was digested with restriction enzymes, wherein restriction enzyme digestion results are shown in **FIG. 3**.

To create a CAR construct without the five additional amino acids to the 4-1BB costimulatory domain, the huGC33 VH-VL-scFv was cloned into a lentiviral vector, pELPS2-CD19-BBz, wherein pELPS2-CD19-BBz is a lentiviral vector including a costimulatory domain 4-1BB without five additional amino acids. The lentivirus vector construct, huGC33(VH-VL)-BBz was digested with restriction enzymes, wherein restriction enzyme digestion results are shown in **FIG. 4**.

Example 2 – Pharmaceutical composition of GPC3 CAR-T cells

PBMCs were thawed and activated by placing the PBMC cryovials (5×10^7 cells/1 mL/vial) in a water bath for 2-3 minutes. 10 mL CAR-T cell culture media and 1 mL PBMC was placed in a 50 mL conical tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the CAR-T cells were counted after resuspending the cells in 5 mL in fresh media. Fresh cell media was added to adjust the cell density to be 1×10^6 cells/mL. 10 μ L of T cell

activation bead was added for every 1×10^6 cells and IL-2 was supplemented to the media. The cell culture medium was cultured in a T75 flask at CO₂ 5% and 37°C in an incubator.

CAR-T cells were then generated by spinoculation of activated T cells with CAR-encoding lentivirus. Activated PBMCs were counted from the cell culture and the cells were seeded in a 24 well plate in the presence of 500 μ L cell media with lentivirus. After spinoculation transduction, the transduced cells from 1 well were cultured in cell culture media supplemented with IL-2.

The cultured CAR-T cells were counted every 2-3 days, and fresh culture media and IL-2 was added after each counting (FIG. 5). On day 12, the cultured CAR-T cells were harvested and stored at -80°C in a freezing container.

CAR expression was analyzed on day 12 of culturing, and results show that the control group did not show expression while the CAR-T cell groups showed 54-66% CAR expression (FIG. 6).

Target cells (GPC3 positive cell line) were harvested and seeded in a 96-well U bottom plate. Effector cells (CAR-T cells) were then added to the wells at Effector cell : Target cell ratios of 10:1, 3:1, 1:1, 0.3:1 and incubated for 24 hours at 37°C. After incubation, CytoTox96 Reagent was added to each well, and cytotoxicity was quantified by measuring absorbance at 490 nm (FIG. 7).

Example 3 - huGC33(VH-VL)-BBz CAR-T cells and huGC33(VH-VL)-euBBz CAR-T cells *in vivo*

In order to verify and compare the efficacy of huGC33(VH-VL)-BBz CAR-T cells and huGC33(VH-VL)-euBBz CAR-T cells, Huh-7_Luf-GFP cells (2×10^6 cells/200 μ L/head) were injected into NSG mice (6-8 weeks old, male) and after 35 days post injection, the mice with a tumor size of about 200mm³ were separated into 5 groups, each group of 4 mice. The control group received injections of 5% HSA and other groups received injections with the CAR-T cells. Growth of the tumor was observed by measuring tumor size twice a week, using TM900 (FIG. 8).

After CAR-T cell administration, orbital blood collection was performed from the mice once a week, wherein 100 μ L of each blood sample was centrifuged at 12,000 rpm for 10 minutes to confirm the proportion of the CAR-T cells and the cell count. 100 μ L of blood was

placed in a FACS tube, and live/dead cell staining was performed using Zombie NIR™ Fixable Viability Kit. After reaching a concentration of 0.1 μL/100 μ DPBS/tube, staining was performed at room temperature for 10 minutes. Counting beads 25 μL, CD45 0.5 μL, CD8 0.5 μL, CD45RO 1.0 μL, CD62L 1.0 μL, PD-1 1.0 μL, Tim-3 1.0 μL, CD4 0.5 μL, CD69 0.5 μL, and Flag 0.0125 μL were added to FACS buffer 100 μL and stained at room temperature for 30 minutes. After 30 minutes, 1X RBC lysis buffer was added and reacted at room temperature for 5 minutes. After centrifugation for 4 minutes at 2,000 rpm, all supernatant was discarded. 2 mL of FACS buffer was added to the tubes and centrifugation was performed for 4 minutes at 2,000 rpm, and analysis was performed using FACSCelesta (**FIG. 9**).

After 6 weeks post injection of CAR-T injection, the mouse spleen, liver and bone marrow were harvested to evaluate the ratio of huGC33(VH-VL)-BBz CAR-T cells and huGC33(VH-VL)-euBBz CAR-T cells. Tissue samples were processed and filtered through a 40 μm cell strainer, then centrifuged at 2,000 rpm for 5 minutes and all supernatant was discarded. 5 μL 1X ACK buffer was added and reacted for 10 minutes. Then, 10 mL DPBS was added and centrifuged at 2,000 rpm for 5 minutes. FACS staining was performed as described above (**FIG. 10**).

