Chimeric Antigen Receptors Targeting Epidermal Growth Factor Receptor Variant III

Field

The invention relates to chimeric antigen receptors (CAR). CARs are able to redirect immune cell specificity and reactivity toward a selected target exploiting the ligand-binding domain properties. In particular, the invention relates to CARs that specifically bind to Epidermal Growth Factor Receptor Variant III (EGFRvIII specific CARs). The invention further relates to polynucleotides encoding EGFRvIII specific CAR and isolated cells expressing EGFRvIII specific CARs at their surface. The invention further relates to methods for engineering immune cells expressing EGFRvIII specific CARs at their surface. The invention is particularly useful for the treatment of solid tumors such as glioblastoma multiforme (GBM), non-small cell lung cancer, head and neck cancer, breast cancer, ovarian cancer, and prostate cancer. The invention further relates to immune cells comprising the EGFRvIII specific CARs (EGFRvIII specific CAR-T cells), compositions comprising the EGFRvIII specific CAR-T cells, and methods of using the EGFRvIII specific CAR-T cells for treating EGFRvIII-mediated pathologies.

Background

EGFR variant III (EGFRvIII), a tumor specific mutant of EGFR, is a product of genomic rearrangement which is often associated with wild-type EGFR gene amplification. EGFRvIII is formed by an in-frame deletion of exons 2-7, leading to deletion of 267 amino acids with a glycine substitution at the junction. The truncated receptor loses its ability to bind ligands but acquires constitutive kinase activity. Interestingly, EGFRvIII always co-expresses with full length wild-type EGFR in the same tumor cells. Moreover, EGFRvIII expressing cells exhibit increased proliferation, invasion, angiogenesis and resistance to apoptosis.

EGFRvIII is most often found in glioblastoma multiforme (GBM). It is estimated that 25-35% of GBM carries this truncated receptors. Moreover, its expression often reflects a more aggressive phenotype and poor prognosis. Besides GBM, expression of EGFRvIII has also been reported in other solid tumors such as non-small cell lung cancer, head and neck cancer, breast cancer, ovarian cancer and prostate cancer. In

contrast, EGFRvIII is not expressed in healthy tissues. The lack of expression in normal tissues makes EGFRvIII an ideal target for developing tumor specific targeted therapy.

Adoptive transfer of T cells genetically modified to recognize malignancy-associated antigens has shown promise as a new approach to treating cancer (see, e.g., Brenner et al., Current Opinion in Immunology, 22(2): 251-257 (2010); Rosenberg et al., Nature Reviews Cancer, 8(4): 299-308 (2008)). T cells can be genetically modified to express chimeric antigen receptors (CARs), which are fusion proteins comprised of an antigen recognition moiety and T cell activation domains (see, e.g., Eshhar et al., Proc. Natl. Acad. Sci. USA, 90(2): 720-724 (1993), and Sadelain et al., Curr. Opin. Immunol, 21(2): 215-223 (2009)). Accordingly, treatment to a solid tumor such as glioblastoma multiforme using an anti-EGFRvIII antagonist including EGFRvIII specific CARs and EGFRvIII specific CAR-T cells would make a promising therapeutic agent.

Summary

Chimeric antigen receptors (CARs) that bind to EGFRvIII are provided. It is demonstrated that certain EGFRvIII specific CARs are effective when expressed in T cells to activate T cells upon contact with EGFRvIII. Advantageously, the EGFRvIII specific CARs provided herein bind human EGFRvIII. Also advantageously, the EGFRvIII specific CAR-T cells provided herein exhibit degranulation activity, increased interferon gamma production, and/or cytotoxic activity upon contact with EGFRvIII-expressing cells.

In one aspect, the invention provides an EGFRvIII specific CAR comprising an extracellular ligand-binding domain, a first transmembrane domain, and an intracellular signaling domain, wherein the extracellular ligand-binding domain comprises (a) a heavy chain variable (VH) region comprising (i) a VH complementarity determining region one (CDR1) comprising the sequence shown in SEQ ID NO: 62, 63, 64, 74, 75, 76, 80, 81, 82, 88, 89, 90, 109, 110, 111, 115, 116, 117, 121, 122, 123, 137, 138, or 139; (ii) a VH CDR2 comprising the sequence shown in SEQ ID NO: 70, 71, 77, 78, 83, 84, 86, 87, 91, 92, 112, 113, 118, 119, 124, 125, 127, 128, 140, or 141; and iii) a VH CDR3 comprising the sequence shown in SEQ ID NO: 73, 79, 85, 114, 120, 126, 129, or 142, and/or (b) a light chain variable (VL) region comprising (i) a VL CDR1 comprising the sequence shown in SEQ ID NO: 149, 156, 159, 162, 165, 182, 185, 187,

or 195; (ii) a VL CDR2 comprising the sequence shown in SEQ ID NO: 152, 157, 160, 163, 183, 186, 188, or 196; and (iii) a VL CDR3 comprising the sequence shown in SEQ ID NO: 153, 158, 161, 164, 184, 189, or 197.

In another aspect, the invention provides an EGFRvIII specific CAR comprising an extracellular ligand-binding domain, a first transmembrane domain, and an intracellular signaling domain, wherein the extracellular ligand-binding domain comprises a single chain Fv fragment (scFv) comprising a heavy chain variable (VH) region comprising three CDRs from the VH region comprising the sequence shown in SEQ ID NO: 5, 9, 11, 13, 15, 37, 39, 41, 43, or 48; and/or a light chain variable (VL) region comprising three CDRs from the VL region comprising the sequence shown in SEQ ID NO: 6, 10, 12, 14, 16, 38, 40, 42, or 49. In some embodiments, the VH region can comprise the sequence shown in SEQ ID NO: 5, 9, 11, 13, 15, 37, 39, 41, 43, or 48, or a variant thereof with one or several conservative amino acid substitutions in residues that are not within a CDR and/or the VL region can comprise the amino acid sequence shown in SEQ ID NO: 6, 10, 12, 14, 16, 38, 40, 42, or 49, or a variant thereof with one or several amino acid substitutions in amino acids that are not within a CDR. For example, in some embodiments, the VH or VL region of the scFv can comprise an amino acid sequence described above or a variant thereof with no more than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 conservative substitutions in residues that are not within a CDR.

In some embodiments, the invention provides an EGFRvIII specific CAR comprising an extracellular ligand-binding domain, a first transmembrane domain, and an intracellular signaling domain, wherein the extracellular ligand-binding domain comprises a single chain Fv fragment (scFv) comprising a heavy chain variable (VH) region comprising the sequence shown in SEQ ID NO: 11, 15, 30, 37, or 41; and/or a light chain variable (VL) region comprising the sequence shown in SEQ ID NO: 12, 16, 31, 38, or 42. In some embodiments, the VH comprises the sequence shown in SEQ ID NO: 11 and the VL comprises the sequence shown in SEQ ID NO: 12. In some embodiments, the VH comprises the sequence shown in SEQ ID NO: 15 and the VL comprises the sequence shown in SEQ ID NO: 16. In some embodiments, the VH comprises the sequence shown in SEQ ID NO: 30 and the VL comprises the sequence shown in SEQ ID NO: 31. In some embodiments, the VH comprises the sequence shown in SEQ ID NO: 37 and the VL comprises the sequence shown in SEQ ID NO:

38. In some embodiments, the VH comprises the sequence shown in SEQ ID NO: 41 and the VL comprises the sequence shown in SEQ ID NO: 42.

In some embodiments, the intracellular signaling domain comprises a CD3zeta signaling domain. In some embodiments, the intracellular signaling domain comprises a 4-1BB signaling domain. In some embodiments, the CAR can further comprise a second intracellular signaling domain. In some embodiments, the second intracellular signaling domain can comprise a 4-1BB signaling domain. In some embodiments the first intracellular signaling domain comprises a CD3zeta signaling domain and the second intracellular signaling domain comprises a 4-1BB signaling domain.

In some embodiments, the CAR can comprise a stalk domain between the extracellular ligand-binding domain and the first transmembrane domain. In some embodiments, the stalk domain can be selected from the group consisting of: a human CD8α hinge, a human CD28 hinge, an IgG1 hinge, and an FcγRIIIα hinge.

In some embodiments, the first transmembrane domain can comprise a CD8α chain transmembrane domain.

In some embodiments, the CAR can comprise another extracellular ligand-binding domain which is not specific for EGFRvIII.

In some embodiments of a CAR, the extracellular ligand-binding domain(s), the first transmembrane domain, and intracellular signaling domain(s) are on a single polypeptide.

In some embodiments, the CAR can comprise a second transmembrane domain, wherein the first transmembrane domain and the extracellular ligand-binding domain(s) are on a first polypeptide, and wherein the second transmembrane domain and the intracellular signaling domain(s) are on a second polypeptide, wherein the first transmembrane domain comprises a transmembrane domain from the α chain of the high-affinity IgE receptor (FcεRI) and the second transmembrane domain comprises a transmembrane domain from the γ or β chain of FcεRI. In some embodiments, the CAR can comprise a third polypeptide comprising a third transmembrane domain fused to an intracellular signaling domain from a co-stimulatory molecule, wherein the third transmembrane domain comprises a transmembrane domain from the γ or β chain of FcεRI.

In another aspect, the invention provides an isolated polynucleotide comprising a nucleic acid sequence encoding an EGFRvIII specific CAR as described herein.

In another aspect, the invention provides an expression vector comprising a nucleic acid sequence encoding an EGFRvIII specific CAR antibody as described herein.

In another aspect, the invention provides an engineered immune cell expressing at its cell surface membrane an EGFRvIII specific CAR as described herein. In some embodiments, the engineered immune cell can comprise another CAR which is not specific for EGFRvIII.

In some embodiments, the engineered immune cell can comprise a polynucleotide encoding a suicide polypeptide. In some embodiments, the suicide polypeptide is RQR8. In some embodiments, the polynucleotide encoding the suicide polypeptide is in a different nucleic acid molecule than the polynucleotide comprising a nucleic acid sequence encoding the EGFRvIII specific CAR. In some embodiments, the polynucleotide encoding the suicide polypeptide is part of the same nucleic acid molecule as the polynucleotide comprising a nucleic acid sequence encoding the EGFRvIII specific CAR.

In some embodiments, an engineered immune cell containing an EGFRvIII-specific CAR can comprise a suicide polypeptide in a separate polypeptide chain from the polypeptide chain of the EGFRvIII-specific CAR.

In some embodiments, an EGFRvIII specific CAR as described herein also comprises a suicide polypeptide in the same polypeptide chain as the CAR. For example, the suicide polypeptide may be between the scFv and hinge sequence of the CAR. In some embodiments, a suicide polypeptide in a CAR may have the R2 format as provided herein. In some embodiments, a suicide polypeptide comprises an epitope that is recognized by rituximab.

Also provided herein is a polynucleotide encoding an EGFRvIII specific CAR which also encodes a suicide polypeptide in the CAR.

In some embodiments, the immune cell can be derived from an inflammatory T-lymphocyte, a cytotoxic T-lymphocyte, a regulatory T- lymphocyte, a memory T-lymphocyte, a helper T-lymphocyte, a natural killer T-lymphocyte, or a natural killer cell.

In some embodiments, the engineered immune cell can comprise a disruption in one or more endogenous genes, wherein the endogenous gene encodes TCRα, TCRβ, CD52, glucocorticoid receptor (GR), deoxycytidine kinase (dCK), or an immune checkpoint protein such as for example programmed death-1 (PD-1).

In some embodiments, the immune cell is obtained from a healthy donor. In some embodiments, the immune cell is obtained from a patient.

In another aspect, the invention provides an engineered immune cell expressing at its cell surface membrane an EGFRvIII specific CAR as described herein for use as a medicament. In some embodiments, the medicament is for use in treatment of an EGFRvIII related cancer (e.g., any cancer with EGFRvIII expression) selecting from the group consisting of glioblastoma multiform, anaplastic astrocytoma, giant cell glioblastoma, gliosarcoma, anaplastic oligodendroglioma, anaplastic ependymoma, anaplastic oligoastrocytoma, choroid plexus carcinoma, anaplastic ganglioglioma, pineoblastoma, pineocytoma, meningioma, medulloepithelioma, ependymoblastoma, medulloblastoma, supraentorial primitive neuroectodermal tumor, atypical teratoid/rhabdoid tumor, mixed glioma, head and neck cancer, non-small cell lung cancer, breast cancer, ovarian cancer, prostate cancer, medullobastoma, colorectal cancer, anal cancer, cervical cancer, renal cancer, skin cancer, pancreatic cancer, liver cancer, bladder cancer, gastric cancer, thyroid cancer, mesothelioma, uterine cancer, lymphoma, and leukemia.

In another aspect, the invention provides a method of engineering an immune cell comprising: providing an immune cell; and expressing at the surface of the cell at least one EGFRvIII specific CAR as described herein.

In some embodiments, the method comprises: providing an immune cell; introducing into the cell at least one polynucleotide encoding said EGFRvIII specific CAR; and expressing said polynucleotide into the cell.

In some embodiments, the method comprises providing an immune cell; introducing into the cell at least one polynucleotide encoding said EGFRvIII specific CAR; and introducing at least one other CAR which is not specific for EGFRvIII.

In another aspect, the invention provides a method of treating a subject suffering from a condition associated with malignant cells, the method comprising: providing an immune cell expressing at the surface an EGFRvIII specific CAR as described herein; and administering said immune cells to said patient.

In another aspect, the invention provides a pharmaceutical composition comprising an engineered immune cell as described herein.

In another aspect, the invention provides a method of treating a condition associated with malignant cells expressing EGFRvIII in a subject comprising

administering to a subject in need thereof an effective amount of a pharmaceutical composition of claim comprising an engineered immune cell as described herein. In some embodiments, the condition is a cancer. In some embodiments, the cancer is an EGFRvIII related cancer selecting from the group consisting of glioblastoma multiform, anaplastic astrocytoma, giant cell glioblastoma, gliosarcoma, anaplastic oligodendroglioma, anaplastic ependymoma, anaplastic oligoastrocytoma, choroid plexus carcinoma, anaplastic ganglioglioma, pineoblastoma, pineocytoma, meningioma, medulloepithelioma, ependymoblastoma, medulloblastoma, supraentorial primitive neuroectodermal tumor, atypical teratoid/rhabdoid tumor, mixed glioma, head and neck cancer, non-small cell lung cancer, breast cancer, ovarian cancer, prostate cancer, medullobastoma, colorectal cancer, anal cancer, renal cancer, cervical cancer, liver cancer, pancreatic cancer, gastric cancer, thyroid cancer, mesothelioma, uterine cancer, and bladder cancer.

In another aspect, the invention provides a method of inhibiting tumor growth or progression in a subject who has malignant cells expressing EGFRvIII, comprising administering to the subject in need thereof an effective amount of a pharmaceutical composition comprising an engineered immune cell as described herein.

In another aspect, the invention provides a method of inhibiting metastasis of malignant cells expressing EGFRvIII in a subject, comprising administering to the subject in need thereof an effective amount of a pharmaceutical composition comprising an engineered immune cell as described herein.

In another aspect, the invention provides a method inducing tumor regression in a subject who has malignant cells expressing EGFRvIII, comprising administering to the subject in need thereof an effective amount of a pharmaceutical composition comprising an engineered immune cell as described herein.

Brief Description of the Figures/Drawings

FIG. 1A, FIG. 1B, and FIG. 1C show examples of FACS binding histograms of three EGFRvIII antibodies: mAb 42G9 (FIG. 1A), 32A10 (FIG. 1B) and 32G8 (FIG. 1C), to three F98 cell lines: F98 (EGFR negative), F98-EGFRwt, and F98-EGFRvIII. The X-axis is fluorescence intensity; the Y-axis is percentage of maximum / normalized to mode.

FIG. 2A shows a bar graph summarizing EGFRvIII specific CAR expression for CARs containing different EGFRvIII specific clones.

FIG. 2B shows a bar graph summarizing the percentage of EGFRvIII specific CAR T cells that bound to recombinant EGFR-mFc and EGFRvIII-mFc.

FIG. 2C shows a bar graph summarizing the expression of CARs containing 10 different EGFRvIII specific clones in CAR T cells.

FIG. 3 shows a bar graph summarizing degranulation activities of EGFRvIII specific CAR T cells expressing different EGFRvIII specific clones, alone or upon co-culture with various cell lines: cells that do not express any EGFR protein (NCI-H522 and U87-KO), cells express high level of wild-type EGFR (U87-KO-EGFRwt), or cells that express low (NCI-H522-EGFRvIII) and high (U87-KO-EGFRvIII) levels of EGFRvIII.

FIG. 4 shows a bar graph summarizing IFNγ secretion by EGFRvIII specific CAR T cells expressing different EGFRvIII specific clones, alone or upon co-culture with various cell lines: cells that do not express any EGFR protein (NCI-H522 and U87-KO), cells express high level of wild-type EGFR (U87-KO-EGFRwt), or cells that express low (NCI-H522-EGFRvIII) and high (U87-KO-EGFRvIII) levels of EGFRvIII.

FIG. 5 shows a bar graph comparing the cytotoxicity of EGFRvIII specific CAR T cells expressing different EGFRvIII specific clones towards a wild-type EGFR expressing cell line (U87-KO-EGFRwt) vs high (U87-KO-EGFRvIII) and low EGFRvIII (NCI-H522-EGFRvIII) expressing cell lines.

FIG. 6 shows a graph summarizing the anti-tumor activities of EGFRvIII specific CAR T cells expressing different EGFRvIII specific clones against GBM cells in a subcutaneous GBM xenograft model.

FIG. 7 shows a bar graph summarizing the CAR expression by CAR T cells expressing four different EGFRvIII specific clones in the CAR and carrying the intra-CAR suicide sequence R2 in the CAR on Day 4, Day 9/10, and Day 14/15 post-T cell transduction.

FIG. 8 shows a bar graph summarizing the CAR expression by CAR T cells expressing four different EGFRvIII specific clones in the CAR and carrying the intra-CAR suicide sequence R2 on Day 4 and Day 14/15 post-T cell transduction.

FIG. 9 shows a bar graph summarizing the cytotoxicity of CAR T cells expressing four different EGFRvIII specific clones and carrying the intra-CAR suicide sequence R2 against a high level (U87-KO-EGFRvIII) and a low level EGFRvIII (NCI-H522-EGFRvIII) expressing cell line.

Detailed Description

The invention disclosed herein provides chimeric antigen receptors (CARs) and immune cells comprising CARs (e.g. CAR-T cells) that specifically bind to EGFRvIII (e.g., human EGFRvIII). The invention also provides polynucleotides encoding these CARs, compositions comprising these CAR-T cells, and methods of making and using these CARs and CAR-T cells. The invention also provides methods for treating a condition associated with EGFRvIII-mediated pathologies in a subject, such as cancer.

General Techniques

The practice of the invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, immunology, virology, monoclonal antibody generation and engineering, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D.M. Weir and C.C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols in Immunology (J.E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C.A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practical approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J.D. Capra, eds., Harwood Academic Publishers, 1995).

Definitions

The term "extracellular ligand-binding domain" as used herein refers to an oligo- or polypeptide that is capable of binding a ligand. Preferably, the domain will be capable of interacting with a cell surface molecule. For example, the extracellular ligand-binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state.

The term “stalk domain” or “hinge domain” are used interchangeably herein to refer to any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular ligand- binding domain. In particular, stalk domains are used to provide more flexibility and accessibility for the extracellular ligand-binding domain.

The term "intracellular signaling domain" refers to the portion of a protein which transduces the effector function signal and directs the cell to perform a specialized function.

A "co-stimulatory molecule" as used herein refers to the cognate binding partner on a T cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the cell, such as, but not limited to proliferation. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and Toll ligand receptor. Examples of costimulatory molecules include CD27, CD28, CD8, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3 and a ligand that specifically binds with CD83 and the like.

A "co-stimulatory ligand" refers to a molecule on an antigen presenting cell that specifically binds a cognate co-stimulatory signal molecule on a T cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation activation, differentiation, cytokine production, and the like. A co-stimulatory ligand can include but is not limited to CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory igand (ICOS-L), intercellular adhesion molecule (ICAM, CD30L, CD40, CD70, CD83, HLA-G, MICA, M1CB, HVEM, lymphotoxin β receptor, 3/TR6, ILT3, ILT4, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also encompasses, inter alia, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as but not

limited to, CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83.

An “antibody” is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, but also antigen binding fragments thereof (such as Fab, Fab', $F(ab')_2$, Fv), single chain (scFv) and domain antibodies (including, for example, shark and camelid antibodies), and fusion proteins comprising an antibody, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site. An antibody includes an antibody of any class, such as IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant region of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant regions that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antigen binding fragment" or “antigen binding portion” of an antibody, as used herein, refers to one or more fragments of an intact antibody that retain the ability to specifically bind to a given antigen (e.g., EGFRvIII). Antigen binding functions of an antibody can be performed by fragments of an intact antibody. Examples of binding fragments encompassed within the term "antigen binding fragment" of an antibody include Fab; Fab'; $F(ab')_2$; an Fd fragment consisting of the VH and CH1 domains; an Fv fragment consisting of the VL and VH domains of a single arm of an antibody; a single domain antibody (dAb) fragment (Ward et al., Nature 341:544-546, 1989), and an isolated complementarity determining region (CDR).

An antibody, an antibody conjugate, or a polypeptide that “preferentially binds” or “specifically binds” (used interchangeably herein) to a target (e.g., EGFRvIII protein) is a term well understood in the art, and methods to determine such specific or

preferential binding are also well known in the art. A molecule is said to exhibit "specific binding" or "preferential binding" if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular cell or substance than it does with alternative cells or substances. An antibody "specifically binds" or "preferentially binds" to a target if it binds with greater affinity, avidity, more readily, and/or with greater duration than it binds to other substances. For example, an antibody that specifically or preferentially binds to an EGFRvIII epitope is an antibody that binds this epitope with greater affinity, avidity, more readily, and/or with greater duration than it binds to other EGFRvIII epitopes or non- EGFRvIII epitopes. It is also understood that by reading this definition, for example, an antibody (or moiety or epitope) that specifically or preferentially binds to a first target may or may not specifically or preferentially bind to a second target. As such, "specific binding" or "preferential binding" does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding.

A "variable region" of an antibody refers to the variable region of the antibody light chain or the variable region of the antibody heavy chain, either alone or in combination. As known in the art, the variable regions of the heavy and light chain each consist of four framework regions (FR) connected by three complementarity determining regions (CDRs) also known as hypervariable regions. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (i.e., Kabat et al. Sequences of Proteins of Immunological Interest, (5th ed., 1991, National Institutes of Health, Bethesda MD)); and (2) an approach based on crystallographic studies of antigen-antibody complexes (Al-lazikani et al., 1997, J. Molec. Biol. 273:927-948). As used herein, a CDR may refer to CDRs defined by either approach or by a combination of both approaches.

A "CDR" of a variable domain are amino acid residues within the variable region that are identified in accordance with the definitions of the Kabat, Chothia, the accumulation of both Kabat and Chothia, AbM, contact, and/or conformational definitions or any method of CDR determination well known in the art. Antibody CDRs may be identified as the hypervariable regions originally defined by Kabat et al. See,

e.g., Kabat et al., 1992, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, NIH, Washington D.C. The positions of the CDRs may also be identified as the structural loop structures originally described by Chothia and others. See, e.g., Chothia et al., Nature 342:877-883, 1989. Other approaches to CDR identification include the "AbM definition," which is a compromise between Kabat and Chothia and is derived using Oxford Molecular's AbM antibody modeling software (now Accelrys®), or the "contact definition" of CDRs based on observed antigen contacts, set forth in MacCallum et al., J. Mol. Biol., 262:732-745, 1996. In another approach, referred to herein as the "conformational definition" of CDRs, the positions of the CDRs may be identified as the residues that make enthalpic contributions to antigen binding. See, e.g., Makabe et al., Journal of Biological Chemistry, 283:1156-1166, 2008. Still other CDR boundary definitions may not strictly follow one of the above approaches, but will nonetheless overlap with at least a portion of the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. As used herein, a CDR may refer to CDRs defined by any approach known in the art, including combinations of approaches. The methods used herein may utilize CDRs defined according to any of these approaches. For any given embodiment containing more than one CDR, the CDRs may be defined in accordance with any of Kabat, Chothia, extended, AbM, contact, and/or conformational definitions.

As used herein, "monoclonal antibody" refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the invention may be made by the hybridoma method first described by Kohler and Milstein, Nature 256:495, 1975, or may be made by recombinant DNA methods such as

described in U.S. Pat. No. 4,816,567. The monoclonal antibodies may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature 348:552-554, 1990, for example.

As used herein, "humanized" antibody refers to forms of non-human (*e.g.* murine) antibodies that are chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. Preferably, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences, but are included to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Preferred are antibodies having Fc regions modified as described in WO 99/58572. Other forms of humanized antibodies have one or more CDRs (CDR L1, CDR L2, CDR L3, CDR H1, CDR H2, or CDR H3) which are altered with respect to the original antibody, which are also termed one or more CDRs “derived from” one or more CDRs from the original antibody.

As used herein, “human antibody” means an antibody having an amino acid sequence corresponding to that of an antibody produced by a human and/or which has been made using any of the techniques for making human antibodies known to those skilled in the art or disclosed herein. This definition of a human antibody includes antibodies comprising at least one human heavy chain polypeptide or at least one human light chain polypeptide. One such example is an antibody comprising murine light chain and human heavy chain polypeptides. Human antibodies can be produced

using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al., Nature Biotechnology, 14:309-314, 1996; Sheets et al., Proc. Natl. Acad. Sci. (USA) 95:6157-6162, 1998; Hoogenboom and Winter, J. Mol. Biol., 227:381, 1991; Marks et al., J. Mol. Biol., 222:581, 1991). Human antibodies can also be made by immunization of animals into which human immunoglobulin loci have been transgenically introduced in place of the endogenous loci, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. This approach is described in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016. Alternatively, the human antibody may be prepared by immortalizing human B lymphocytes that produce an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or from single cell cloning of the cDNA, or may have been immunized *in vitro).* See, e.g., Cole et al. Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77, 1985; Boerner et al., J. Immunol., 147 (1):86-95, 1991; and U.S. Pat. No. 5,750,373.

The term “chimeric antibody” is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody.

The terms “polypeptide”, “oligopeptide”, “peptide” and “protein” are used interchangeably herein to refer to chains of amino acids of any length. For example, the chain may be relatively short (e.g., 10-100 amino acids), or longer. The chain may be linear or branched, it may comprise modified amino acids, and/or may be interrupted by non-amino acids. The terms also encompass an amino acid chain that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that the polypeptides can occur as single chains or associated chains.

A “monovalent antibody” comprises one antigen binding site per molecule (e.g., IgG or Fab). In some instances, a monovalent antibody can have more than one antigen binding sites, but the binding sites are from different antigens.

A “bivalent antibody” comprises two antigen binding sites per molecule (e.g., IgG). In some instances, the two binding sites have the same antigen specificities. However, bivalent antibodies may be bispecific.

A “bispecific” or “dual-specific” is a hybrid antibody having two different antigen binding sites. The two antigen binding sites of a bispecific antibody bind to two different epitopes, which may reside on the same or different protein targets.

A “bifunctional” is an antibody having identical antigen binding sites (i.e., identical amino acid sequences) in the two arms but each binding site can recognize two different antigens.

Antibodies of the invention can be produced using techniques well known in the art, *e.g.*, recombinant technologies, phage display technologies, synthetic technologies or combinations of such technologies or other technologies readily known in the art (see, for example, Jayasena, S.D., Clin. Chem., 45: 1628-50, 1999 and Fellouse, F.A., et al, J. Mol. Biol., 373(4):924-40, 2007).

As known in the art, “polynucleotide,” or “nucleic acid,” as used interchangeably herein, refer to chains of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a chain by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the chain. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, “caps”, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.),

those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha- or beta-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

As known in the art a "constant region" of an antibody refers to the constant region of the antibody light chain or the constant region of the antibody heavy chain, either alone or in combination.

As used herein, "substantially pure" refers to material which is at least 50% pure (i.e., free from contaminants), more preferably, at least 90% pure, more preferably, at least 95% pure, yet more preferably, at least 98% pure, and most preferably, at least 99% pure.

A "host cell" includes an individual cell or cell culture that can be or has been a recipient for vector(s) for incorporation of polynucleotide inserts. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent cell due

to natural, accidental, or deliberate mutation. A host cell includes cells transfected in vivo with a polynucleotide(s) of this invention.

As used herein, “immune cell” refers to a cell of hematopoietic origin functionally involved in the initiation and/or execution of innate and/or adaptative immune response.

As known in the art, the term "Fc region" is used to define a C-terminal region of an immunoglobulin heavy chain. The "Fc region" may be a native sequence Fc region or a variant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The numbering of the residues in the Fc region is that of the EU index as in Kabat. Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., 1991. The Fc region of an immunoglobulin generally comprises two constant regions, CH2 and CH3.

As used in the art, "Fc receptor" and “FcR” describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. FcRs are reviewed in Ravetch and Kinet, Ann. Rev. Immunol., 9:457-92, 1991; Capel et al., Immunomethods, 4:25-34, 1994; and de Haas et al., J. Lab. Clin. Med., 126:330-41, 1995. “FcR” also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol., 117:587, 1976; and Kim et al., J. Immunol., 24:249, 1994).

The term “compete”, as used herein with regard to an antibody, means that a first antibody, or an antigen binding fragment (or portion) thereof, binds to an epitope in a manner sufficiently similar to the binding of a second antibody, or an antigen binding portion thereof, such that the result of binding of the first antibody with its cognate epitope is detectably decreased in the presence of the second antibody compared to the binding of the first antibody in the absence of the second antibody. The alternative, where the binding of the second antibody to its epitope is also detectably decreased in the presence of the first antibody, can, but need not be the case. That is, a first

antibody can inhibit the binding of a second antibody to its epitope without that second antibody inhibiting the binding of the first antibody to its respective epitope. However, where each antibody detectably inhibits the binding of the other antibody with its cognate epitope or ligand, whether to the same, greater, or lesser extent, the antibodies are said to "cross-compete" with each other for binding of their respective epitope(s). Both competing and cross-competing antibodies are encompassed by the invention. Regardless of the mechanism by which such competition or cross-competition occurs (e.g., steric hindrance, conformational change, or binding to a common epitope, or portion thereof), the skilled artisan would appreciate, based upon the teachings provided herein, that such competing and/or cross-competing antibodies are encompassed and can be useful for the methods disclosed herein.

As used herein "autologous" means that cells, a cell line, or population of cells used for treating patients are originating from said patient or from a Human Leucocyte Antigen (HLA) compatible donor.

As used herein "allogeneic" means that cells or population of cells used for treating patients are not originating from said patient but from a donor.

As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, one or more of the following: reducing the proliferation of (or destroying) neoplastic or cancerous cells, inhibiting metastasis of neoplastic cells, shrinking or decreasing the size of EGFRvIII expressing tumor, remission of an EGFRvIII associated disease (e.g., cancer), decreasing symptoms resulting from an EGFRvIII associated disease (e.g., cancer), increasing the quality of life of those suffering from an EGFRvIII associated disease (e.g., cancer), decreasing the dose of other medications required to treat an EGFRvIII associated disease (e.g., cancer), delaying the progression of an EGFRvIII associated disease (e.g., cancer), curing an EGFRvIII associated disease (e.g., cancer), and/or prolong survival of patients having an EGFRvIII associated disease (e.g., cancer).

"Ameliorating" means a lessening or improvement of one or more symptoms as compared to not administering an EGFRvIII specific CAR. "Ameliorating" also includes shortening or reduction in duration of a symptom.

As used herein, an "effective dosage" or "effective amount" of drug, compound, or pharmaceutical composition is an amount sufficient to effect any one or more

beneficial or desired results. For prophylactic use, beneficial or desired results include eliminating or reducing the risk, lessening the severity, or delaying the outset of the disease, including biochemical, histological and/or behavioral symptoms of the disease, its complications and intermediate pathological phenotypes presenting during development of the disease. For therapeutic use, beneficial or desired results include clinical results such as reducing incidence or amelioration of one or more symptoms of various EGFRvIII associated diseases or conditions (such as for example glioblastoma multiform), decreasing the dose of other medications required to treat the disease, enhancing the effect of another medication, and/or delaying the progression of the EGFRvIII associated disease of patients. An effective dosage can be administered in one or more administrations. For purposes of this invention, an effective dosage of drug, compound, or pharmaceutical composition is an amount sufficient to accomplish prophylactic or therapeutic treatment either directly or indirectly. As is understood in the clinical context, an effective dosage of a drug, compound, or pharmaceutical composition may or may not be achieved in conjunction with another drug, compound, or pharmaceutical composition. Thus, an “effective dosage” may be considered in the context of administering one or more therapeutic agents, and a single agent may be considered to be given in an effective amount if, in conjunction with one or more other agents, a desirable result may be or is achieved.

An “individual” or a "subject" is a mammal, more preferably, a human. Mammals also include, but are not limited to primates, horses, dogs, cats, mice and rats.

As used herein, "vector" means a construct, which is capable of delivering, and, preferably, expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

As used herein, "expression control sequence" means a nucleic acid sequence that directs transcription of a nucleic acid. An expression control sequence can be a promoter, such as a constitutive or an inducible promoter, or an enhancer. The expression control sequence is operably linked to the nucleic acid sequence to be transcribed.

As used herein, "pharmaceutically acceptable carrier" or "pharmaceutical acceptable excipient" includes any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Preferred diluents for aerosol or parenteral administration are phosphate buffered saline (PBS) or normal (0.9%) saline. Compositions comprising such carriers are formulated by well known conventional methods (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. Gennaro, ed., Mack Publishing Co., Easton, PA, 1990; and Remington, The Science and Practice of Pharmacy 21st Ed. Mack Publishing, 2005).

The term "k_{on}" or "k_a", as used herein, refers to the rate constant for association of an antibody to an antigen. Specifically, the rate constants (k_{on}/k_a and k_{off}/k_d) and equilibrium dissociation constants may be measured using, for example, full-length antibodies and/or Fab antibody fragments and corresponding antigen.

The term "k_{off}" or "k_d", as used herein, refers to the rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "K_D", as used herein, refers to the equilibrium dissociation constant of an antibody-antigen interaction.

Determinations of the association and dissociation rate constants, k_{on} and k_{off} respectively, may be made using a surface plasmon resonance-based biosensor to characterize an analyte/ligand interaction under conditions where the analyte is monovalent with respect to binding a ligand that is immobilized at low capacity onto a sensor surface via a capture reagent. The analysis is performed using a kinetic titration methodology as described in Karlsson et al., Anal. Biochem 349, 136-147, 2006. The sensor chip, capturing reagent, and assay buffer employed for a given assay are chosen to give stable capture of ligand onto the sensor surface, minimize non-specific binding of the analyte to the surfaces, and yield analyte-binding responses that are appropriate for kinetic analysis, per the recommendations in Myszka, J. Mol. Recognit 12, 279-284, 1999. The analyte-binding responses per analyte/ligand interaction are double referenced and fit to a 1:1 Langmuir "mass transport limited model" with k_a, k_d and R_{max} as global parameters as described in Myszka & Morton et al., *Biophys. Chem* 64, 127-137 (1997). The equilibrium dissociation constant, K_D, is deduced from the ratio

of the kinetic rate constants, $K_D = k_{off}/k_{on}$. Such determinations preferably take place at 25°C or 37°C.

Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X." Numeric ranges are inclusive of the numbers defining the range. Generally speaking, the term "about" refers to the indicated value of the variable and to all values of the variable that are within the experimental error of the indicated value (e.g. within the 95% confidence interval for the mean) or within 10 percent of the indicated value, whichever is greater. Where the term "about" is used within the context of a time period (years, months, weeks, days etc.), the term "about" means that period of time plus or minus one amount of the next subordinate time period (e.g. about 1 year means 11-13 months; about 6 months means 6 months plus or minus 1 week; about 1 week means 6-8 days; etc.), or within 10 per cent of the indicated value, whichever is greater.

It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

Where aspects or embodiments of the invention are described in terms of a Markush group or other grouping of alternatives, the invention encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, but also the main group absent one or more of the group members. The invention also envisages the explicit exclusion of one or more of any of the group members in the claimed invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present specification, including definitions, will control. Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

Exemplary methods and materials are described herein, although methods and materials similar or equivalent to those described herein can also be used in the

practice or testing of the invention. The materials, methods, and examples are illustrative only and not intended to be limiting.

EGFRvIII specific CARs and Methods of Making Thereof

The invention provides CARs that bind to EGFRvIII (e.g., human EGFRvIII (e.g., SEQ ID NO: 201, accession number: P00533 Feature Identifier VAR_066493, or GenBank Acession No. AJN69267)). EGFRvIII specific CARs provided herein include single chain CARS and multichain CARs. The CARs have the ability to redirect T cell specificity and reactivity toward EGFRvIII in a non-MHC-restricted manner, exploiting the antigen-binding properties of monoclonal antibodies. The non-MHC-restricted antigen recognition gives T cells expressing CARs the ability to recognize an antigen independent of antigen processing, thus bypassing a major mechanism of tumor escape.

In some embodiments, CARs provided herein comprise an extracellular ligand-binding domain (e.g., a single chain variable fragment (scFv)), a transmembrane domain, and an intracellular signaling domain. In some embodiments, the extracellular ligand-binding domain, transmembrane domain, and intracellular signaling domain are in one polypeptide, i.e., in a single chain. Multichain CARs and polypeptides are also provided herein. In some embodiments, the multichain CARs comprise: a first polypeptide comprising a transmembrane domain and at least one extracellular ligand-binding domain, and a second polypeptide comprising a transmembrane domain and at least one intracellular signaling domain, wherein the polypeptides assemble together to form a multichain CAR.

In some embodiments, an EGFRvIII specific multichain CAR is based on the high affinity receptor for IgE (FcεRI). The FcεRI expressed on mast cells and basophiles triggers allergic reactions. FcεRI is a tetrameric complex composed of a single α subunit, a single β subunit, and two disulfide-linked γ subunits. The α subunit contains the IgE-binding domain. The β and γ subunits contain ITAMs that mediate signal transduction. In some embodiments, the extracellular domain of the FcRα chain is deleted and replaced by an EGFRvIII specific extracellular ligand-binding domain. In some embodiments, the multichain EGFRvIII specific CAR comprises an scFv that binds specifically to EGFRvIII, the CD8α hinge, and the ITAM of the FcRβ chain. In some embodiments, the CAR may or may not comprise the FcRγ chain.

In some embodiments, the extracellular ligand-binding domain comprises an scFv comprising the light chain variable (VL) region and the heavy chain variable (VH) region of a target antigen specific monoclonal antibody joined by a flexible linker. Single chain variable region fragments are made by linking light and/or heavy chain variable regions by using a short linking peptide (Bird et al., Science 242:423-426, 1988). An example of a linking peptide is the GS linker having the amino acid sequence $(GGGGS)_4$ (SEQ ID NO: 202), which bridges approximately 3.5 nm between the carboxyl terminus of one variable region and the amino terminus of the other variable region. Linkers of other sequences have been designed and used (Bird et al., 1988, supra). In general, linkers can be short, flexible polypeptides and preferably comprised of about 20 or fewer amino acid residues. Linkers can in turn be modified for additional functions, such as attachment of drugs or attachment to solid supports. The single chain variants can be produced either recombinantly or synthetically. For synthetic production of scFv, an automated synthesizer can be used. For recombinant production of scFv, a suitable plasmid containing polynucleotide that encodes the scFv can be introduced into a suitable host cell, either eukaryotic, such as yeast, plant, insect or mammalian cells, or prokaryotic, such as E. coli. Polynucleotides encoding the scFv of interest can be made by routine manipulations such as ligation of polynucleotides. The resultant scFv can be isolated using standard protein purification techniques known in the art.