Example 4 - Construction of CD19-euBBz CAR

In order to increase immunological efficacy in the cytoplasmic signal domain 4-1BB, 5 amino acids were added in the 4-1BB cytoplasmic domain used in CAR-T, as a co-stimulatory signal factor, to newly construct a CAR expression vector (CD19-euBBz CAR). The completed construct comprises an anti-CD19, which is an scFv, including the EF1 alpha promoter, a hinge region and a transmembrane domain of human CD8, and an intracellular signaling domain. In particular, the intracellular signaling domain consists of a stimulatory domain and a co-stimulatory signaling domain. The intracellular signaling domain is 4-1BB, a co-stimulatory signal domain to which 5 consecutive amino acids were added, to which CD3 zeta was linked. The CAR gene fragment ultimately produced was conjugated to ELPS lentiviral expression vectors cleaved with BamH I and Sal I. In addition, cloning was performed using BamH I / Nhe I restriction enzyme to replace only the scFv part.

Example 5 – CD19-euBBz and CD19-BBz CAR-T cells

The 293T cell culture used for the production of recombinant lentiviruses contains medium comprising 10% FBS (Millipore, TMS-013-BKR) and $1 \times$ P/S (Gibco, 15140-122) in high glucose DMEM (Welgene, LM001-05). 293T cells were incubated in DMEM medium comprising 10% FBS for 24 hours prior to transduction in a 37°C 5% CO₂ incubator. The next day, for transfection, the transfection reagent and lentiviral plasmids were mixed at an appropriate proportion and incubated for 48 hours. The supernatant containing the lentivirus was then collected and centrifuged at 400 × g for 10 minutes. In addition, the supernatant was filtered with a 0.45 µm syringe filter using a 50 mL syringe. The obtained supernatant was mixed 3:1 with a lentiviral enrichment kit (Clontech, 631231), and reacted at 4°C for 24 to 48 hours. This was followed by centrifugation for 2 hours at 4°C and 4,000 rpm to obtain a virus, which was resuspended in 0.5 mL RPMI (Welgene, LM001-01) not comprising FBS to produce a lentivirus.

To determine transduction efficiency of mammalian cells, Transformation Units (TU/mL) were measured by analyzing the particle count of the actual transduction-capable lentiviruses using Jurkat cells. CAR expression can be assayed by FACS. On the first day, Jurkat cells were seeded in 96-well plates at 1×10^5 cells/100 µL per well. On the second day, the lentivirus was serially diluted by 1/3 in 96-well plates, and the lentiviral transduction was performed on the already seeded Jurkat cells. At this time, by introducing polybrene (Millipore) into RPMI medium (10% FBS and 1x P/S), transduction of lentivirus was further increased. After centrifugation at 1200xg and 32°C for 2 hours, the cells were incubated for 3 hours in a 37°C 5% CO₂ incubator, and only 100 µL of RPMI only was added per well. On day 5, the flag of the lentivirus infected into the cell was stained with anti-Flag-DYKDDDDK (Biolegend, Cat No. 637310) to analyze the percentage of cells transduced with a flow cytometer. Using this, the titer was calculated as described in Follenzi and Naldini, 2002 (Follenzi and Naldini, 2002).

FACS staining was performed to confirm the production proportion of the two species of CAR-T cells after 14 days of incubation. For each CAR-T cell type, 2×10^5 cells were collected in FACS tubes (FALCON, Cat. No. 352052), and then 2 mL of FACS buffer was added and centrifugation was performed for 5 minutes at 2,000 rpm using a centrifuge (Thermo, ST16). After discarding the supernatant, 0.5 µL/tube anti-CD8 APC (SKI, Biolegend, Cat.No. 344722), 0.5 µL/tube anti-CD4 BV650 (RPA-T4, Biolegend, Cat. No. 300536) and 0.125 µL/tube anti-flag PE (L5, Biolegend, Cat. No. 637310) was added and staining was performed at room

temperature for 30 minutes. After adding 2 mL of FACS buffer and centrifuging at 2,000 rpm for 5 minutes, this process was repeated one more time. For staining surviving/dead cells, 1 μ L/tube 7-AAD (Biolegend, Cat. No. 420404) was added and the mixture was left for 5 minutes at room temperature and then analyzed using FACS (BD, FACSCelesta).