In some embodiments, the extracellular ligand-binding domain comprises (a) a VH region comprising (i) a VH complementarity determining region one (CDR1) comprising the sequence shown in SEQ ID NO: 62, 63, 64, 74, 75, 76, 80, 81, 82, 88, 89, 90, 93, 94, 95, 99, 100, 101, 109, 110, 111, 115, 116, 117, 121, 122, 123, 132, 133,134, 137, 138, 139, 143, 144, or 145; (ii) a VH CDR2 comprising the sequence shown in SEQ ID NO: 65, 66, 68, 69, 70, 71, 77, 78, 83, 84, 86, 87, 91, 92, 96, 97, 98, 102, 103, 105, 106, 112, 113, 118, 119, 124, 125, 127, 128, 130, 131, 135, 136, 140, 141, 146, or 147; and iii) a VH CDR3 comprising the sequence shown in SEQ ID NO: 67, 72, 73, 79, 85, 104, 107, 108, 114, 120, 126, 129, 142, or 148; and/or a VL region comprising (i) a VL CDR1 comprising the sequence shown in SEQ ID NO: 149, 154, 156, 159, 162, 165, 166, 168, 169, 170, 171, 173, 174, 176, 178, 181, 182, 185, 187, 190, 192, 195, or 198; (ii) a VL CDR2 comprising the sequence shown in SEQ ID NO: 150, 152, 155, 157, 160, 163, 172, 175, 179, 183, 186, 188, 191, 193, 196, or 199; and

(iii) a VL CDR3 comprising the sequence shown in SEQ ID NO: 151, 153, 158, 161, 164, 167, 177, 180, 184, 189, 194, 197, or 200. In some embodiments, the VH and VL are linked together by a flexible linker. In some embodiments a flexible linker comprises the amino acid sequence shown in SEQ ID NO: 202.

In some embodiments, the extracellular ligand-binding domain comprises (a) a VH region comprising (i) a VH complementarity determining region one (CDR1) comprising the sequence shown in SEQ ID NO: 62, 63, 64, 74, 75, 76, 80, 81, 82, 88, 89, 90, 109, 110, 111, 115, 116, 117, 121, 122, 123, 137, 138, or 139; (ii) a VH CDR2 comprising the sequence shown in SEQ ID NO: 70, 71, 77, 78, 83, 84, 86, 87, 91, 92, 112, 113, 118, 119, 124, 125, 127, 128, 140, or 141; and iii) a VH CDR3 comprising the sequence shown in SEQ ID NO: 73, 79, 85, 114, 120, 126, 129, or 142, and/or (b) a VL region comprising (i) a VL CDR1 comprising the sequence shown in SEQ ID NO: 149, 156, 159, 162, 165, 182, 185, 187, or 195; (ii) a VL CDR2 comprising the sequence shown in SEQ ID NO: 152, 157, 160, 163, 183, 186, 188, or 196; and (iii) a VL CDR3 comprising the sequence shown in SEQ ID NO: 153, 158, 161, 164, 184, 189, or 197.

In another aspect, provided is CAR, which specifically binds to EGFRvIII, wherein the CAR comprises an extracellular ligand-binding domain comprising**:** a VH region comprising a VH CDR1, VH CDR2, and VH CDR3 of the VH sequence shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 30, 32, 34, 35, 37, 39, 41, 43, 44, 46, 48, or 50; and/or a VL region comprising VL CDR1, VL CDR2, and VL CDR3 of the VL sequence shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 29, 31, 33, 36, 38, 40, 42, 45, 47, 49, or 51. In some embodiments, the VH and VL are linked together by a flexible linker. In some embodiments a flexible linker comprises the amino acid sequence shown in SEQ ID NO: 202.

In some embodiments, the CAR comprises an extracellular ligand-binding domain comprising**:** a VH region comprising a VH CDR1, VH CDR2, and VH CDR3 of the VH sequence shown in SEQ ID NO: 5, 9, 11, 13, 15, 37, 39, 41, 43, or 48; and/or a VL region comprising VL CDR1, VL CDR2, and VL CDR3 of the VL sequence shown in SEQ ID NO: 6, 10, 12, 14, 16, 38, 40, 42, or 49. In some embodiments, the VH and VL are linked together by a flexible linker. In some embodiments a flexible linker comprises the amino acid sequence shown in SEQ ID NO: 202.

In some embodiments, a CAR of the invention comprises an extracellular ligand-binding domain having any one of partial light chain sequence as listed in Table 1

and/or any one of partial heavy chain sequence as listed in Table 1. In Table 1, the underlined sequences are CDR sequences according to Kabat and in bold according to Chothia. The different mAbs of Table 1 may also be referred to herein as different anti-EGFRvIII antibody "clones".

Table 1

mAb	Light Chain	Heavy Chain
m62G7	DVVMTQTPLTLSVTIGQPASISC**K** **SSQSLLYSNGKTYLN**WLLQRPG QSPKRLIY**LVSKLDS**GVPDRFTG SGSGTDFTLKISRVEAEDLGFYY C**VQDTHFPLT**FGAGTKLELK (SEQ ID NO: 2)	EVQLQQSGPELVKPGASVKISCKT S**GYTFTD**YTLHWVKQSHVKSLEWI G**GIDPINGGTTY**NQKFKGKATLTV DKSSSTAYMELRSLTSEDSAVYYC AR**GEAMDS**WGQGTSVTVSS (SEQ ID NO: 1)
h62G7	DVVMTQSPLSLPVTLGQPASISC **KSSQSLLYSNGKTYLN**WFQQRP GQSPRRLIY**LVSKLDS**GVPDRFS GSGSGTDFTLKISRVEAEDVGVY YC**VQDTHFPLT**FGGGTKVEIK (SEQ ID NO: 4)	QVQLVQSGAEVKKPGASVKVSCK AS**GYTFTD**YTLHWVRQAPGQGLE WMG**GINPINGGTTY**NQKFKGRVT MTRDTSTSTVYMELSSLRSEDTAV YYCAR**GEAMDS**WGQGTLVTVSS (SEQ ID NO: 3)
h62G7 -L6/EQ	DVVMTQSPLSLPVTLGQPASISC **KSSQSLLYSNGKTYLN**WFQQRP GQSPRRLIY**QVSKLDS**GVPDRFS GSGSGTDFTLKISRVEAEDVGVY YC**GQDTHFPLT**FGGGTKVEIK (SEQ ID NO: 6)	QVQLVQSGAEVKKPGASVKVSCK AS**GYTFTD**YTLHWVRQAPGQGLE WMG**GIWPITGGTTY**NQKFKGRVT MTRDTSTSTVYMELSSLRSEDTAV YYCAR**GEAQGS**WGQGTLVTVSS (SEQ ID NO: 5)
h62G7 - H14/L1 -DV	DVVMTQSPLSLPVTLGQPASISC **KSSQSLLYSNDKTYTN**WFQQRP GQSPRRLIY**EVSKLDV**GVPDRFS GSGSGTDFTLKISRVEAEDVGVY YC**GQDTHFPLT**FGGGTKVEIK (SEQ ID NO: 8)	QVQLVQSGAEVKKPGASVKVSCK AS**GYTFTD**YTLHWVRQAPGQGLE WMG**GIWPITGGTTY**NQKFKGRVT MTRDTSTSTVYMELSSLRSEDTAV YYCAR**GEAEGS**WGQGTLVTVSS (SEQ ID NO: 7)
42G9	EVVLTQSPATLSVSPGERATLSC	QVTLKESGPVLLKPTETLTLTCTVS

mAb	Light Chain	Heavy Chain
	RASQSVRSNLAWYQQKSGQAP RLLIY**GSTIRAT**GVPARFSGSGS GTEFTLTISSLQSEDFAVYYC**QQ YSDWPFT**FGPGTKVDIK (SEQ ID NO: 10)	**GFSLSNPR**MGVSWIRQPPGKALE WFA**HIFSTDEKSL**KLSLRSRLTLSK DTSKSQVVLTMTNMAPVDSATYY CAR**DSSNYEGYFDF**WGQGTLVTV SS (SEQ ID NO: 9)
32A10	EVVMTQSPATLSVSPGERVTLSC **RASQSVSSNFA**WYQQRPGQAP RLLLY**GATTRAT**GLPGRFSGSGS GTENILTISSLQSEDFAIYFC**QQY KDWPFT**FGPGSKVDIK (SEQ ID NO: 12)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLSNAR**MGVSWIRQPPGKAL EWLA**HIFSTDEKSI**RRSLRSRLTLS KDTSKSQVVLTMTNMDPVDTATY FCAR**DSSNYEGYFDY**WGQGTLVT VSS (SEQ ID NO: 11)
20B9	EIVMTQSPATLSVSPGERATLSC **RVSQSIGANLA**WYQQKFGQAPR LLIY**GASTRAT**GIPVRFSGGGSG TEFTLTISSLQSEDFAIYSC**QQYIY WPFT**FGPGTTVDIK (SEQ ID NO: 14)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLSNAR**MGVSWIRQPPGKAL EWLG**HIFSTDEKSY**STSLRGRITIS KDTSRGLVVLTLTNMDPVDTATYY CAR**DSSNYEGYFDF**WGPGFLVTV SS (SEQ ID NO: 13)
14C11	EIVMTQSPATLSVSPGERATLSC **RASQSVSNNLA**WYQQKPGQAP RLLIY**GASTRAT**GVPARFSGSDS GTEFSLTISSLQSEDFAVYFC**QQ YKDWPFT**FGPGTKVEIK (SEQ ID NO: 16)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLNNAR**MGVSWIRQPPGKAL EWFA**HIFSTDEKSF**RTSLRSRLTL SKDTSKSQVVLTMTNMDPVDTAT YYCAR**DSSNYEGYFDY**WGQGILV TVSS (SEQ ID NO: 15)
21E11	DMVVTQSPATLSVSPGERATLSC **RASQSVGSDLA**WYQQPPGQSP RLLIY**GASTRAT**GVPARFSGSGS GTDFTLTITSLESEDFAVYYC**QQY NDWPFT**FGPGTKVDIK (SEQ ID NO: 18)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLSNVR**MGVSWIRQPPGKAL EWFA**HIFSSDEKSI**RRSLRSRLTLS KDTSKSQVVLTMTNMDPVDTATY YCAR**DSSNYEGYFDF**WGQGTLVT VSSN (SEQ ID NO: 17)

mAb	Light Chain	Heavy Chain
49B11	EMEVTQSPATLSVSPGERATLSC **RASQNIGSDLA**WYQQQSGQAP RLLIS**GASTRAT**GVPTRFSGSGS GTDFTLTITSLQSEDFAVYYC**QQ YNDWPFT**FGPGTKVDIK (SEQ ID NO: 20)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLSNVR**MGVSWIRQPPGKAL EWFA**HIFSSDEKSI**RRSLRSRLTLS KDTSKSQVVLTMTNMDPVDTATY YCAR**DSSNYEGYFDY**WGQGTLVT VSS (SEQ ID NO: 19)
46E10	EVVMTQSPPNLSVSPGERATLSC **RASQSVTSNFA**WYQQRPGQSP RLLLY**GASTRAT**GVPGRFSGSG SGTENILTISSLQSEDFAVYFC**QQ YKDWPFT**FGPGSKVDIK (SEQ ID NO: 22)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLSNAR**MGVSWIRQPPGKAL EWLA**HIFSTDEKSI**RRSLRSRLTLS KDTSKSQVVLIMTNMDPVDTATYY CAR**DSSNYEGYFDY**WGQGTLVTV SS (SEQ ID NO: 21)
12H6	EVVMTQSPATLSVSPGERATLSC **RASQGVSSNFA**WYQQRPGQSP RLLLY**GASTRAT**GVPGRFSGSG SGTENILTISSLQSEDFAIYFC**QQ YKDWPFT**FGPGSKVDIK (SEQ ID NO: 24)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLSNAR**MGVSWIRQPPGKAL EWLA**HIFSTDEKSI**RRSLRSRLTLS KDTSKSQVVLTMTNMDPVDTATY YCAR**DSSNYEGYFDY**WGQGTLVT VSS (SEQ ID NO: 23)
19A9	EVVMTQSPATLSVSPGERATLSC **RASQSVNRNLA**WYQQKPGQAP RLLIF**GTSTRAT**GIPARFSGSGSG TEFTLTIDSLQSEHSGLYYC**QQY NDWPFT**FGPGTKVDIK (SEQ ID NO: 26)	QVTLEESGPVLVKPTETLTLTCTV S**GFSLSNAR**MGVSWIRQPPGKAP EWFA**HIFSTDEKSL**RLSLRSRLTL SKDTSKSQVVLTMTNMDPVDTAT YYCAR**DSSNYEGYFDY**WGQGTLV TVSS (SEQ ID NO: 25)
11B11	EVLMTQSPATLSVSPGERATLSC **RASQSVSTNFA**WYQQRPGQAP RLLLF**GASTRAT**GIPGRFSGSGS GTENILTISSLQSEDFAIYFC**QQY KDWPFT**FGPGSKVEIK (SEQ ID NO: 28)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLSNAK**MGVSWIRQPPGKAL EWLA**HIFSTDEKSI**RRSLRSRLTM SKDTSKSQVVLTMTNMDPVDTAT YYCVR**DSSNYEGYFDY**WGQGTLV TVSS (SEQ ID NO: 27)
21E7	DVVLTQSPATLSVSPGERATLSC	QVTLEESGPVLVKPTETLTLTCTV

mAb	Light Chain	Heavy Chain
	RASQSVNSNLAWYQQNPGQAP RLLIF**GSSTRAT**GIPASFSGSGSG TEFTLTINSLQSEHSAVYYC**QQY NDWPFT**FGPGTKVDIK (SEQ ID NO: 29)	S**GFSLSNAR**MGVSWIRQPPGKAP EWFA**HIFSTDEKSL**RLSLRSRLTL SKDTSKSQVVLTMTNMDPVDTAT YYCAR**DSSNYEGYFDY**WGQGTLV TVSS (SEQ ID NO: 25)
12B2	EVVMTQSPATLSVSPGERATLSC **RASQSVINNLA**WYQQKPGQAPR LLIY**GTSTRAT**DIPARFSGSGSGT EFTLTISSLQSEDFAVYYC**QDYN NWPFT**FGPGTKVDIK (SEQ ID NO: 31)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLSNPR**MGVSWIRQPPGKAL EWLG**HIFSSDEKSY**RLSLRSRLSIS KDTSKSQVVLTMTNMDPVDTATY YCVR**DSSNYGGYFDY**WGQGTLV TVSS (SEQ ID NO: 30)
11F10	EIVMTQSPATLSVSPGERTTLSC **RASQSVGSNLA**WYQQKPGQAP RLLIY**GASTRASG**VPARFSGSGS GTEFTLTISSLQSEDFAVYSC**QEY NNWPFT**FGQGTKVEIK (SEQ ID NO: 33)	QVTLKESGPVLVKPIETLTLTCTVC **GFSLSNPR**MGVSWIRQPPGKALE WLG**HIFSSDEKSY**RLFLRSRLSISK DTSKSQVVLTMTNMDPVDTATYY CAR**DSSDYEGYFDY**WGQGTLVTV SS (SEQ ID NO: 32)
17G11	EVVMTQSPATLSVSPGERATLSC **RASQSVINNLA**WYQQKPGQAPR LLIY**GTSTRAT**DIPARFSGSGSGT EFTLTISSLQSEDFAVYYC**QDYN NWPFT**FGPGTKVDIK (SEQ ID NO: 31)	QVTLKESGPVLVKPTETLTLTCTVF **GFSLSNPR**MGVSWIRQPPGKAPE WLG**HIFSSDEKSY**RLSLRSRLSISK DTSKSQVVFXMTNMDPGDPATYY CVR**DSSNYEEYFDY**WGQGTLVTV SS (SEQ ID NO: 34)
29D5	KIVMTQSPATLSVSPGERATLSC **RANQIVSSNLA**WYQQKPGQAPR LLVF**GTSTRAT**GIPIRFSGSGSGT EFTLTVSSLQSEDFAVYVC**QQYN DWPFT**FGPGTKVDIK (SEQ ID NO: 36)	QVTLKESGPVLVKPTETLTLTCTV S**GFSLSNPR**MGVSWLRQPPGKAL EWFA**HIFSTDEKSY**SPSLRGRLTV SKDTSKSQVVLTLTNMDPVDTATY YCAR**DSSNYEGYFDY**WGQGTLVT VSS (SEQ ID NO: 35)
30D8	DIVMTQSPLSLPVTPGEPASISC**R SSQSLLHNKRNNYLD**WFLQKPG	EVQLVESGGGLVKPGGSLRLSCE AS**GFTFSD**AWMSWVRQAPGKGL

mAb	Light Chain	Heavy Chain
	QSPQLLIY**LASNRAS**GVPDRFSG GGSGTDFTLKISRVEAEDVGVYY C**MQAQQTPIT**FGQGTRLEIK (SEQ ID NO: 38)	EWVG**RIKSKTDGGTTDY**VVPLNG RFIISRDDSRNTLYLQLNNLKTEDT AVYYCTT**VPGSYGY**WGQGTLVTV SS (SEQ ID NO: 37)
20E12	DIVLTQSPLSLSVTPGEPASISC**R SSQSLLYSNGKNYLD**WFLHKPG QSPQLLIY**LGSNRAS**GVPDRFSG SGSGIDFILKISRVEAEDVGVYYC **MQAQQTPIT**FGQGTRLEIK (SEQ ID NO: 40)	EVNLVESGGGLVKPGGSLRLSCE ASGFTFSYAWMSWVRQAPGKGL EWVG**RIKSIADGGATDY**AAPVRN RFTISRDDSRNTLYLEMHSLKTED TAVYYCTT**IPGNDAFDM**WGQGTM VTVSS (SEQ ID NO: 39)
26B9	DIVLTQSPLSLPVTPGEPASISC**R SSQSLLHRDGFNYLD**WFLQKPG QSPQLLIY**LASSRAS**GVPDRFSG SDSGTDFTLKISRVEAEDVGVYY C**MQALQTPIT**FGQGTRLEIK (SEQ ID NO: 42)	EVQLVESWGVLVKPGGSLRLSCA ASGFIF**NN**AWMSWVRQAPGKGLE WIG**RIKSKSDGGTTDY**AAPVKDRF TISRDDSKDTLYLQMNGLKTEDTA VYFCTT**APGGPFDY**WGQGTLVTV SS (SEQ ID NO: 41)
32G8	DIVLTQSPLSLSVTPGEPASISC**R SSQSLLYSNGKNYLD**WFLHKPG QSPQLLIY**LGSNRAS**GVPDRFSG SGSGIDFILKISRVEAEDVGVYYC **MQAQQTPIT**FGQGTRLEIK (SEQ ID NO: 40)	EVNLVESGGGLVKPGGSLRLSCE ASGFTFSYAWMSWVRQAPGKGL EWVG**RIKSITDGGVIDY**AAPVRNR CTISRDDSRNTLYLEMHSLKTEDT AVYYCTT**IPGNDDFDM**WGQGRM VTVSS (SEQ ID NO: 43)
34E7	DIVLTQSPLSLSVTPGEPASISC**R STQSLLYSNGKNYLD**WFLHKPG QSPQLLIF**LGSIRAS**GVPDRFSG SGSGIDFILKISRVEAEDVGVYYC **MQAQQTPIT**FGQGTRLEIK (SEQ ID NO: 45)	EVNLVESGGGLVKPGGSLRLSCE ASGFTFSYAWMSWVRQAPGKGL EWVG**RIKSINDGGATDY**ASPVRN RFTISRDDSRNMLYLEMHSLKTED TAVYYCTT**IPGNDAFDM**WGQGTL VTVSS (SEQ ID NO: 44)
20G5	DIVLTQSPLSLPVTPGEPASISC**R SSQSLLYSDRRNYLD**WFLQKPG QSPHLLIY**LGSYRAS**GVPDRFSG	EVQLVESGGDLVKPGGSLRLSCA ASGFTF**TN**AWMSWVRQAPGKGL EWVG**RIKSKIDGGTTDY**AAPVKG

mAb	Light Chain	Heavy Chain
	SGSGTDFTLKISRVEAEDVGVYY C**MQALQIPIT**FGQGTRLEIK (SEQ ID NO: 47)	RFIISRDDSKNTLSLQMNSLKTEDT AMYYCTT**APGGPFDY**WGQGSLV TVSS (SEQ ID NO: 46)
C6	ELQSVLTQPPSASGTPGQRVTIS C**SGSSSNIGSNYVY**WYQQLPGT APKILIY**RNNQRPS**GVPDRFSGS KSGTSASLAISGLRSEDEADYYC **AAWDDNLSGWV**FGTGTKLTVL (SEQ ID NO: 49)	QVQLVQSGAEVKKPGSSVKVSCK AS**GDTFSS**NAISWVRQAPGQGLE WMG**VIIPIFGTADY**AQKFQGRVTIT ADESTSTAYMELSSLRSEDTAVYY CAR**HTYHEYAGGYYGGAMDP**WG QGTLVTVSS (SEQ ID NO: 48)
B5	DIQMTQSPSSLSASVGDRVTITC **RASQSISSYLN**WYQQKPGKAPK LLIY**AASSLQS**GVPSRFSGSGSG TDFTLTISSLQPEDFATYYC**QQSY STPLT**FGQGTKVEIK (SEQ ID NO: 51)	EVQLLESGGGLVQPGGSLRLSCA AS**GFTFSN**YAMSWVRQAPGKGLE WVS**DISGGGGRTYY**ADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAV YYCAR**AGLLYGGGVYPMDI**WGQ GTLVTVSS (SEQ ID NO: 50)

Also provided herein are CDR portions of extracellular ligand-binding domains of CARs to EGFRvIII (including Chothia, Kabat CDRs, and CDR contact regions). Determination of CDR regions is well within the skill of the art. It is understood that in some embodiments, CDRs can be a combination of the Kabat and Chothia CDR (also termed "combined CRs" or "extended CDRs"). In some embodiments, the CDRs are the Kabat CDRs. In other embodiments, the CDRs are the Chothia CDRs. In other words, in embodiments with more than one CDR, the CDRs may be any of Kabat, Chothia, combination CDRs, or combinations thereof. Table 2 provides examples of CDR sequences provided herein.

Table 2

Heavy Chain			
mAb	**CDRH1**	**CDRH2**	**CDRH3**
m62G7	TDYTLH (SEQ ID NO: 62) (Kabat); GYTFTD (SEQ ID NO: 63) (Chothia);	GIDPINGGTTYNQKFK G (SEQ ID NO: 65) (Kabat) GIDPINGGTTY (SEQ	GEAMDS (SEQ ID NO: 67)

	GYTFTDYTLH (SEQ ID NO: 64) (extended)	ID NO: 66) (Chothia)	
h62G7	TDYTLH (SEQ ID NO: 62) (Kabat); GYTFTD (SEQ ID NO: 63) (Chothia); GYTFTDYTLH (SEQ ID NO: 64) (extended)	GINPINGGTTYNQKFKG (SEQ ID NO: 68) (Kabat) GINPINGGTTY (SEQ ID NO: 69) (Chothia)	GEAMDS (SEQ ID NO: 67)
h62G7-H14	TDYTLH (SEQ ID NO: 62) (Kabat); GYTFTD (SEQ ID NO: 63) (Chothia); GYTFTDYTLH (SEQ ID NO: 64) (extended)	GIWPITGGTTYNQKFKG (SEQ ID NO: 70) (Kabat) GIWPITGGTTY (SEQ ID NO: 71) (Chothia)	GEAEGS (SEQ ID NO: 72)
h62G7-EQ	TDYTLH (SEQ ID NO: 62) (Kabat); GYTFTD (SEQ ID NO: 63) (Chothia); GYTFTDYTLH (SEQ ID NO: 64) (extended)	GIWPITGGTTYNQKFKG (SEQ ID NO: 70) (Kabat) GIWPITGGTTY (SEQ ID NO: 71) (Chothia)	GEAQGS (SEQ ID NO: 73)
42G9	SNPRMGVS (SEQ ID NO: 74) (Kabat); GFSLSNPR (SEQ ID NO: 75) (Chothia); GFSLSNPRMGVS (SEQ ID NO: 76) (extended)	HIFSTDEKSLKLSLRS(SEQ ID NO: 77) (Kabat) HIFSTDEKSL (SEQ ID NO: 78) (Chothia)	DSSNYEGYFDF (SEQ ID NO: 79)
32A10	SNARMGVS (SEQ ID NO: 80) (Kabat); GFSLSNAR (SEQ ID NO: 81) (Chothia); GFSLSNARMGVS (SEQ ID	HIFSTDEKSIRRSLRS (SEQ ID NO: 83) (Kabat) HIFSTDEKSI (SEQ ID NO: 84) (Chothia)	DSSNYEGYFDY (SEQ ID NO: 85)

	NO: 82) (extended)		
20B9	SNARMGVS (SEQ ID NO: 80) (Kabat); GFSLSNAR (SEQ ID NO: 81) (Chothia); GFSLSNARMGVS (SEQ ID NO: 82) (extended)	HIFSTDEKSYSTSLRG(SEQ ID NO: 86) (Kabat) HIFSTDEKSY (SEQ ID NO: 87) (Chothia)	DSSNYEGYFDF (SEQ ID NO: 79)
14C11	NNARMGVS (SEQ ID NO: 88) (Kabat); GFSLNNAR (SEQ ID NO: 89) (Chothia); GFSLNNARMGVS (SEQ ID NO: 90) (extended)	HIFSTDEKSFRTSLRS(SEQ ID NO: 91) (Kabat) HIFSTDEKSF (SEQ ID NO: 92) (Chothia)	DSSNYEGYFDY (SEQ ID NO: 85)
21E11	SNVRMGVS (SEQ ID NO: 93) (Kabat); GFSLSNVR (SEQ ID NO: 94) (Chothia); GFSLSNVRMGVS (SEQ ID NO: 95) (extended)	HIFSSDEKSIRRSLRS(SEQ ID NO: 96) (Kabat) HIFSSDEKSI (SEQ ID NO: 97) (Chothia)	DSSNYEGYFDF (SEQ ID NO: 79)
49B11	SNVRMGVS (SEQ ID NO: 93) (Kabat); GFSLSNVR (SEQ ID NO: 94) (Chothia); GFSLSNVRMGVS (SEQ ID NO: 95) (extended)	HIFSSDEKSIRRSLRS(SEQ ID NO: 96) (Kabat) HIFSSDEKSI (SEQ ID NO: 97) (Chothia)	DSSNYEGYFDY (SEQ ID NO: 85)
46E10 12H6	SNARMGVS (SEQ ID NO: 80) (Kabat); GFSLSNAR (SEQ ID NO: 81) (Chothia); GFSLSNARMGVS (SEQ ID NO: 82) (extended)	HIFSTDEKSIRRSLRS (SEQ ID NO: 83) (Kabat) HIFSTDEKSI (SEQ ID NO: 84) (Chothia)	DSSNYEGYFDY (SEQ ID NO: 85)

19A9 21E7	SNARMGVS (SEQ ID NO: 80) (Kabat); GFSLSNAR (SEQ ID NO: 81) (Chothia); GFSLSNARMGVS (SEQ ID NO: 82) (extended)	HIFSTDEKSLRLSLRS (SEQ ID NO: 98) (Kabat) HIFSTDEKSL (SEQ ID NO: 78) (Chothia)	DSSNYEGYFDY (SEQ ID NO: 85)
11B11	SNAKMGVS (SEQ ID NO: 99) (Kabat); GFSLSNAK (SEQ ID NO: 100) (Chothia); GFSLSNAKMGVS (SEQ ID NO: 101) (extended)	HIFSTDEKSIRRSLRS (SEQ ID NO: 83) (Kabat) HIFSTDEKSI (SEQ ID NO: 84) (Chothia)	DSSNYEGYFDY (SEQ ID NO: 85)
12B2	SNPRMGVS (SEQ ID NO: 74) (Kabat); GFSLSNPR (SEQ ID NO: 75) (Chothia); GFSLSNPRMGVS (SEQ ID NO: 76) (extended)	HIFSSDEKSYRLSLRS (SEQ ID NO: 102) (Kabat) HIFSSDEKSY (SEQ ID NO: 103) (Chothia)	DSSNYGGYFDY (SEQ ID NO: 104)
11F10	SNPRMGVS (SEQ ID NO: 74) (Kabat); GFSLSNPR (SEQ ID NO: 75) (Chothia); GFSLSNPRMGVS (SEQ ID NO: 76) (extended)	HIFSSDEKSYRLFLRS (SEQ ID NO: 105) (Kabat) HIFSSDEKSY (SEQ ID NO: 103) (Chothia)	DSSDYEGYFDY (SEQ ID NO: 107)
17G11	SNPRMGVS (SEQ ID NO: 74) (Kabat); GFSLSNPR (SEQ ID NO: 75) (Chothia); GFSLSNPRMGVS (SEQ ID NO: 76) (extended)	HIFSSDEKSYRLSLRS (SEQ ID NO: 102) (Kabat) HIFSSDEKSY (SEQ ID NO: 103) (Chothia)	DSSNYEEYFDY (SEQ ID NO: 108)
29D5	SNPRMGVS (SEQ ID NO: 74)	HIFSTDEKSYSPSLRG(S	DSSNYEGYFDY

	(Kabat); GFSLSNPR (SEQ ID NO: 75) (Chothia); GFSLSNPRMGVS (SEQ ID NO: 76) (extended)	EQ ID NO: 106) (Kabat) HIFSTDEKSY (SEQ ID NO: 87) (Chothia)	(SEQ ID NO: 85)
30D8	SDAWMS (SEQ ID NO: 109) (Kabat); GFTFSD (SEQ ID NO: 110) (Chothia); GFTFSDAWMS (SEQ ID NO: 111) (extended)	RIKSKTDGGTTDYVVPL NG (SEQ ID NO: 112) (Kabat) RIKSKTDGGTTDY (SEQ ID NO: 113) (Chothia)	VPGSYGY (SEQ ID NO: 114)
20E12	SYAWMS (SEQ ID NO: 115) (Kabat); GFTFSY (SEQ ID NO: 116) (Chothia); GFTFSYAWMS (SEQ ID NO: 117) (extended)	RIKSIADGGATDYAAP VRN (SEQ ID NO: 118) (Kabat) RIKSIADGGATDY (SEQ ID NO: 119) (Chothia)	IPGNDAFDM (SEQ ID NO: 120)
26B9	NNAWMS (SEQ ID NO: 121) (Kabat); GFIFNN (SEQ ID NO: 122) (Chothia); GFIFNNAWMS (SEQ ID NO: 123) (extended)	RIKSKSDGGTTDYAAP VKD (SEQ ID NO: 124) (Kabat) RIKSKSDGGTTDY (SEQ ID NO: 125) (Chothia)	APGGPFDY (SEQ ID NO: 126)
32G8	SYAWMS (SEQ ID NO: 115) (Kabat); GFTFSY (SEQ ID NO: 116) (Chothia); GFTFSYAWMS (SEQ ID NO: 117) (extended)	RIKSITDGGVIDYAAPV RN (SEQ ID NO: 127) (Kabat) RIKSITDGGVIDY (SEQ ID NO: 128) (Chothia)	IPGNDDFDM (SEQ ID NO: 129)
34E7	SYAWMS (SEQ ID NO: 115)	RIKSINDGGATDYASPV	IPGNDAFDM (SEQ

	(Kabat); GFTFSY (SEQ ID NO: 116) (Chothia); GFTFSYAWMS (SEQ ID NO: 117) (extended)	RN (SEQ ID NO: 130) (Kabat) RIKSINDGGATDY (SEQ ID NO: 131 (Chothia)	ID NO: 120)
20G5	TNAWMS (SEQ ID NO: 132) (Kabat); GFTFTN (SEQ ID NO: 133) (Chothia); GFTFTNAWMS (SEQ ID NO: 134) (extended)	RIKSKIDGGTTDYAAPVKG (SEQ ID NO: 135) (Kabat) RIKSKIDGGTTDY (SEQ ID NO: 136) (Chothia)	APGGPFDY (SEQ ID NO: 126)
C6	SSNAIS (SEQ ID NO: 137) (Kabat); GDTFSS (SEQ ID NO: 138) (Chothia); GDTFSSNAIS (SEQ ID NO: 139) (extended)	VIIPIFGTADYAQKFQG (SEQ ID NO: 140) (Kabat) VIIPIFGTADY (SEQ ID NO: 141) (Chothia)	HTYHEYAGGYYGGAMDP (SEQ ID NO: 142)
B5	SNYAMS (SEQ ID NO: 143) (Kabat); GFTFSN (SEQ ID NO: 144) (Chothia); GFTFSNYAMS (SEQ ID NO: 145) (extended)	DISGGGGRTYYADSVKG (SEQ ID NO: 146) (Kabat) DISGGGGRTYY (SEQ ID NO: 147) (Chothia)	AGLLYGGGVYPMDI (SEQ ID NO: 148)
Light Chain			
mAb	**CDRL1**	**CDRL2**	**CDRL3**
m62G7 h62G7	KSSQSLLYSNGKTYLN (SEQ ID NO: 149)	LVSKLDS (SEQ ID NO: 150)	VQDTHFPLT (SEQ ID NO: 151)
h62G7-L6	KSSQSLLYSNGKTYLN (SEQ ID NO: 149)	QVSKLDS (SEQ ID NO: 152)	GQDTHFPLT (SEQ ID NO: 153)
h62G7-L1-DV	KSSQSLLYSNDKTYTN (SEQ	EVSKLDV (SEQ ID NO:	GQDTHFPLT (SEQ

	ID NO: 154)	155)	ID NO: 153)
42G9	RASQSVRSNLA (SEQ ID NO: 156)	GSTIRAT (SEQ ID NO: 157)	QQYSDWPFT (SEQ ID NO: 158)
32A10	RASQSVSSNFA (SEQ ID NO: 159)	GATTRAT (SEQ ID NO: 160)	QQYKDWPFT (SEQ ID NO: 161)
20B9	RVSQSIGANLA (SEQ ID NO: 162)	GASTRAT (SEQ ID NO: 163)	QQYIYWPFT (SEQ ID NO: 164)
14C11	RASQSVSNNLA (SEQ ID NO: 165)	GASTRAT (SEQ ID NO: 163)	QQYKDWPFT (SEQ ID NO: 161)
21E11	RASQSVGSDLA (SEQ ID NO: 166)	GASTRAT (SEQ ID NO: 163)	QQYNDWPFT (SEQ ID NO: 167)
49B11	RASQNIGSDLA (SEQ ID NO: 168)	GASTRAT (SEQ ID NO: 163)	QQYNDWPFT (SEQ ID NO: 167)
46E10	RASQSVTSNFA (SEQ ID NO: 169)	GASTRAT (SEQ ID NO: 163)	QQYKDWPFT (SEQ ID NO: 161)
12H6	RASQGVSSNFA (SEQ ID NO: 170)	GASTRAT (SEQ ID NO: 163)	QQYKDWPFT (SEQ ID NO: 161)
19A9	RASQSVNRNLA (SEQ ID NO: 171)	GTSTRAT (SEQ ID NO: 172)	QQYNDWPFT (SEQ ID NO: 167)
11B11	RASQSVSTNFA (SEQ ID NO: 173)	GASTRAT (SEQ ID NO: 163)	QQYKDWPFT (SEQ ID NO: 161)
21E7	RASQSVNSNLA (SEQ ID NO: 174)	GSSTRAT (SEQ ID NO: 175)	QQYNDWPFT (SEQ ID NO: 167)
12B2 17G11	RASQSVINNLA (SEQ ID NO: 176)	GTSTRAT (SEQ ID NO: 172)	QDYNNWPFT (SEQ ID NO: 177)
11F10	RASQSVGSNLA (SEQ ID NO: 178)	GASTRASG (SEQ ID NO: 179)	QEYNNWPFT (SEQ ID NO: 180)
29D5	RANQIVSSNLA (SEQ ID NO: 181)	GTSTRAT (SEQ ID NO: 172)	QQYNDWPFT (SEQ ID NO: 167)
30D8	RSSQSLLHNKRNNYLD (SEQ	LASNRAS (SEQ ID NO:	MQAQQTPIT (SEQ

	ID NO: 182)	183)	ID NO: 184)
20E12 32G8	RSSQSLLYSNGKNYLD (SEQ ID NO: 185)	LGSNRAS (SEQ ID NO: 186)	MQAQQTPIT (SEQ ID NO: 184)
26B9	RSSQSLLHRDGFNYLD (SEQ ID NO: 187)	LASSRAS (SEQ ID NO: 188)	MQALQTPIT (SEQ ID NO: 189)
34E7	RSTQSLLYSNGKNYLD (SEQ ID NO: 190)	LGSIRAS (SEQ ID NO: 191)	MQAQQTPIT (SEQ ID NO: 184)
20G5	RSSQSLLYSDRRNYLD (SEQ ID NO: 192)	LGSYRAS (SEQ ID NO: 193)	MQALQIPIT(SEQ ID NO: 194)
C6	SGSSSNIGSNYVY (SEQ ID NO: 195)	RNNQRPS (SEQ ID NO: 196)	AAWDDNLSGWV (SEQ ID NO: 197)
B5	RASQSISSYLN (SEQ ID NO: 198)	AASSLQS (SEQ ID NO: 199)	QQSYSTPLT(SEQ ID NO: 200)

The invention encompasses modifications to the CARs and polypeptides of the invention variants shown in Table 1, including functionally equivalent CARs having modifications which do not significantly affect their properties and variants which have enhanced or decreased activity and/or affinity. For example, the amino acid sequence may be mutated to obtain an antibody with the desired binding affinity to EGFRvIII. Modification of polypeptides is routine practice in the art and need not be described in detail herein. Examples of modified polypeptides include polypeptides with conservative substitutions of amino acid residues, one or more deletions or additions of amino acids which do not significantly deleteriously change the functional activity, or which mature (enhance) the affinity of the polypeptide for its ligand, or use of chemical analogs.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody of an

enzyme or a polypeptide which increases the half-life of the antibody in the blood circulation.

Substitution variants have at least one amino acid residue in the antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 3 under the heading of "conservative substitutions." If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 3, or as further described below in reference to amino acid classes, may be introduced and the products screened. In some embodiments, substitution variants of antibodies provided herein have no more than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 conservative substitution in the VH or VL region as compared to the reference parent antibody. In some embodiments, the substitutions are not within a CDR of the VH or VL region.

Table 3: Amino Acid Substitutions

Original Residue (naturally occurring amino acid)	Conservative Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Asp, Lys; Arg
Asp (D)	Glu	Glu; Asn
Cys (C)	Ser	Ser; Ala
Gln (Q)	Asn	Asn; Glu
Glu (E)	Asp	Asp; Gln
Gly (G)	Ala	Ala
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; Norleucine
Leu (L)	Ile	Norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Tyr	Leu; Val; Ile; Ala; Tyr

Original Residue (naturally occurring amino acid)	Conservative Substitutions	Exemplary Substitutions
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr; Phe
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; Norleucine

In some embodiments, the invention provides a CAR comprising an extracellular ligand-binding domain that binds to EGFRvIII and competes for binding to EGFRvIII with the antibodies described herein or the CARs described herein (e.g., Table 5A), including m62G7, h62G7, h62G7-H14/L1-DV, h62G7-L6/EQ, 42G9, 32A10, 20B9, 14C11, 21E11, 49B11, 46E10, 12H6, 19A9, 21E7, 11B11, 12B2, 11F10, 17G11, 29D5, 30D8, 20E12, 26B9, 32G8, 34E7, 20G5, C6, and B5.

In some embodiments, the invention provides CARs comprising CDR portions of antibodies to EGFRvIII antibodies based on CDR contact regions. CDR contact regions are regions of an antibody that imbue specificity to the antibody for an antigen. In general, CDR contact regions include the residue positions in the CDRs and Vernier zones which are constrained in order to maintain proper loop structure for the antibody to bind a specific antigen. See, e.g., Makabe et al., J. Biol. Chem., 283:1156-1166, 2007. Determination of CDR contact regions is well within the skill of the art.