- 5 Confirmation of the proportion of the produced CD19 CAR-T cells using FACS staining confirmed that in the case of the improved-construct CD19-euBBZ CAR-T cells, the cell ratios were CD4+/CAR+ 29.4%, CD8+/CAR+ 50.8%, and total CAR-T 80.2%. It was confirmed that for the non-construct-improved CD19-BBz CAR-T cells, the cell ratios were 42.7% for CD4+/CAR+, 29.3% for CD8+/CAR+, and 72.0% for total CAR-T. Accordingly, it was
- 10 confirmed that CD19-euBBZ CAR-T cells had 8.2% higher expression of CAR than CD19-BBz CAR-T cells, and the CD8+/CAR+ cell was 21.5%, or about 2 times as many. (FIG. 11A)

Example 6 - Confirmation of Cytotoxicity of Produced CD19 CAR-T Cells

- To determine the cytotoxicity of the two CAR-T cell types cultured for 14 days, the
- 15 CAR-T (E): LCL (T) proportion was brought to 30:1, 10:1, 3:1, and 1: 1 in 96-well white plates (Corning, Cat. No. 3917). First, CAR-T cells were placed in wells at 6×10^5 cells / 50 μ L, 2×10^5 cells / 50 μ L, 9×10^4 cells / 50 μ L, and 2×10^4 cells / 50 μ L, respectively. Next, the target cell line, namely the CBK LCL-Luc cell line, was added to 2×10^4 cells/50 μ L in a 37°C CO₂ incubator (Mammert, INCO153med) and reacted for 4 hours. After 4 hours, 100 μ L of Bright-
- 20 Glo™ (Promega, Cat. No. E2620) was added to each well, and 5 minutes later, the relative light unit (RLU) value was measured using Luminometer (Thermo, Fluoroskan FL).

There was found to be no difference in cytotoxicity between CAR-T cells in which conventional 4-1BB was introduced and CAR-T cells in which euBBz containing five amino acids added to 4-1BB domain was introduced.

- 25 The results show, when two CAR-T cells and CBK LCL-Luc cell lines were incubated together at a proportion of 30:1, the cytotoxicity was found to be about 80% after 4 hours, and when they were incubated at 10:1, the cytotoxicity was about 50%. As the respective number of CAR-T cells incubated with cancer cells decreased by a factor of 3, the cytotoxicity decreased by

about a factor of 3; in addition, when 5 amino acids were added to the 4-1BB domain in vitro, this was confirmed not to affect the in vitro cytotoxicity. (FIG. 11B)

Example 7 - Induction of subcutaneous animal models and validation of CAR-T through automatic caliper and IVIS imaging

For the experimental animals, NSG (NOD-scid IL2 γ μ L1) mice (The Jackson Laboratory) were used and managed under constant conditions in an animal nursery. The temperature was $23 \pm 2^\circ\text{C}$ with a 12 hour light/dark cycle and $50 \pm 10\%$ humidity; feed and drink were provided ad libitum. In efficacy experiments using CD19-euBBz CAR-T with five amino acids added to the 4-1BB domain, CBK LCL-Luc cell lines were prepared at 2×10^6 cells/100 μL DPBS/head and injected subcutaneously in 6-week-old female mice to induce a subcutaneous animal model. When the cancer size reached between 50 and 100 mm^3 as measured using an automatic caliper (Youngbio, TM900), CD19-euBBz CAR-T cells and CD19-BBz CAR-T cells at 2×10^6 cells/100 μL DPBS/head (dose 1) and 6×10^6 cells/100 μL DPBS/head (dose 2) were administered once through the tail vein to confirm efficacy. In all animal experiments, cancer size and viability were confirmed periodically.

More specifically, cancer size and photon values were measured using automatic calipers and IVIS imaging equipment (PerkinElmer, Luna III), at 3 and 4-day intervals after CAR-T administration (FIG. 12, 13). In the case of using TM900, the cancer size was determined after placing the equipment at the cancer site. When confirming imaging and photon values using an IVIS imaging device, 150 mg/kg XenoLight™ D-luciferin (PerkinElmer, Cat. No. 122799) was first administered intraperitoneally in mice. After 15 minutes, inhalation anesthesia was induced using isoflurane, and 5 minutes later, IVIS was used for imaging the presence of cancer cells. After imaging, normalization was performed and the luciferase values (photon values) were then confirmed and graphed. After constructing a subcutaneous animal model using a CBK LCL-Luc cell line, the effects of CD19-BBz CAR-T and CD19-euBBz CAR-T cells were compared using IVIS imaging. As shown in FIG. 12, the effect could be confirmed within 1 week after administering the 2 species of CAR-T cells.

In the experimental group in which CD19-euBBz CAR-T cells was administered at 2×10^6 cells/100 μL DPBS/head and 6×10^6 cells/100 μL DPBS/head, cancer cells were observed on IVIS imaging 1 week after administration. In addition, in the group treated with CD19-BBz

CAR-T, when 6×10^6 cells/100 μ L DPBS/head was administered, cancer cells were rarely observed by imaging within 1 week of administration. However, cancer cells were identified in the experimental group administered CD19-BBz CAR-T after 1 week.