The binding affinity (K_D) of the EGFRvIII specific CAR as described herein to EGFRvIII (such as human EGFRvIII (e.g., (SEQ ID NO: 201)) can be about 0.001 to about 5000 nM. In some embodiments, the binding affinity is about any of 5000 nM, 4500 nM, 4000 nM, 3500 nM, 3000 nM, 2500 nM, 2000 nM, 1789 nM, 1583 nM, 1540 nM, 1500 nM, 1490 nM, 1064 nM, 1000 nM, 933 nM, 894 nM, 750 nM, 705 nM, 678 nM, 532 nM, 500 nM, 494 nM, 400 nM, 349 nM, 340 nM, 353 nM, 300 nM, 250 nM, 244 nM, 231 nM, 225 nM, 207 nM, 200 nM, 186 nM, 172 nM, 136 nM, 113 nM, 104 nM, 101 nM, 100 nM, 90 nM, 83 nM, 79 nM, 74 nM, 54 nM, 50 nM, 45 nM, 42 nM, 40 nM, 35 nM, 32 nM, 30 nM, 25 nM, 24 nM, 22 nM, 20 nM, 19 nM, 18 nM, 17 nM, 16 nM, 15 nM, 12 nM, 10 nM, 9 nM, 8 nM, 7.5 nM, 7 nM, 6.5 nM, 6 nM, 5.5 nM, 5 nM, 4 nM, 3 nM, 2

nM, 1 nM, 0.5 nM, 0.3 nM, 0.1 nM, 0.01 nM, or 0.001 nM. In some embodiments, the binding affinity is less than about any of 5000 nM, 4000 nM, 3000 nM, 2000 nM, 1000 nM, 900 nM, 800 nM, 250 nM, 200 nM, 100 nM, 50 nM, 30 nM, 20 nM, 10 nM, 7.5 nM, 7 nM, 6.5 nM, 6 nM, 5 nM, 4.5 nM, 4 nM, 3.5 nM, 3 nM, 2.5 nM, 2 nM, 1.5 nM, 1 nM, or 0.5 nM.

The intracellular signaling domain of a CAR according to the invention is responsible for intracellular signaling following the binding of extracellular ligand-binding domain to the target resulting in the activation of the immune cell and immune response. The intracellular signaling domain has the ability to activate of at least one of the normal effector functions of the immune cell in which the CAR is expressed. For example, the effector function of a T cell can be a cytolytic activity or helper activity including the secretion of cytokines.

In some embodiments, an intracellular signaling domain for use in a CAR can be the cytoplasmic sequences of, for example without limitation, the T cell receptor and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant of these sequences and any synthetic sequence that has the same functional capability. Intracellular signaling domains comprise two distinct classes of cytoplasmic signaling sequences: those that initiate antigen-dependent primary activation, and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal. Primary cytoplasmic signaling sequences can comprise signaling motifs which are known as immunoreceptor tyrosine-based activation motifs of ITAMs. ITAMs are well defined signaling motifs found in the intracytoplasmic tail of a variety of receptors that serve as binding sites for syk/zap70 class tyrosine kinases. Examples of ITAM used in the invention can include as non limiting examples those derived from TCRζ, FcRγ, FcRβ, FcRε, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b and CD66d. In some embodiments, the intracellular signaling domain of the CAR can comprise the CD3ζ (zeta) signaling domain which has amino acid sequence with at least about 70%, preferably at least 80%, more preferably at least 90%, 95%, 97%, or 99% sequence identity with an amino acid sequence shown in SEQ. ID NO: 205. In some embodiments the intracellular signaling domain of the CAR of the invention comprises a domain of a co-stimulatory molecule.

In some embodiments, the intracellular signaling domain of a CAR of the invention comprises a part of co-stimulatory molecule selected from the group

consisting of a fragment of 41BB (GenBank: AAA53133.) and CD28 (NP_006130.1). In some embodiments, the intracellular signaling domain of the CAR of the invention comprises amino acid sequence which comprises at least 70%, preferably at least 80%, more preferably at least 90%, 95%, 97%, or 99% sequence identity with an amino acid sequence shown in SEQ. ID NO: 213 (CD28 signaling domain) or SEQ. ID NO: 204 (4-1BB signaling domain).

CARs are expressed on the surface membrane of the cell. Thus, the CAR can comprise a transmembrane domain. Suitable transmembrane domains for a CAR disclosed herein have the ability to (a) be expressed at the surface of a cell, preferably an immune cell such as, for example without limitation, lymphocyte cells or Natural killer (NK) cells, and (b) interact with the ligand-binding domain and intracellular signaling domain for directing cellular response of immune cell against a predefined target cell. The transmembrane domain can be derived either from a natural or from a synthetic source. The transmembrane domain can be derived from any membrane-bound or transmembrane protein. As non-limiting examples, the transmembrane polypeptide can be a subunit of the T cell receptor such as α, β, γ or δ, polypeptide constituting CD3 complex, IL-2 receptor p55 (a chain), p75 (β chain) or γ chain, subunit chain of Fc receptors, in particular Fcγ receptor III or CD proteins. Alternatively, the transmembrane domain can be synthetic and can comprise predominantly hydrophobic residues such as leucine and valine. In some embodiments said transmembrane domain is derived from the human CD8α chain (e.g., NP_001139345.1). The CAR can further comprise a stalk domain between the extracellular ligand-binding domain and said transmembrane domain. A stalk domain may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids. Stalk region may be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, CD4, or CD28, or from all or part of an antibody constant region. Alternatively the stalk domain may be a synthetic sequence that corresponds to a naturally occurring stalk sequence, or may be an entirely synthetic stalk sequence. In some embodiments said stalk domain is a part of human CD8α chain (e.g., NP_001139345.1). In another particular embodiment, said transmembrane and hinge domains comprise a part of human CD8α chain, preferably which comprise at least 70%, preferably at least 80%, more preferably at least 90%, 95%, 97%, or 99% sequence identity with the amino acid sequence shown in SEQ ID NO: 210 and SEQ ID

NO: 208, respectively. In some embodiments, CARs disclosed herein can comprise an extracellular ligand-binding domain that specifically binds EGFRvIII, CD8α human hinge and transmembrane domains, the CD3ζ signaling domain, and the 4-1BB signaling domain.

Table 4 provides exemplary sequences of domains which can be used in the CARs disclosed herein.

Table 4: Exemplary sequences of CAR Components

Domain	Amino Acid Sequence	SEQ ID NO:
CD8α signal peptide	MALPVTALLLPLALLLHAARP	206
FcγRIIIα hinge	GLAVSTISSFFPPGYQ	207
CD8α hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD	208
IgG1 hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	209
CD8α transmembrane (TM) domain	IYIWAPLAGTCGVLLLSLVITLYC	210
41BB intracellular signaling domain (ISD)	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	203
CD3ζ intracellular signaling domain (ISD)	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMG GKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPR	205

Domain	Amino Acid Sequence	SEQ ID NO:
FcεRI α-TM-IC (FcεRI α chain transmembrane and intracellular domain)	FFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNN	211
FcεRIβ-ΔITAM (FcεRI β chain without ITAM)	MDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEGDIFSSFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNKVPE	212
41BB-IC (41BB co-signaling domain)	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	204
CD28-IC (CD28 co-signaling domain)	RSKRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	213
FcεRIγ-SP (signal peptide)	MIPAVVLLLLLLVEQAAA	214
FcεRI γ-ΔITAM (FcεRI γ chain without ITAM)	LGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKS	215
GSG-P2A (GSG-P2A ribosomal skip polypeptide)	GSGATNFSLLKQAGDVEENPGP	216
GSG-T2A (GSG-T2A ribosomal skip polypeptide)	GSGEGRGSLLTCGDVEENPGP	217

Table 5A provides amino acid sequences of exemplary EGFRvIII specific CARs of the present invention. In Table 5A, the signal/leader peptide sequence is in bold, and GS linker [$(GGGGS)_4$ (SEQ ID NO: 202)] is underlined.

Table 5A: Amino acid sequences of exemplary EGFRvIII specific CARs

CAR	CAR Amino Acid Sequence	Components (in order, N-terminus to C-terminus)
h62G7-L6/EQ	**MALPVTALLLPLALLLHAARP**DVVMTQSPLSLPVTLGQP ASISCKSSQSLLYSNGKTYLNWFQQRPGQSPRRLIYQVS KLDSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQD THFPLTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSQVQ LVQSGAEVKKPGASVKVSCKASGYTFTDYTLHWVRQAPG QGLEWMGGIWPITGGTTYNQKFKGRVTMTRDTSTSTVYM ELSSLRSEDTAVYYCARGEAQGSWGQGTLVTVSSTTTPA PRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFAC DIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAP AYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPR RKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGL YQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 52)	CD8α signal peptide; h62G7-L6/EQ VL (Table 1 SEQ ID NO: 6) ; GS linker [$(GGGGS)_4$ (SEQ ID NO: 202)]; h62G7-L6/EQ VH (Table 1 SEQ ID NO: 5) ; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
42G9	**MALPVTALLLPLALLLHAARP**QVTLKESGPVLLKPTETL TLTCTVSGFSLSNPRMGVSWIRQPPGKALEWFAHIFSTD EKSLKLSLRSRLTLSKDTSKSQVVLTMTNMAPVDSATYY CARDSSNYEGYFDFWGQGTLVTVSSGGGGSGGGGSGGGG SGGGGSEVVLTQSPATLSVSPGERATLSCRASQSVRSNL AWYQQKSGQAPRLLIYGSTIRATGVPARFSGSGSGTEFT LTISSLQSEDFAVYYCQQYSDWPFTFGPGTKVDIKTTTP APRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFA CDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQ PFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADA PAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG LYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 53)	CD8α signal peptide; 42G9 VH (Table 1 SEQ ID NO: 9); GS linker; 42G9 VL (Table 1 SEQ ID NO: 10); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
32A10	**MALPVTALLLPLALLLHAARP**QVTLKESGPVLVKPTETL TLTCTVSGFSLSNARMGVSWIRQPPGKALEWLAHIFSTD EKSIRRSLRSRLTLSKDTSKSQVVLTMTNMDPVDTATYF	CD8α signal peptide; 32A10 VH (Table 1 SEQ

CAR	CAR Amino Acid Sequence	Components (in order, N-terminus to C-terminus)
	CARDSSNYEGYFDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSEVVMTQSPATLSVSPGERVTLSCRASQSVSSNFAWYQQRPGQAPRLLLYGATTRATGLPGRFSGSGSGTENILTISSLQSEDFAIYFCQQYKDWPFTFGPGSKVDIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR(SEQ ID NO: 54)	ID NO: 11); GS linker; 32A10 VL (Table 1 SEQ ID NO: 12); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
20B9	**MALPVTALLLPLALLLHAARP**QVTLKESGPVLVKPTETLTLTCTVSGFSLSNARMGVSWIRQPPGKALEWLGHIFSTDEKSYSTSLRGRITISKDTSRGLVVLTLTNMDPVDTATYYCARDSSNYEGYFDFWGPGFLVTVSSGGGGSGGGGSGGGGSGGGGSEIVMTQSPATLSVSPGERATLSCRVSQSIGANLAWYQQKFGQAPRLLIYGASTRATGIPVRFSGGGSGTEFTLTISSLQSEDFAIYSCQQYIYWPFTFGPGTTVDIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 55)	CD8α signal peptide; 20B9 VH (Table 1, SEQ ID NO: 13); GS linker; 20B9 VL (Table 1, SEQ ID NO: 14); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
14C11	**MALPVTALLLPLALLLHAARP**QVTLKESGPVLVKPTETLTLTCTVSGFSLNNARMGVSWIRQPPGKALEWFAHIFSTDEKSFRTSLRSRLTLSKDTSKSQVVLTMTNMDPVDTATYYCARDSSNYEGYFDYWGQGILVTVSSGGGGSGGGGSGGGGSGGGGSEIVMTQSPATLSVSPGERATLSCRASQSVSNNLAWYQQKPGQAPRLLIYGASTRATGVPARFSGSDSGTEFSLTISSLQSEDFAVYFCQQYKDWPFTFGPGTKVEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQ	CD8α signal peptide; 14C11 VH (Table 1, SEQ ID NO: 15); GS linker; 14C11 VL (Table 1, SEQ

CAR	CAR Amino Acid Sequence	Components (in order, N-terminus to C-terminus)
	PFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADA PAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG LYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 56)	ID NO: 16); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
20E12	**MALPVTALLLPLALLLHAARP**EVNLVESGGGLVKPGGSL RLSCEASGFTFSYAWMSWVRQAPGKGLEWVGRIKSIADG GATDYAAPVRNRFTISRDDSRNTLYLEMHSLKTEDTAVY YCTTIPGNDAFDMWGQGTMVTVSSGGGGSGGGGSGGGGS GGGGSDIVLTQSPLSLSVTPGEPASISCRSSQSLLYSNG KNYLDWFLHKPGQSPQLLIYLGSNRASGVPDRFSGSGSG IDFILKISRVEAEDVGVYYCMQAQQTPITFGQGTRLEIK TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRG LDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLY IFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSR SADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEM GGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK GHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 57)	CD8α signal peptide; 20E12 VH (Table 1, SEQ ID NO: 39); GS linker; 20E12 VL (Table 1, SEQ ID NO: 40); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
32G8	**MALPVTALLLPLALLLHAARP**EVNLVESGGGLVKPGGSL RLSCEASGFTFSYAWMSWVRQAPGKGLEWVGRIKSITDG GVIDYAAPVRNRCTISRDDSRNTLYLEMHSLKTEDTAVY YCTTIPGNDDFDMWGQGRMVTVSSGGGGSGGGGSGGGGS GGGGSDIVLTQSPLSLSVTPGEPASISCRSSQSLLYSNG KNYLDWFLHKPGQSPQLLIYLGSNRASGVPDRFSGSGSG IDFILKISRVEAEDVGVYYCMQAQQTPITFGQGTRLEIK TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRG LDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLY IFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSR SADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEM GGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK GHDGLYQGLSTATKDTYDALHMQALPPR	CD8α signal peptide; 32G8 VH (Table 1, SEQ ID NO: 43); GS linker; 32G8 VL (Table 1, SEQ ID NO: 40); CD8α hinge; CD8α TM;

CAR	CAR Amino Acid Sequence	Components (in order, N-terminus to C-terminus)
	(SEQ ID NO: 58)	4-1BB signaling domain; CD3zeta signaling domain
26B9	**MALPVTALLLPLALLLHAARP**EVQLVESWGVLVKPGGSL RLSCAASGFIFNNAWMSWVRQAPGKGLEWIGRIKSKSDG GTTDYAAPVKDRFTISRDDSKDTLYLQMNGLKTEDTAVY FCTTAPGGPFDYWGQGTLVTVSSGGGGSGGGGSGGGGSG GGGSDIVLTQSPLSLPVTPGEPASISCRSSQSLLHRDGF NYLDWFLQKPGQSPQLLIYLASSRASGVPDRFSGSDSGT DFTLKISRVEAEDVGVYYCMQALQTPITFGQGTRLEIK TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRG LDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLY IFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSR SADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEM GGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK GHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 59)	CD8α signal peptide; 26B9 VH (Table 1, SEQ ID NO: 41); GS linker; 26B9 VL (Table 1, SEQ ID NO: 42); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
30D8	**MALPVTALLLPLALLLHAARP**EVQLVESGGGLVKPGGSL RLSCEASGFTFSDAWMSWVRQAPGKGLEWVGRIKSKTDG GTTDYVVPLNGRFIISRDDSRNTLYLQLNNLKTEDTAVY YCTTVPGSYGYWGQGTLVTVSSGGGGSGGGGSGGGGSGG GGSDIVMTQSPLSLPVTPGEPASISCRSSQSLLHNKRNN YLDWFLQKPGQSPQLLIYLASNRASGVPDRFSGGGSGTD FTLKISRVEAEDVGVYYCMQAQQTPITFGQGTRLEIKTT TPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIF KQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSA DAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH DGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 60)	CD8α signal peptide; 30D8 VH (Table 1, SEQ ID NO: 37); GS linker; 30D8 VL (Table 1, SEQ ID NO: 38); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling

CAR	CAR Amino Acid Sequence	Components (in order, N-terminus to C-terminus)
		domain
C6	**MALPVTALLLPLALLLHAARP**QVQLVQSGAEVKKPGSSV KVSCKASGDTFSSNAISWVRQAPGQGLEWMGVIIPIFGT ADYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYC ARHTYHEYAGGYYGGAMDPWGQGTLVTVSSGGGGSGGGG SGGGGSGGGGSELQSVLTQPPSASGTPGQRVTISCSGSS SNIGSNYVYWYQQLPGTAPKILIYRNNQRPSGVPDRFSG SKSGTSASLAISGLRSEDEADYYCAAWDDNLSGWVFGTG TKLTVLTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRG RKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRR GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKG ERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 61)	CD8α signal peptide; C6 VH (Table 1, SEQ ID NO: 48); GS linker; C6 VL (Table 1, SEQ ID NO: 49); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

Table 5B provides nucleic acid sequences of exemplary scFvs of EGFRvIII specific CARs of the present invention. In Table 5B, the sequence encoding the CD8α signal/leader peptide is underlined.

Table 5B: Nucleic acid sequences of exemplary EGFRvIII specific scFvs

CAR	scFv Nucleic Acid Sequence
h62G7-L6/EQ scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCCCTGGCTCTGCTGCTGCACG CTGCTCGCCCTGATGTGGTCATGACTCAGTCTCCCCTGTCTCTGCCCGTCAC CCTGGGACAGCCCGCCAGCATCTCCTGCAAGAGCTCCCAGAGCCTGCTGTAC TCCAACGGCAAGACCTATCTGAATTGGTTCCAGCAGAGACCCGGCCAGAGCC CTCGGAGACTGATCTACCAGGTGTCTAAGCTGGACAGCGGCGTGCCTGATCG CTTCTCTGGAAGCGGATCCGGAACCGACTTTACACTGAAGATCAGCCGGGTG GAGGCAGAGGACGTGGGCGTGTACTATTGCGGCCAGGATACCCACTTCCCAC TGACATTTGGCGGCGGCACCAAGGTGGAGATCAAGGGAGGAGGAGGAAGCGG AGGAGGAGGAAGCGGCGGCGGCGGCTCTGGCGGCGGCGGCAGCCAGGTGCAG CTGGTGCAGAGCGGAGCAGAGGTGAAGAAGCCTGGCGCCTCCGTGAAGGTGT CTTGTAAGGCCAGCGGCTACACATTCACCGATTATACACTGCACTGGGTGCG GCAGGCCCCTGGCCAGGGACTGGAGTGGATGGGAGGAATCTGGCCTATCACC

CAR	scFv Nucleic Acid Sequence
	GGAGGAACCACATACAACCAGAAGTTTAAGGGCAGAGTGACAATGACCAGGG ACACATCTACCAGCACAGTGTATATGGAGCTGTCTAGCCTGCGCTCCGAGGA TACAGCCGTGTACTATTGCGCCAGAGGCGAGGCACAGGGATCTTGGGGACAG GGCACCCTGGTGACAGTGTCCTCT(SEQ ID NO:228)
42G9 scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACG CAGCAAGGCCTCAGGTGACCCTGAAGGAGAGCGGCCCTGTGCTGCTGAAGCC AACAGAGACCCTGACACTGACCTGCACAGTGTCTGGCTTCAGCCTGTCCAAC CCCCGGATGGGCGTGAGCTGGATCAGACAGCCCCCTGGCAAGGCCCTGGAGT GGTTCGCCCACATCTTTTCTACCGATGAGAAGAGCCTGAAGCTGTCCCTGAG ATCTAGGCTGACCCTGAGCAAGGACACATCTAAGAGCCAGGTGGTGCTGACC ATGACAAACATGGCCCCTGTGGACTCCGCCACATACTATTGCGCCAGAGACA GCTCCAATTACGAGGGCTATTTCGACTTTTGGGGCCAGGGCACCCTGGTGAC AGTGTCTAGCGGCGGAGGAGGATCCGGAGGAGGAGGATCTGGCGGCGGCGGC TCCGGCGGCGGCGGCTCCGAGGTGGTGCTGACCCAGAGCCCTGCCACACTGT CCGTGTCTCCAGGCGAGAGAGCCACCCTGTCTTGTAGGGCCAGCCAGTCCGT GCGCAGCAATCTGGCCTGGTACCAGCAGAAGTCCGGCCAGGCCCCAAGACTG CTGATCTATGGCTCCACCATCAGGGCCACAGGAGTGCCAGCACGCTTCTCTG GAAGCGGATCCGGCACAGAGTTTACCCTGACAATCTCCTCTCTGCAGTCCGA GGATTTCGCCGTGTACTATTGCCAGCAGTACTCTGACTGGCCCTTCACCTTT GGCCCTGGCACAAAGGTGGATATCAAG (SEQ ID NO: 229)
32A10 scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACG CAGCAAGGCCACAGGTGACCCTGAAGGAGTCCGGCCCCGTGCTGGTGAAGCC TACAGAGACCCTGACACTGACCTGCACAGTGTCCGGCTTCTCTCTGAGCAAC GCCCGCATGGGCGTGTCTTGGATCAGGCAGCCCCCTGGCAAGGCCCTGGAGT GGCTGGCCCACATCTTTTCCACCGACGAGAAGTCTATCCGGAGAAGCCTGCG CTCCAGGCTGACCCTGAGCAAGGATACATCCAAGTCTCAGGTGGTGCTGACC ATGACAAACATGGACCCCGTGGATACCGCCACATACTTCTGCGCCAGAGACA GCTCCAATTACGAGGGCTATTTTGATTACTGGGGCCAGGGCACCCTGGTGAC AGTGTCTAGCGGAGGAGGAGGAAGCGGAGGAGGAGGATCTGGCGGCGGCGGC TCTGGCGGCGGCGGCAGCGAGGTGGTCATGACCCAGAGCCCAGCCACACTGA GCGTGTCCCCTGGCGAGAGGGTGACCCTGTCCTGTAGGGCATCTCAGAGCGT GTCCTCTAACTTCGCCTGGTATCAGCAGAGACCAGGCCAGGCACCAAGGCTG CTGCTGTACGGAGCAACCACAAGAGCCACAGGACTGCCCGGCAGGTTTTCCG GATCTGGAAGCGGCACCGAGAATATCCTGACAATCAGCTCCCTGCAGTCTGA GGACTTCGCCATCTATTTTTGCCAGCAGTACAAGGATTGGCCATTCACCTTT GGCCCCGGCAGCAAGGTGGACATCAAG (SEQ ID NO: 230)
20B9 scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACG CAGCAAGACCTCAGGTGACCCTGAAGGAGTCCGGCCCTGTGCTGGTGAAGCC AACAGAGACCCTGACACTGACCTGCACAGTGTCTGGCTTCAGCCTGTCCAAC GCAAGGATGGGCGTGAGCTGGATCAGGCAGCCCCCTGGCAAGGCCCTGGAGT GGCTGGGCCACATCTTTAGCACCGACGAGAAGTCTTACAGCACATCCCTGAG AGGCAGGATCACCATCTCTAAGGATACAAGCAGAGGCCTGGTGGTGCTGACC CTGACAAACATGGACCCCGTGGATACCGCCACATACTATTGCGCCAGGGACA GCTCCAATTACGAGGGCTATTTCGATTTTTGGGGCCCTGGCTTCCTGGTGAC CGTGTCTAGCGGCGGCGGCGGCTCTGGAGGAGGAGGAAGCGGAGGAGGAGGA TCCGGCGGCGGCGGCTCTGAGATCGTGATGACCCAGTCCCCTGCCACACTGT CTGTGAGCCCAGGCGAGAGAGCCACCCTGTCTTGTAGGGTGTCCCAGTCTAT CGGCGCCAATCTGGCCTGGTACCAGCAGAAGTTCGGCCAGGCCCCAAGGCTG

CAR	scFv Nucleic Acid Sequence
	CTGATCTATGGAGCATCCACCAGAGCCACAGGAATCCCCGTGAGGTTCTCCG GAGGAGGATCTGGAACCGAGTTTACCCTGACAATCTCCTCTCTGCAGAGCGA GGACTTTGCCATCTACTCCTGCCAGCAGTACATCTATTGGCCCTTCACATTT GGCCCTGGCACCACAGTGGATATCAAG (SEQ ID NO: 231)
14C11 scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACG CAGCAAGACCACAGGTGACCCTGAAGGAGAGCGGACCCGTGCTGGTGAAGCC TACAGAGACCCTGACACTGACCTGCACAGTGAGCGGCTTCTCCCTGAACAAT GCAAGGATGGGCGTGTCCTGGATCAGGCAGCCCCCTGGCAAGGCCCTGGAGT GGTTCGCCCACATCTTTAGCACCGACGAGAAGTCCTTTCGCACATCTCTGAG AAGCAGGCTGACCCTGAGCAAGGATACAAGCAAGTCCCAGGTGGTGCTGACC ATGACAAACATGGACCCCGTGGATACCGCCACATACTATTGCGCCAGAGACA GCTCCAATTACGAGGGCTATTTCGATTACTGGGGCCAGGGCATCCTGGTGAC CGTGTCTAGCGGCGGCGGCGGCTCTGGAGGAGGAGGAAGCGGAGGAGGAGGA TCCGGCGGCGGCGGCTCTGAGATCGTGATGACCCAGTCTCCCGCCACACTGT CTGTGAGCCCTGGCGAGAGAGCCACACTGAGCTGTAGGGCCTCCCAGTCTGT GAGCAACAATCTGGCCTGGTATCAGCAGAAGCCAGGCCAGGCACCAAGGCTG CTGATCTACGGAGCATCCACCAGAGCCACAGGAGTGCCAGCAAGGTTCTCCG GATCTGACAGCGGCACCGAGTTTAGCCTGACAATCTCCTCTCTGCAGTCCGA GGACTTCGCCGTGTATTTTTGCCAGCAGTACAAGGATTGGCCATTCACCTTT GGCCCCGGCACAAAGGTGGAGATCAAG (SEQ ID NO: 232)
20E12 scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACG CAGCAAGGCCAGAGGTGAACCTGGTGGAGTCCGGCGGCGGCCTGGTGAAGCC TGGCGGATCCCTGAGGCTGTCTTGCGAGGCAAGCGGCTTCACCTTCAGCTAC GCCTGGATGTCCTGGGTGCGCCAGGCCCCCGGCAAGGGACTGGAGTGGGTGG GACGGATCAAGTCCATCGCAGACGGAGGAGCAACCGATTACGCAGCCCCTGT GAGAAACAGGTTCACAATCTCCAGAGACGATTCTAGGAATACCCTGTATCTG GAGATGCACTCTCTGAAGACAGAGGACACCGCCGTGTACTATTGCACCACAA TCCCTGGCAACGACGCCTTTGATATGTGGGGCCAGGGCACAATGGTGACCGT GAGCTCCGGCGGCGGCGGCTCTGGAGGAGGAGGAAGCGGAGGAGGAGGAAGC GGGGGCGGCGGCTCTGACATCGTGCTGACACAGTCCCCACTGTCCCTGTCTG TGACCCCCGGCGAGCCTGCAAGCATCTCCTGTAGATCTAGCCAGAGCCTGCT GTACTCCAACGGCAAGAATTATCTGGATTGGTTCCTGCACAAGCCAGGCCAG TCTCCCCAGCTGCTGATCTACCTGGGATCTAATAGGGCAAGCGGAGTGCCAG ACCGGTTCTCTGGAAGCGGATCCGGCATCGACTTCATCCTGAAGATCAGCAG GGTGGAGGCCGAGGATGTGGGCGTGTACTATTGCATGCAGGCCCAGCAGACA CCCATCACCTTCGGCCAGGGCACAAGACTGGAGATCAAG (SEQ ID NO: 233)
32G8 scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACG CAGCAAGGCCAGAGGTGAACCTGGTGGAGTCCGGCGGCGGCCTGGTGAAGCC TGGCGGATCCCTGAGGCTGTCTTGCGAGGCAAGCGGCTTCACCTTCAGCTAC GCCTGGATGTCCTGGGTGCGCCAGGCCCCCGGCAAGGGACTGGAGTGGGTGG GCCGGATCAAGTCCATCACCGACGGAGGCGTGATCGATTACGCAGCACCTGT GAGAAACAGGTGCACAATCTCCAGAGACGATTCTAGGAATACCCTGTATCTG GAGATGCACTCTCTGAAGACAGAGGACACCGCCGTGTACTATTGTACCACAA TCCCTGGCAACGACGATTTCGATATGTGGGGCCAGGGCAGAATGGTGACCGT GAGCTCCGGCGGCGGCGGCTCTGGAGGAGGAGGAAGCGGAGGAGGAGGAAGC GGGGGCGGCGGCTCTGACATCGTGCTGACACAGTCCCCACTGTCCCTGTCTG TGACCCCCGGCGAGCCTGCAAGCATCTCCTGTAGGTCTAGCCAGAGCCTGCT

CAR	scFv Nucleic Acid Sequence
	GTACTCCAACGGCAAGAATTATCTGGATTGGTTTCTGCACAAGCCAGGCCAG TCTCCCCAGCTGCTGATCTACCTGGGATCTAATAGGGCAAGCGGAGTGCCAG ACCGGTTCTCTGGAAGCGGATCCGGCATCGACTTCATCCTGAAGATCAGCCG CGTGGAGGCAGAGGACGTGGGCGTGTACTATTGCATGCAGGCCCAGCAGACA CCCATCACCTTCGGCCAGGGCACAAGACTGGAGATCAAG (SEQ ID NO: 234)
26B9 scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACG CAGCAAGGCCAGAGGTGCAGCTGGTGGAGTCTTGGGGCGTGCTGGTGAAGCC TGGCGGATCTCTGAGGCTGAGCTGCGCAGCATCCGGCTTCATCTTTAACAAT GCCTGGATGTCCTGGGTGCGCCAGGCCCCCGGCAAGGGACTGGAGTGGATCG GCCGGATCAAGAGCAAGTCCGACGGAGGAACCACAGATTACGCAGCACCTGT GAAGGACCGCTTCACAATCTCTCGGGACGATAGCAAGGATACCCTGTATCTG CAGATGAACGGCCTGAAGACAGAGGACACCGCCGTGTACTTCTGCACCACAG CCCCTGGCGGCCCTTTTGATTATTGGGGCCAGGGCACACTGGTGACCGTGAG CTCCGGAGGAGGAGGAAGCGGCGGAGGAGGCAGCGGCGGCGGCGGCTCTGGC GGCGGCGGCAGCGACATCGTGCTGACACAGAGCCCTCTGTCCCTGCCAGTGA CCCCCGGCGAGCCTGCCTCTATCAGCTGTCGCTCTAGCCAGAGCCTGCTGCA CCGGGACGGCTTCAATTACCTGGATTGGTTTCTGCAGAAGCCAGGCCAGTCC CCCCAGCTGCTGATCTATCTGGCCTCCTCTAGAGCCTCTGGCGTGCCAGACA GGTTCTCCGGCTCTGACAGCGGCACAGACTTCACCCTGAAGATCAGCAGAGT GGAGGCCGAGGATGTGGGCGTGTACTATTGCATGCAGGCCCTGCAGACACCC ATCACCTTCGGCCAGGGCACAAGACTGGAGATCAAG (SEQ ID NO: 235)
30D8 scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACG CAGCAAGGCCTGAGGTGCAGCTGGTGGAGAGCGGCGGCGGCCTGGTGAAGCC TGGCGGATCCCTGAGGCTGTCTTGCGAGGCAAGCGGCTTCACCTTTAGCGAC GCATGGATGTCCTGGGTGCGCCAGGCCCCTGGCAAGGGACTGGAGTGGGTGG GACGGATCAAGAGCAAGACAGACGGCGGCACCACAGATTACGTGGTGCCACT GAACGGCCGCTTCATCATCTCCCGCGACGATTCTCGGAATACCCTGTATCTG CAGCTGAACAATCTGAAGACAGAGGATACCGCCGTGTACTATTGCACCACAG TGCCAGGCTCCTACGGCTATTGGGGCCAGGGCACACTGGTGACCGTGAGCTC CGGCGGCGGCGGCTCTGGAGGAGGAGGAAGCGGAGGAGGAGGAAGCGGGGGC GGCGGCTCTGACATCGTGATGACACAGTCTCCACTGAGCCTGCCAGTGACCC CTGGCGAGCCAGCCTCCATCTCTTGTCGCTCTAGCCAGAGCCTGCTGCACAA CAAGCGGAACAATTACCTGGATTGGTTTCTGCAGAAGCCTGGCCAGTCCCCT CAGCTGCTGATCTATCTGGCCAGCAATAGAGCCTCCGGAGTGCCAGACAGGT TCTCTGGAGGAGGAAGCGGAACAGACTTCACCCTGAAGATCAGCAGAGTGGA GGCCGAGGACGTGGGCGTGTACTATTGCATGCAGGCCCAGCAGACACCTATC ACCTTCGGCCAGGGCACAAGACTGGAGATCAAG (SEQ ID NO: 236)
C6 scFv	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCTCTGGCCCTGCTGCTGCACG CAGCAAGGCCACAGGTGCAGCTGGTGCAGTCCGGAGCAGAGGTGAAGAAGCC TGGCAGCTCCGTGAAGGTGAGCTGCAAGGCCTCCGGCGACACATTCTCTAGC AACGCAATCAGCTGGGTGCGCCAGGCCCCTGGCCAGGGACTGGAGTGGATGG GCGTGATCATCCCTATCTTCGGCACCGCCGACTATGCCCAGAAGTTTCAGGG CCGGGTGACAATCACCGCCGATGAGTCTACAAGCACCGCCTACATGGAGCTG TCCTCTCTGAGATCCGAGGACACAGCCGTGTACTATTGTGCCAGGCACACCT ATCACGAGTACGCAGGAGGATACTATGGAGGAGCAATGGATCCTTGGGGACA GGGCACACTGGTGACCGTGAGCTCCGGCGGCGGCGGCTCTGGAGGAGGAGGA

CAR	scFv Nucleic Acid Sequence
	AGCGGAGGAGGAGGAAGCGGGGGCGGCGGCTCTGAGCTGCAGAGCGTGCTGA CCCAGCCACCTTCCGCCTCTGGAACACCAGGCCAGAGGGTGACCATCAGCTG CTCCGGATCTAGCTCCAACATCGGCTCCAATTACGTGTATTGGTACCAGCAG CTGCCAGGCACAGCCCCCAAGATCCTGATCTACCGCAACAATCAGCGGCCTT CTGGCGTGCCAGATAGATTCTCTGGCAGCAAGTCCGGCACCTCTGCCAGCCT GGCAATCTCCGGCCTGAGGTCTGAGGACGAGGCCGATTACTATTGCGCCGCC TGGGACGATAACCTGAGCGGCTGGGTGTTTGGCACAGGCACCAAGCTGACAG TGCTG (SEQ ID NO: 237)

Table 5C provides exemplary nucleic acid sequences of exemplary EGFRvIII specific CARs of the present invention.

Table 5C: Nucleic acid sequences of exemplary EGFRvIII specific CARs

CAR	CAR Nucleic Acid Sequence	Components
h62G7-L6/EQ	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCCCTGGCT CTGCTGCTGCACGCTGCTCGCCCTGATGTGGTCATGACT CAGTCTCCCCTGTCTCTGCCCGTCACCCTGGGACAGCCC GCCAGCATCTCCTGCAAGAGCTCCCAGAGCCTGCTGTAC TCCAACGGCAAGACCTATCTGAATTGGTTCCAGCAGAGA CCCGGCCAGAGCCCTCGGAGACTGATCTACCAGGTGTCT AAGCTGGACAGCGGCGTGCCTGATCGCTTCTCTGGAAGC GGATCCGGAACCGACTTTACACTGAAGATCAGCCGGGTG GAGGCAGAGGACGTGGGCGTGTACTATTGCGGCCAGGAT ACCCACTTCCCACTGACATTTGGCGGCGGCACCAAGGTG GAGATCAAGGGAGGAGGAGGAAGCGGAGGAGGAGGAAGC GGCGGCGGCGGCTCTGGCGGCGGCGGCAGCCAGGTGCAG CTGGTGCAGAGCGGAGCAGAGGTGAAGAAGCCTGGCGCC TCCGTGAAGGTGTCTTGTAAGGCCAGCGGCTACACATTC ACCGATTATACACTGCACTGGGTGCGGCAGGCCCCTGGC CAGGGACTGGAGTGGATGGGAGGAATCTGGCCTATCACC GGAGGAACCACATACAACCAGAAGTTTAAGGGCAGAGTG ACAATGACCAGGGACACATCTACCAGCACAGTGTATATG GAGCTGTCTAGCCTGCGCTCCGAGGATACAGCCGTGTAC TATTGCGCCAGAGGCGAGGCACAGGGATCTTGGGGACAG GGCACCCTGGTGACAGTGTCCTCTACCACAACCCCAGCA CCAAGACCACCTACCCCTGCACCAACAATCGCCTCCCAG CCTCTGTCTCTGCGCCCAGAGGCATGTAGGCCAGCAGCA GGAGGAGCAGTGCACACCAGGGGCCTGGACTTTGCCTGC GATATCTACATCTGGGCACCACTGGCAGGAACATGTGGC GTGCTGCTGCTGAGCCTGGTCATCACCCTGTACTGCAAG CGCGGCCGGAAGAAGCTGCTGTATATCTTCAAGCAGCCC TTCATGAGACCCGTGCAGACAACCCAGGAGGAGGACGGC TGCTCCTGTAGGTTCCCAGAAGAAGAGGAGGGCGGCTGT GAGCTGAGAGTGAAGTTTTCCAGGTCTGCCGATGCACCA	CD8α signal peptide; h62G7-L6/EQ VL (Table 1 SEQ ID NO: 6); GS linker [$(GGGGS)_4$ (SEQ ID NO: 202)]; h62G7-L6/EQ VH (Table 1 SEQ ID NO: 5) ; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

CAR	CAR Nucleic Acid Sequence	Components
	GCATACCAGCAGGGACAGAATCAGCTGTATAACGAGCTG AATCTGGGCAGGCGCGAGGAGTATGACGTGCTGGATAAG AGGAGAGGAAGGGACCCTGAGATGGGAGGCAAGCCTAGG CGCAAGAACCCACAGGAGGGCCTGTACAATGAGCTGCAG AAGGATAAGATGGCCGAGGCCTATTCCGAGATCGGCATG AAGGGCGAGCGGAGAAGGGGCAAGGGCCACGACGGGCTG TACCAGGGACTGTCAACCGCTACCAAGGATACTTACGAC GCCCTGCATATGCAGGCACTGCCTCCAAGGTGA (SEQ ID NO:238)	
42G9	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCC CTGCTGCTGCACGCAGCAAGGCCTCAGGTGACCCTGAAG GAGAGCGGCCCTGTGCTGCTGAAGCCAACAGAGACCCTG ACACTGACCTGCACAGTGTCTGGCTTCAGCCTGTCCAAC CCCCGGATGGGCGTGAGCTGGATCAGACAGCCCCCTGGC AAGGCCCTGGAGTGGTTCGCCCACATCTTTTCTACCGAT GAGAAGAGCCTGAAGCTGTCCCTGAGATCTAGGCTGACC CTGAGCAAGGACACATCTAAGAGCCAGGTGGTGCTGACC ATGACAAACATGGCCCCTGTGGACTCCGCCACATACTAT TGCGCCAGAGACAGCTCCAATTACGAGGGCTATTTCGAC TTTTGGGGCCAGGGCACCCTGGTGACAGTGTCTAGCGGC GGAGGAGGATCCGGAGGAGGAGGATCTGGCGGCGGCGGC TCCGGCGGCGGCGGCTCCGAGGTGGTGCTGACCCAGAGC CCTGCCACACTGTCCGTGTCTCCAGGCGAGAGAGCCACC CTGTCTTGTAGGGCCAGCCAGTCCGTGCGCAGCAATCTG GCCTGGTACCAGCAGAAGTCCGGCCAGGCCCCAAGACTG CTGATCTATGGCTCCACCATCAGGGCCACAGGAGTGCCA GCACGCTTCTCTGGAAGCGGATCCGGCACAGAGTTTACC CTGACAATCTCCTCTCTGCAGTCCGAGGATTTCGCCGTG TACTATTGCCAGCAGTACTCTGACTGGCCCTTCACCTTT GGCCCTGGCACAAAGGTGGATATCAAGACCACAACCCCT GCACCAAGGCCACCAACCCCAGCACCTACAATCGCAAGC CAGCCACTGTCCCTGAGACCCGAGGCCTGTAGGCCTGCA GCAGGAGGAGCAGTGCACACCCGCGGCCTGGACTTTGCC TGCGATATCTATATCTGGGCACCACTGGCAGGAACCTGT GGCGTGCTGCTGCTGAGCCTGGTCATCACCCTGTACTGC AAGCGCGGCCGGAAGAAGCTGCTGTATATCTTCAAGCAG CCCTTCATGCGGCCCGTGCAGACAACCCAGGAGGAGGAT GGCTGCTCCTGTAGATTCCCTGAGGAGGAGGAGGGAGGA TGTGAGCTGAGGGTGAAGTTTTCTCGGAGCGCCGACGCA CCAGCATACCAGCAGGGACAGAACCAGCTGTATAACGAG CTGAATCTGGGCCGGAGAGAGGAGTACGACGTGCTGGAT AAGAGGAGGGGAAGAGACCCAGAGATGGGAGGCAAGCCA CGGAGAAAGAACCCCCAGGAGGGCCTGTACAATGAGCTG CAGAAGGATAAGATGGCCGAGGCCTATTCTGAGATCGGC ATGAAGGGAGAGAGGCGCCGGGGCAAGGGACACGACGGA CTGTACCAGGGACTGTCCACCGCAACAAAGGACACCTAT GATGCCCTGCATATGCAGGCACTGCCTCCAAGGTGA (SEQ ID NO: 239)	CD8α signal peptide; 42G9 VH (Table 1 SEQ ID NO: 9); GS linker; 42G9 VL (Table 1 SEQ ID NO: 10); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