After 1 week after CAR-T administration, as shown in imaging, the value of luciferase imaged in each subject after the imaging process was determined; luciferase values were confirmed only in the group administered CD19 CAR-T at 2×10^6 cells/100 μ L DPBS/head. When observed 3 weeks or more thereafter, the tumors continued to grow in mice not administered CAR-T cells, and no cancer cells were identified in the three experimental groups in which cancer cells disappeared initially. However, after 10 days, luciferase levels were decreased in the group receiving 2×10^6 cells/100 μ L DPBS/head for CD19 CAR-T, but cancer cells did not disappear completely after 3 weeks. When administering the CD19-euBBz CAR-T 2×10^6 cells/100 μ L DPBS/head, it was found via experimental imaging of cancer cells that the efficacy was similar to the group treated with CD19-BBz CAR-T at 6×10^6 cells/100 μ L DPBS/head. The results showed that there was no difference in cytotoxicity of CD19-euBBz CAR-T and CD19-BBz CAR-T in vitro, but it was confirmed that the efficacy was 5 times better in CD19-euBBz CAR-T in animal models. (FIG. 12)

Example 8 - Confirmation of proportion of CD19-euBBz CAR-T in *in vivo* animal model

After CAR-T cell administration in a subcutaneous animal model to verify the potency of the improved-construct CAR-T cells, the presence of CAR-T was confirmed from mouse blood. More specifically, orbital blood collection was performed from mice at 3 and 4-day intervals after CAR-T administration. At a time of each blood collection, 70 μ L of blood was collected and 60 μ L of the blood was used to confirm the proportion of the CAR-T cells and the cell count. 60 μ L blood was placed in a 5 mL FACS tube, and live/dead cell staining was performed using Zombie Aqua BV510 (Biolegend, Cat. No. 423101). After reaching a concentration of 0.1 μ L / 100 μ L DPBS/tube, staining was performed at room temperature for 10 minutes. Since counting beads (Molecularprobes, Cat. No. C36950), anti-CD45 FITC (HI30, Biolegend, Cat. No. 304006), anti-CD8 BV786 (SK-1, Biolegend, Cat. No. 344740), anti-CD4 BV650 and anti-flag PE were added and stained at room temperature for 30 minutes. Each antibody was mixed in at 0.5 μ L/100 μ L FACS buffer/tube, and 25 μ L of counting beads were added thereto. After 30 minutes 2 mL of 1X RBC lysis buffer (Biolegend, Cat. No. 422401) was added and reacted at

room temperature for 5 minutes. After centrifugation for 5 minutes at 2,000 rpm using a centrifuge, all supernatant was discarded. 2 mL of FACS wash buffer was added to this tube and centrifugation was performed for 5 min at 2,000 rpm. This process was repeated one more time, and then 50 μ L of FACS buffer was added and analyzed using FACS.

5 One week after CAR-T cell administration, in the CD19-euBBz CAR-T treatment group, approximately 20% CD19-euBBz CAR-T cells were confirmed in the blood in both the 2×10^6 cells/100 μ L DPBS/head and 6×10^6 cells/100 μ L DPBS/head groups; but in the CD19-BBz CAR-T-administered group, when 6×10^6 cells/100 μ L DPBS/head was administered only 5% CD19 CAR-T was confirmed. 3 days later, the number and proportion of CAR-T cells in the
10 mouse body reached a maximum and decreased. Within one week, in the 3 experimental groups in which CAR-T cells were identified (CD19-euBBz CAR-T; 2×10^6 cells/100 μ L DPBS/head and 6×10^6 cells/100 μ L DPBS/head, CD19-BBz CAR-T; 6×10^6 cells/100 μ L DPBS/head), the cancer cells were killed quickly because it was possible for them to contact relatively many CAR-T cells before they proliferated in the mouse body. However, in the experimental group
15 administered CD19-BBz CAR-T at 2×10^6 cells/100 μ L DPBS/head, the proportion and number of CAR-T cells reached a maximum at 2 weeks, and the CAR-T proportion was about 25% with cancer cells proliferating relatively well. The CD19-euBBz CAR-T was more stable in quantity than the CD19-BBz CAR-T, after the CAR-T proportion initially increased and later decreased. In the case of CD19-BBz CAR-T, however, the proportion of CAR-T cells increased and
20 decreased at a later time, and consequently it took longer for the tumor to disappear in the mouse body. As a result, as in the results of this experiment, the group administered CD19-euBBz CAR-T at 2×10^6 cells/100 μ L DPBS/head exhibited similar CAR-T levels and effects in mice as the group administered CD19-BBz CAR-T at 6×10^6 cells/100 μ L DPBS/head, indicating that CD19-euBBz CAR-T has a superior effect. (FIG. 14)

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