CAR	CAR Nucleic Acid Sequence	Components
32A10	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCC CTGCTGCTGCACGCAGCAAGGCCACAGGTGACCCTGAAG GAGTCCGGCCCCGTGCTGGTGAAGCCTACAGAGACCCTG ACACTGACCTGCACAGTGTCCGGCTTCTCTCTGAGCAAC GCCCGCATGGGCGTGTCTTGGATCAGGCAGCCCCCTGGC AAGGCCCTGGAGTGGCTGGCCCACATCTTTTCCACCGAC GAGAAGTCTATCCGGAGAAGCCTGCGCTCCAGGCTGACC CTGAGCAAGGATACATCCAAGTCTCAGGTGGTGCTGACC ATGACAAACATGGACCCCGTGGATACCGCCACATACTTC TGCGCCAGAGACAGCTCCAATTACGAGGGCTATTTTGAT TACTGGGGCCAGGGCACCCTGGTGACAGTGTCTAGCGGA GGAGGAGGAAGCGGAGGAGGAGGATCTGGCGGCGGCGGC TCTGGCGGCGGCGGCAGCGAGGTGGTCATGACCCAGAGC CCAGCCACACTGAGCGTGTCCCCTGGCGAGAGGGTGACC CTGTCCTGTAGGGCATCTCAGAGCGTGTCCTCTAACTTC GCCTGGTATCAGCAGAGACCAGGCCAGGCACCAAGGCTG CTGCTGTACGGAGCAACCACAAGAGCCACAGGACTGCCC GGCAGGTTTTCCGGATCTGGAAGCGGCACCGAGAATATC CTGACAATCAGCTCCCTGCAGTCTGAGGACTTCGCCATC TATTTTTGCCAGCAGTACAAGGATTGGCCATTCACCTTT GGCCCCGGCAGCAAGGTGGACATCAAGACCACAACCCCT GCACCAAGACCACCAACCCCAGCACCTACAATCGCCTCT CAGCCTCTGAGCCTGCGCCCAGAGGCATGTAGGCCAGCA GCAGGAGGAGCAGTGCACACAAGGGGCCTGGACTTCGCC TGCGATATCTATATCTGGGCACCTCTGGCAGGAACCTGT GGCGTGCTGCTGCTGAGCCTGGTCATCACCCTGTATTGC AAGAGAGGCAGGAAGAAGCTGCTGTACATCTTCAAGCAG CCTTTTATGCGCCCAGTGCAGACAACCCAGGAGGAGGAC GGCTGCAGCTGTCGGTTCCCTGAAGAGGAGGAGGGCGGC TGTGAGCTGAGAGTGAAGTTTTCCAGGTCTGCCGATGCC CCAGCCTATCAGCAGGGCCAGAATCAGCTGTACAACGAG CTGAATCTGGGCAGGCGCGAGGAGTACGACGTGCTGGAT AAGAGGAGAGGAAGGGATCCAGAGATGGGAGGCAAGCCT AGGCGCAAGAACCCACAGGAGGGCCTGTATAATGAGCTG CAGAAGGACAAGATGGCCGAGGCCTACTCCGAGATCGGC ATGAAGGGAGAGCGGAGAAGGGGCAAGGGACACGATGGC CTGTATCAGGGCCTGTCTACCGCCACAAAGGACACCTAC GATGCCCTGCATATGCAGGCACTGCCTCCAAGGTGA (SEQ ID NO: 240)	CD8α signal peptide; 32A10 VH (Table 1 SEQ ID NO: 11) ; GS linker; 32A10 VL (Table 1 SEQ ID NO: 12); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
20B9	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCC CTGCTGCTGCACGCAGCAAGACCTCAGGTGACCCTGAAG GAGTCCGGCCCTGTGCTGGTGAAGCCAACAGAGACCCTG ACACTGACCTGCACAGTGTCTGGCTTCAGCCTGTCCAAC GCAAGGATGGGCGTGAGCTGGATCAGGCAGCCCCCTGGC AAGGCCCTGGAGTGGCTGGGCCACATCTTTAGCACCGAC GAGAAGTCTTACAGCACATCCCTGAGAGGCAGGATCACC ATCTCTAAGGATACAAGCAGAGGCCTGGTGGTGCTGACC CTGACAAACATGGACCCCGTGGATACCGCCACATACTAT	CD8α signal peptide; 20B9 VH (Table 1, SEQ ID NO: 13); GS linker; 20B9 VL (Table 1, SEQ

CAR	CAR Nucleic Acid Sequence	Components
	TGCGCCAGGGACAGCTCCAATTACGAGGGCTATTTCGAT TTTTGGGGCCCTGGCTTCCTGGTGACCGTGTCTAGCGGC GGCGGCGGCTCTGGAGGAGGAGGAAGCGGAGGAGGAGGA TCCGGCGGCGGCGGCTCTGAGATCGTGATGACCCAGTCC CCTGCCACACTGTCTGTGAGCCCAGGCGAGAGAGCCACC CTGTCTTGTAGGGTGTCCCAGTCTATCGGCGCCAATCTG GCCTGGTACCAGCAGAAGTTCGGCCAGGCCCCAAGGCTG CTGATCTATGGAGCATCCACCAGAGCCACAGGAATCCCC GTGAGGTTCTCCGGAGGAGGATCTGGAACCGAGTTTACC CTGACAATCTCCTCTCTGCAGAGCGAGGACTTTGCCATC TACTCCTGCCAGCAGTACATCTATTGGCCCTTCACATTT GGCCCTGGCACCACAGTGGATATCAAGACCACAACCCCT GCACCAAGGCCACCAACCCCAGCACCTACAATCGCAAGC CAGCCACTGTCCCTGAGACCAGAGGCATGTAGGCCTGCA GCAGGAGGAGCCGTGCACACCAGAGGCCTGGACTTTGCC TGCGATATCTATATCTGGGCACCACTGGCAGGAACCTGT GGCGTGCTGCTGCTGAGCCTGGTCATCACCCTGTACTGC AAGCGCGGCCGGAAGAAGCTGCTGTATATCTTCAAGCAG CCCTTCATGCGCCCCGTGCAGACAACCCAGGAGGAGGAC GGCTGCAGCTGTCGGTTCCCTGAAGAGGAGGAGGGAGGA TGTGAGCTGAGGGTGAAGTTTAGCCGGTCCGCCGATGCA CCAGCATACCAGCAGGGCCAGAACCAGCTGTATAACGAG CTGAATCTGGGCCGGAGAGAGGAGTACGACGTGCTGGAT AAGAGGAGGGGAAGAGACCCAGAGATGGGAGGCAAGCCA CGGAGAAAGAACCCCCAGGAGGGCCTGTACAATGAGCTG CAGAAGGACAAGATGGCCGAGGCCTATAGCGAGATCGGC ATGAAGGGAGAGAGGCGCCGGGGCAAGGGACACGATGGC CTGTACCAGGGCCTGTCCACCGCCACAAAGGACACCTAT GATGCCCTGCATATGCAGGCACTGCCTCCAAGGTGA (SEQ ID NO: 241)	ID NO: 14); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
14C11	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCC CTGCTGCTGCACGCAGCAAGACCACAGGTGACCCTGAAG GAGAGCGGACCCGTGCTGGTGAAGCCTACAGAGACCCTG ACACTGACCTGCACAGTGAGCGGCTTCTCCCTGAACAAT GCAAGGATGGGCGTGTCCTGGATCAGGCAGCCCCCTGGC AAGGCCCTGGAGTGGTTCGCCCACATCTTTAGCACCGAC GAGAAGTCCTTTCGCACATCTCTGAGAAGCAGGCTGACC CTGAGCAAGGATACAAGCAAGTCCCAGGTGGTGCTGACC ATGACAAACATGGACCCCGTGGATACCGCCACATACTAT TGCGCCAGAGACAGCTCCAATTACGAGGGCTATTTCGAT TACTGGGGCCAGGGCATCCTGGTGACCGTGTCTAGCGGC GGCGGCGGCTCTGGAGGAGGAGGAAGCGGAGGAGGAGGA TCCGGCGGCGGCGGCTCTGAGATCGTGATGACCCAGTCT CCCGCCACACTGTCTGTGAGCCCTGGCGAGAGAGCCACA CTGAGCTGTAGGGCCTCCCAGTCTGTGAGCAACAATCTG GCCTGGTATCAGCAGAAGCCAGGCCAGGCACCAAGGCTG CTGATCTACGGAGCATCCACCAGAGCCACAGGAGTGCCA GCAAGGTTCTCCGGATCTGACAGCGGCACCGAGTTTAGC	CD8α signal peptide; 14C11 VH (Table 1, SEQ ID NO: 15); GS linker; 14C11 VL (Table 1, SEQ ID NO: 16); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling

CAR	CAR Nucleic Acid Sequence	Components
	CTGACAATCTCCTCTCTGCAGTCCGAGGACTTCGCCGTG TATTTTTGCCAGCAGTACAAGGATTGGCCATTCACCTTT GGCCCCGGCACAAAGGTGGAGATCAAGACCACAACCCCT GCACCAAGACCACCAACCCCAGCACCTACAATCGCATCC CAGCCTCTGTCTCTGAGACCAGAGGCATGTAGGCCAGCA GCAGGAGGAGCAGTGCACACCAGGGGCCTGGACTTTGCC TGCGATATCTATATCTGGGCACCTCTGGCAGGAACCTGT GGCGTGCTGCTGCTGAGCCTGGTCATCACCCTGTATTGC AAGCGCGGCCGGAAGAAGCTGCTGTACATCTTCAAGCAG CCTTTTATGCGCCCAGTGCAGACAACCCAGGAGGAGGAC GGCTGCTCCTGTCGGTTCCCTGAAGAGGAGGAGGGAGGA TGTGAGCTGAGGGTGAAGTTTTCCCGGTCTGCCGATGCC CCAGCCTATCAGCAGGGCCAGAACCAGCTGTACAACGAG CTGAATCTGGGCCGGAGAGAGGAGTACGACGTGCTGGAT AAGAGGAGGGGAAGAGATCCAGAGATGGGAGGCAAGCCT CGGAGAAAGAACCCACAGGAGGGCCTGTATAATGAGCTG CAGAAGGACAAGATGGCCGAGGCCTACTCCGAGATCGGC ATGAAGGGAGAGAGGCGCCGGGGCAAGGGACACGATGGC CTGTATCAGGGCCTGTCTACCGCCACAAAGGACACCTAC GATGCCCTGCATATGCAGGCACTGCCTCCAAGGTGA (SEQ ID NO: 242)	domain
20E12	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCC CTGCTGCTGCACGCAGCAAGGCCAGAGGTGAACCTGGTG GAGTCCGGCGGCGGCCTGGTGAAGCCTGGCGGATCCCTG AGGCTGTCTTGCGAGGCAAGCGGCTTCACCTTCAGCTAC GCCTGGATGTCCTGGGTGCGCCAGGCCCCCGGCAAGGGA CTGGAGTGGGTGGGACGGATCAAGTCCATCGCAGACGGA GGAGCAACCGATTACGCAGCCCCTGTGAGAAACAGGTTC ACAATCTCCAGAGACGATTCTAGGAATACCCTGTATCTG GAGATGCACTCTCTGAAGACAGAGGACACCGCCGTGTAC TATTGCACCACAATCCCTGGCAACGACGCCTTTGATATG TGGGGCCAGGGCACAATGGTGACCGTGAGCTCCGGCGGC GGCGGCTCTGGAGGAGGAGGAAGCGGAGGAGGAGGAAGC GGGGGCGGCGGCTCTGACATCGTGCTGACACAGTCCCCA CTGTCCCTGTCTGTGACCCCCGGCGAGCCTGCAAGCATC TCCTGTAGATCTAGCCAGAGCCTGCTGTACTCCAACGGC AAGAATTATCTGGATTGGTTCCTGCACAAGCCAGGCCAG TCTCCCCAGCTGCTGATCTACCTGGGATCTAATAGGGCA AGCGGAGTGCCAGACCGGTTCTCTGGAAGCGGATCCGGC ATCGACTTCATCCTGAAGATCAGCAGGGTGGAGGCCGAG GATGTGGGCGTGTACTATTGCATGCAGGCCCAGCAGACA CCCATCACCTTCGGCCAGGGCACAAGACTGGAGATCAAG ACCACAACCCCAGCACCAAGGCCACCTACACCTGCACCA ACCATCGCATCCCAGCCACTGTCTCTGAGGCCTGAGGCA TGTCGGCCAGCAGCAGGAGGAGCAGTGCACACCCGCGGC CTGGACTTTGCCTGCGATATCTACATCTGGGCACCACTG GCAGGAACATGTGGCGTGCTGCTGCTGAGCCTGGTCATC ACCCTGTACTGCAAGCGCGGCCGGAAGAAGCTGCTGTAT	CD8α signal peptide; 20E12 VH (Table 1, SEQ ID NO: 39); GS linker; 20E12 VL (Table 1, SEQ ID NO: 40); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

CAR	CAR Nucleic Acid Sequence	Components
	ATCTTCAAGCAGCCTTTTATGAGACCAGTGCAGACAACC CAGGAGGAGGACGGCTGCTCCTGTAGGTTCCCTGAAGAG GAGGAGGGCGGCTGTGAGCTGAGAGTGAAGTTTTCTAGG AGCGCCGATGCACCAGCATACCAGCAGGGACAGAATCAG CTGTATAACGAGCTGAATCTGGGCCGGAGAGAGGAGTAT GACGTGCTGGATAAGAGGAGGGGAAGGGACCCTGAGATG GGAGGCAAGCCCCGGAGAAAGAACCCTCAGGAGGGCCTG TACAATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTAT AGCGAGATCGGCATGAAGGGAGAGAGGCGCCGGGGCAAG GGACACGATGGCCTGTACCAGGGCCTGTCCACAGCCACC AAGGACACCTATGATGCCCTGCATATGCAGGCACTGCCT CCAAGGTGA (SEQ ID NO: 243)	
32G8	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCC CTGCTGCTGCACGCAGCAAGGCCAGAGGTGAACCTGGTG GAGTCCGGCGGCGGCCTGGTGAAGCCTGGCGGATCCCTG AGGCTGTCTTGCGAGGCAAGCGGCTTCACCTTCAGCTAC GCCTGGATGTCCTGGGTGCGCCAGGCCCCCGGCAAGGGA CTGGAGTGGGTGGGCCGGATCAAGTCCATCACCGACGGA GGCGTGATCGATTACGCAGCACCTGTGAGAAACAGGTGC ACAATCTCCAGAGACGATTCTAGGAATACCCTGTATCTG GAGATGCACTCTCTGAAGACAGAGGACACCGCCGTGTAC TATTGTACCACAATCCCTGGCAACGACGATTTCGATATG TGGGGCCAGGGCAGAATGGTGACCGTGAGCTCCGGCGGC GGCGGCTCTGGAGGAGGAGGAAGCGGAGGAGGAGGAAGC GGGGGCGGCGGCTCTGACATCGTGCTGACACAGTCCCCA CTGTCCCTGTCTGTGACCCCCGGCGAGCCTGCAAGCATC TCCTGTAGGTCTAGCCAGAGCCTGCTGTACTCCAACGGC AAGAATTATCTGGATTGGTTTCTGCACAAGCCAGGCCAG TCTCCCCAGCTGCTGATCTACCTGGGATCTAATAGGGCA AGCGGAGTGCCAGACCGGTTCTCTGGAAGCGGATCCGGC ATCGACTTCATCCTGAAGATCAGCCGCGTGGAGGCAGAG GACGTGGGCGTGTACTATTGCATGCAGGCCCAGCAGACA CCCATCACCTTCGGCCAGGGCACAAGACTGGAGATCAAG ACCACAACCCCAGCACCAAGGCCACCTACACCTGCACCA ACCATCGCATCCCAGCCACTGTCTCTGAGGCCTGAGGCA TGTAGGCCAGCAGCAGGAGGAGCAGTGCACACCAGAGGC CTGGACTTTGCCTGCGATATCTACATCTGGGCACCACTG GCAGGAACATGTGGCGTGCTGCTGCTGAGCCTGGTCATC ACCCTGTACTGCAAGCGCGGCCGGAAGAAGCTGCTGTAT ATCTTCAAGCAGCCTTTTATGAGACCAGTGCAGACAACC CAGGAGGAGGACGGCTGCTCCTGTAGGTTCCCTGAAGAG GAGGAGGGCGGCTGTGAGCTGAGAGTGAAGTTTTCTAGG AGCGCCGATGCACCAGCATACCAGCAGGGACAGAATCAG CTGTATAACGAGCTGAATCTGGGCCGGAGAGAGGAGTAT GACGTGCTGGATAAGAGGAGGGGAAGGGATCCTGAGATG GGAGGCAAGCCCCGGAGAAAGAACCCTCAGGAGGGCCTG	CD8α signal peptide; 32G8 VH (Table 1, SEQ ID NO: 43); GS linker; 32G8 VL (Table 1, SEQ ID NO: 40); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

CAR	CAR Nucleic Acid Sequence	Components
	TACAATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTAT AGCGAGATCGGCATGAAGGGAGAGAGGCGCCGGGGCAAG GGACACGATGGCCTGTACCAGGGCCTGTCCACAGCCACC AAGGACACCTATGATGCCCTGCATATGCAGGCACTGCCT CCAAGGTGA (SEQ ID NO: 244)	
26B9	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCC CTGCTGCTGCACGCAGCAAGGCCAGAGGTGCAGCTGGTG GAGTCTTGGGGCGTGCTGGTGAAGCCTGGCGGATCTCTG AGGCTGAGCTGCGCAGCATCCGGCTTCATCTTTAACAAT GCCTGGATGTCCTGGGTGCGCCAGGCCCCCGGCAAGGGA CTGGAGTGGATCGGCCGGATCAAGAGCAAGTCCGACGGA GGAACCACAGATTACGCAGCACCTGTGAAGGACCGCTTC ACAATCTCTCGGGACGATAGCAAGGATACCCTGTATCTG CAGATGAACGGCCTGAAGACAGAGGACACCGCCGTGTAC TTCTGCACCACAGCCCCTGGCGGCCCTTTTGATTATTGG GGCCAGGGCACACTGGTGACCGTGAGCTCCGGAGGAGGA GGAAGCGGCGGAGGAGGCAGCGGCGGCGGCGGCTCTGGC GGCGGCGGCAGCGACATCGTGCTGACACAGAGCCCTCTG TCCCTGCCAGTGACCCCCGGCGAGCCTGCCTCTATCAGC TGTCGCTCTAGCCAGAGCCTGCTGCACCGGGACGGCTTC AATTACCTGGATTGGTTTCTGCAGAAGCCAGGCCAGTCC CCCCAGCTGCTGATCTATCTGGCCTCCTCTAGAGCCTCT GGCGTGCCAGACAGGTTCTCCGGCTCTGACAGCGGCACA GACTTCACCCTGAAGATCAGCAGAGTGGAGGCCGAGGAT GTGGGCGTGTACTATTGCATGCAGGCCCTGCAGACACCC ATCACCTTCGGCCAGGGCACAAGACTGGAGATCAAGACC ACAACCCCAGCACCAAGGCCACCTACACCTGCACCAACC ATCGCATCCCAGCCACTGTCTCTGAGACCTGAGGCCTGT AGGCCAGCAGCAGGAGGAGCAGTGCACACCAGGGGCCTG GACTTTGCCTGCGATATCTACATCTGGGCACCTCTGGCA GGAACATGTGGCGTGCTGCTGCTGAGCCTGGTCATCACC CTGTACTGCAAGAGAGGCAGGAAGAAGCTGCTGTATATC TTCAAGCAGCCTTTTATGAGACCAGTGCAGACAACCCAG GAGGAGGACGGCTGCTCCTGTAGGTTCCCTGAAGAGGAG GAGGGAGGATGTGAGCTGAGGGTGAAGTTTTCCCGGTCT GCCGATGCACCAGCATACCAGCAGGGACAGAACCAGCTG TATAACGAGCTGAATCTGGGCCGGAGAGAGGAGTACGAC GTGCTGGATAAGAGGCGCGGCAGAGATCCAGAGATGGGC GGCAAGCCCCGGAGAAAGAACCCTCAGGAGGGCCTGTAC AATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTATAGC GAGATCGGCATGAAGGGAGAGAGGCGCCGGGGCAAGGGA CACGATGGCCTGTACCAGGGCCTGTCCACAGCCACCAAG GACACCTATGATGCCCTGCATATGCAGGCACTGCCTCCA AGGTGA (SEQ ID NO: 245)	CD8α signal peptide; 26B9 VH (Table 1, SEQ ID NO: 41); GS linker; 26B9 VL (Table 1, SEQ ID NO: 42); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

CAR	CAR Nucleic Acid Sequence	Components
30D8	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCACTGGCC CTGCTGCTGCACGCAGCAAGGCCTGAGGTGCAGCTGGTG GAGAGCGGCGGCGGCCTGGTGAAGCCTGGCGGATCCCTG AGGCTGTCTTGCGAGGCAAGCGGCTTCACCTTTAGCGAC GCATGGATGTCCTGGGTGCGCCAGGCCCCTGGCAAGGGA CTGGAGTGGGTGGGACGGATCAAGAGCAAGACAGACGGC GGCACCACAGATTACGTGGTGCCACTGAACGGCCGCTTC ATCATCTCCCGCGACGATTCTCGGAATACCCTGTATCTG CAGCTGAACAATCTGAAGACAGAGGATACCGCCGTGTAC TATTGCACCACAGTGCCAGGCTCCTACGGCTATTGGGGC CAGGGCACACTGGTGACCGTGAGCTCCGGCGGCGGCGGC TCTGGAGGAGGAGGAAGCGGAGGAGGAGGAAGCGGGGGC GGCGGCTCTGACATCGTGATGACACAGTCTCCACTGAGC CTGCCAGTGACCCCTGGCGAGCCAGCCTCCATCTCTTGT CGCTCTAGCCAGAGCCTGCTGCACAACAAGCGGAACAAT TACCTGGATTGGTTTCTGCAGAAGCCTGGCCAGTCCCCT CAGCTGCTGATCTATCTGGCCAGCAATAGAGCCTCCGGA GTGCCAGACAGGTTCTCTGGAGGAGGAAGCGGAACAGAC TTCACCCTGAAGATCAGCAGAGTGGAGGCCGAGGACGTG GGCGTGTACTATTGCATGCAGGCCCAGCAGACACCTATC ACCTTCGGCCAGGGCACAAGACTGGAGATCAAGACCACA ACCCCAGCACCAAGGCCACCTACACCTGCACCAACCATC GCCTCCCAGCCTCTGTCTCTGAGACCAGAGGCATGTAGG CCAGCAGCAGGAGGAGCAGTGCACACCAGGGGCCTGGAC TTTGCCTGCGATATCTACATCTGGGCACCTCTGGCAGGA ACATGTGGCGTGCTGCTGCTGAGCCTGGTCATCACCCTG TACTGCAAGAGAGGCAGGAAGAAGCTGCTGTATATCTTC AAGCAGCCCTTCATGAGACCCGTGCAGACAACCCAGGAG GAGGACGGCTGCTCTTGTAGGTTCCCAGAAGAGGAGGAG GGAGGATGTGAGCTGAGGGTGAAGTTTAGCCGGTCCGCC GATGCACCAGCATACCAGCAGGGACAGAACCAGCTGTAT AACGAGCTGAATCTGGGCCGGAGAGAGGAGTACGACGTG CTGGATAAGAGGAGGGGAAGGGATCCAGAGATGGGAGGC AAGCCTCGGAGAAAGAACCCACAGGAGGGCCTGTACAAT GAGCTGCAGAAGGACAAGATGGCCGAGGCCTATTCTGAG ATCGGCATGAAGGGAGAGAGGCGCCGGGGCAAGGGACAC GATGGCCTGTACCAGGGCCTGAGCACAGCCACCAAGGAC ACCTATGATGCCCTGCATATGCAGGCACTGCCTCCAAGG TGA (SEQ ID NO: 246)	CD8α signal peptide; 30D8 VH (Table 1, SEQ ID NO: 37); GS linker; 30D8 VL (Table 1, SEQ ID NO: 38); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
C6	ATGGCTCTGCCCGTCACCGCTCTGCTGCTGCCTCTGGCC CTGCTGCTGCACGCAGCAAGGCCACAGGTGCAGCTGGTG CAGTCCGGAGCAGAGGTGAAGAAGCCTGGCAGCTCCGTG AAGGTGAGCTGCAAGGCCTCCGGCGACACATTCTCTAGC AACGCAATCAGCTGGGTGCGCCAGGCCCCTGGCCAGGGA CTGGAGTGGATGGGCGTGATCATCCCTATCTTCGGCACC GCCGACTATGCCCAGAAGTTTCAGGGCCGGGTGACAATC ACCGCCGATGAGTCTACAAGCACCGCCTACATGGAGCTG	CD8α signal peptide; C6 VH (Table 1, SEQ ID NO: 48); GS linker; C6 VL (Table 1, SEQ ID

CAR	CAR Nucleic Acid Sequence	Components
	TCCTCTCTGAGATCCGAGGACACAGCCGTGTACTATTGT GCCAGGCACACCTATCACGAGTACGCAGGAGGATACTAT GGAGGAGCAATGGATCCTTGGGGACAGGGCACACTGGTG ACCGTGAGCTCCGGCGGCGGCGGCTCTGGAGGAGGAGGA AGCGGAGGAGGAGGAAGCGGGGGCGGCGGCTCTGAGCTG CAGAGCGTGCTGACCCAGCCACCTTCCGCCTCTGGAACA CCAGGCCAGAGGGTGACCATCAGCTGCTCCGGATCTAGC TCCAACATCGGCTCCAATTACGTGTATTGGTACCAGCAG CTGCCAGGCACAGCCCCCAAGATCCTGATCTACCGCAAC AATCAGCGGCCTTCTGGCGTGCCAGATAGATTCTCTGGC AGCAAGTCCGGCACCTCTGCCAGCCTGGCAATCTCCGGC CTGAGGTCTGAGGACGAGGCCGATTACTATTGCGCCGCC TGGGACGATAACCTGAGCGGCTGGGTGTTTGGCACAGGC ACCAAGCTGACAGTGCTGACCACAACCCCTGCACCAAGA CCACCAACACCAGCACCTACCATCGCAAGCCAGCCACTG TCCCTGAGACCCGAGGCCTGTAGGCCTGCAGCAGGAGGA GCAGTGCACACCAGGGGCCTGGACTTTGCCTGCGATATC TATATCTGGGCACCACTGGCAGGAACATGTGGCGTGCTG CTGCTGAGCCTGGTCATCACCCTGTATTGCAAGAGAGGC AGGAAGAAGCTGCTGTACATCTTCAAGCAGCCCTTTATG CGCCCTGTGCAGACAACCCAGGAGGAGGACGGCTGCAGC TGTCGGTTCCCAGAAGAGGAGGAGGGAGGATGTGAGCTG AGGGTGAAGTTTTCCCGGTCTGCCGATGCACCAGCATAT CAGCAGGGACAGAATCAGCTGTACAACGAGCTGAATCTG GGCCGGAGAGAGGAGTACGACGTGCTGGATAAGAGGAGG GGAAGGGACCCTGAGATGGGAGGCAAGCCACGGAGAAAA ACCCCCAGGAGGGCCTGTATAATGAGCTGCAGAAGGACA AGATGGCCGAGGCCTACTCTGAGATCGGCATGAAGGGAG AGAGGCGCCGGGGCAAGGGACACGATGGCCTGTATCAGG GCCTGAGCACAGCCACCAAGGACACCTACGATGCCCTGC ATATGCAGGCACTGCCTCCAAGGTGA (SEQ ID NO: 247)	NO: 49); CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

Downregulation or mutation of target antigens is commonly observed in cancer cells, creating antigen-loss escape variants. Thus, to offset tumor escape and render immune cell more specific to target, the EGFRvIII specific CAR can comprise one or more additional extracellular ligand-binding domains, to simultaneously bind different elements in target thereby augmenting immune cell activation and function. In one embodiment, the extracellular ligand-binding domains can be placed in tandem on the same transmembrane polypeptide, and optionally can be separated by a linker. In some embodiments, said different extracellular ligand- binding domains can be placed on different transmembrane polypeptides composing the CAR. In some embodiments, the

invention relates to a population of CARs, each CAR comprising a different extracellular ligand-binding domain. In a particular, the invention relates to a method of engineering immune cells comprising providing an immune cell and expressing at the surface of the cell a population of CARs, each CAR comprising different extracellular ligand-binding domains. In another particular embodiment, the invention relates to a method of engineering an immune cell comprising providing an immune cell and introducing into the cell polynucleotides encoding polypeptides composing a population of CARs each one comprising different extracellular ligand-binding domains. By population of CARs, it is meant at least two, three, four, five, six or more CARs each one comprising different extracellular ligand-binding domains. The different extracellular ligand-binding domains according to the invention can preferably simultaneously bind different elements in target thereby augmenting immune cell activation and function. The invention also relates to an isolated immune cell which comprises a population of CARs each one comprising different extracellular ligand-binding domains.

In another aspect, the invention provides polynucleotides encoding any of the CARs and polypeptides described herein. Polynucleotides can be made and expressed by procedures known in the art.

In another aspect, the invention provides compositions (such as a pharmaceutical compositions) comprising any of the cells of the invention. In some embodiments, the composition comprises a cell comprising a polynucleotide encoding any of the CARs described herein.

Expression vectors, and administration of polynucleotide compositions are further described herein.

In another aspect, the invention provides a method of making any of the polynucleotides described herein.

Polynucleotides complementary to any such sequences are also encompassed by the invention. Polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

Polynucleotides may comprise a native sequence (i.e., an endogenous sequence that encodes an antibody or a portion thereof) or may comprise a variant of such a sequence. Polynucleotide variants contain one or more substitutions, additions, deletions and/or insertions such that the immunoreactivity of the encoded polypeptide is not diminished, relative to a native immunoreactive molecule. The effect on the immunoreactivity of the encoded polypeptide may generally be assessed as described herein. Variants preferably exhibit at least about 70% identity, more preferably, at least about 80% identity, yet more preferably, at least about 90% identity, and most preferably, at least about 95% identity to a polynucleotide sequence that encodes a native antibody or a portion thereof.

Two polynucleotide or polypeptide sequences are said to be "identical" if the sequence of nucleotides or amino acids in the two sequences is the same when aligned for maximum correspondence as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, or 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O., 1978, A model of evolutionary change in proteins - Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J., 1990, Unified Approach to Alignment and Phylogenes pp. 626-645 Methods in Enzymology vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M., 1989, CABIOS 5:151-153; Myers, E.W. and Muller W., 1988, CABIOS 4:11-17; Robinson, E.D., 1971, Comb. Theor. 11:105; Santou, N., Nes, M., 1987, Mol. Biol. Evol. 4:406-425; Sneath, P.H.A. and Sokal, R.R., 1973, Numerical Taxonomy the Principles and Practice of Numerical Taxonomy, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J., 1983, Proc. Natl. Acad. Sci. USA 80:726-730.

Preferably, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (i.e. the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

Variants may also, or alternatively, be substantially homologous to a native gene, or a portion or complement thereof. Such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA sequence encoding a native antibody (or a complementary sequence).

Suitable “moderately stringent conditions” include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1 % SDS.

As used herein, "highly stringent conditions" or "high stringency conditions" are those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt’s solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the invention. Further, alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the invention. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein may, but need not, have an altered structure or function. Alleles may be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

The polynucleotides of this invention can be obtained using chemical synthesis, recombinant methods, or PCR. Methods of chemical polynucleotide synthesis are well known in the art and need not be described in detail herein. One of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to produce a desired DNA sequence.

For preparing polynucleotides using recombinant methods, a polynucleotide comprising a desired sequence can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification, as further discussed herein. Polynucleotides may be inserted into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by direct uptake, endocytosis, transfection, F-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell genome. The polynucleotide so amplified can be isolated from the host cell by methods well known within the art. See, e.g., Sambrook et al., 1989.

Alternatively, PCR allows reproduction of DNA sequences. PCR technology is well known in the art and is described in U.S. Patent Nos. 4,683,195, 4,800,159, 4,754,065 and 4,683,202, as well as PCR: The Polymerase Chain Reaction, Mullis et al. eds., Birkauswer Press, Boston, 1994.

RNA can be obtained by using the isolated DNA in an appropriate vector and inserting it into a suitable host cell. When the cell replicates and the DNA is transcribed

into RNA, the RNA can then be isolated using methods well known to those of skill in the art, as set forth in Sambrook et al., 1989, supra, for example.

Suitable cloning vectors may be constructed according to standard techniques, or may be selected from a large number of cloning vectors available in the art. While the cloning vector selected may vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, may possess a single target for a particular restriction endonuclease, and/or may carry genes for a marker that can be used in selecting clones containing the vector. Suitable examples include plasmids and bacterial viruses, e.g., pUC18, pUC19, Bluescript (e.g., pBS SK+) and its derivatives, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3 and pAT28. These and many other cloning vectors are available from commercial vendors such as BioRad, Strategene, and Invitrogen.

Expression vectors generally are replicable polynucleotide constructs that contain a polynucleotide according to the invention. It is implied that an expression vector must be replicable in the host cells either as episomes or as an integral part of the chromosomal DNA. Suitable expression vectors include but are not limited to plasmids, viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, cosmids, and expression vector(s) disclosed in PCT Publication No. WO 87/04462. Vector components may generally include, but are not limited to, one or more of the following: a signal sequence; an origin of replication; one or more marker genes; suitable transcriptional controlling elements (such as promoters, enhancers and terminator). For expression (i.e., translation), one or more translational controlling elements are also usually required, such as ribosome binding sites, translation initiation sites, and stop codons.

The vectors containing the polynucleotides of interest can be introduced into the host cell by any of a number of appropriate means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (e.g., where the vector is an infectious agent such as vaccinia virus). The choice of introducing vectors or polynucleotides will often depend on features of the host cell.

A polynucleotide encoding an EGFRvIII specific CAR disclosed herein may exist in an expression cassette or expression vector (e.g., a plasmid for introduction into a bacterial host cell, or a viral vector such as a baculovirus vector for transfection of an

insect host cell, or a plasmid or viral vector such as a lentivirus for transfection of a mammalian host cell). In some embodiments, a polynucleotide or vector can include a nucleic acid sequence encoding ribosomal skip sequences such as, for example without limitation, a sequence encoding a 2A peptide. 2A peptides, which were identified in the Aphthovirus subgroup of picornaviruses, causes a ribosomal "skip" from one codon to the next without the formation of a peptide bond between the two amino acids encoded by the codons (see (Donnelly and Elliott 2001; Atkins, Wills et al. 2007; Doronina, Wu et al. 2008)). By "codon" is meant three nucleotides on an mRNA (or on the sense strand of a DNA molecule) that are translated by a ribosome into one amino acid residue. Thus, two polypeptides can be synthesized from a single, contiguous open reading frame within an imRNA when the polypeptides are separated by a 2A oligopeptide sequence that is in frame. Such ribosomal skip mechanisms are well known in the art and are known to be used by several vectors for the expression of several proteins encoded by a single messenger RNA.

To direct transmembrane polypeptides into the secretory pathway of a host cell, in some embodiments, a secretory signal sequence (also known as a signal peptide, leader sequence, prepro sequence or pre sequence) is provided in a polynucleotide sequence or vector sequence. The secretory signal sequence is operably linked to the transmembrane nucleic acid sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleic acid sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the nucleic acid sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830). In some embodiments the signal peptide comprises the amino acid sequence shown in SEQ ID NO: 206 or 214. Those skilled in the art will recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. In some embodiments, nucleic acid sequences of the invention are codon-optimized for expression in mammalian cells, preferably for expression in human cells. Codon-optimization refers to the exchange in a sequence of interest of codons that are generally rare in highly expressed genes of a given species by codons that are generally frequent in highly

expressed genes of such species, such codons encoding identical amino acids as the codons that are being exchanged.

Methods of engineering an immune cell

Methods of preparing immune cells for use in immunotherapy are provided herein. In some embodiments, the methods comprise introducing a CAR according to the invention into immune cells, and expanding the cells. In some embodiments, the invention relates to a method of engineering an immune cell comprising: providing a cell and expressing at the surface of the cell at least one CAR as described above. Methods for engineering immune cells are described in, for example, PCT Patent Application Publication Nos. WO/2014/039523, WO/2014/184741, WO/2014/191128, WO/2014/184744, and WO/2014/184143, each of which is incorporated herein by reference in its entirety. In some embodiments, the method comprises: transforming the cell with at least one polynucleotide encoding CAR as described above, and expressing the polynucleotides in the cell.

In some embodiments, the polynucleotides are present in lentiviral vectors for stable expression in the cells.

In some embodiments, the method can further comprise a step of genetically modifying a cell by inactivating at least one gene expressing, for example without limitation, a component of the TCR, a target for an immunosuppressive agent, an HLA gene, and/or an immune checkpoint protein such as, for example, PDCD1 or CTLA-4. By inactivating a gene it is intended that the gene of interest is not expressed in a functional protein form. In some embodiments, the gene to be inactivated is selected from the group consisting of, for example without limitation, TCRα, TCRβ, dCK, CD52, GR, PD-1, and CTLA-4. In some embodiments the method comprises inactivating one or more genes by introducing into the cells a rare-cutting endonuclease able to selectively inactivate a gene by selective DNA cleavage. In some embodiments the rare-cutting endonuclease can be, for example, a transcription activator-like effector nuclease (TALE-nuclease) or Cas9 endonuclease.

In some embodiments, an additional catalytic domain is used with a rare-cutting endonuclease to enhance its capacity to inactivate targeted genes. For example, an additional catalytic domain can be a DNA end-processing enzyme. Non-limiting examples of DNA end-processing enzymes include 5-3′ exonucleases, 3-5′

exonucleases, 5-3′ alkaline exonucleases, 5′ flap endonucleases, helicases, hosphatase, hydrolases and template-independent DNA polymerases. Non-limiting examples of such catalytic domain comprise of a protein domain or catalytically active derivate of the protein domain selected from the group consisting of hExoI (EXO1_HUMAN), Yeast ExoI (EXO1_YEAST), *E. coli* ExoI, Human TREX2, Mouse TREX1, Human TREX1, Bovine TREX1, Rat TREX1, TdT (terminal deoxynucleotidyl transferase) Human DNA2, Yeast DNA2 (DNA2_YEAST). In some embodiments, an additional catalytic domain can have a 3′-5′-exonuclease activity, and in some embodiments, said additional catalytic domain is TREX, more preferably TREX2 catalytic domain (WO2012/058458). In some embodiments, said catalytic domain is encoded by a single chain TREX polypeptide. The additional catalytic domain may be fused to a nuclease fusion protein or chimeric protein. In some embodiments, the additional catalytic domain is fused using, for example, a peptide linker.

In some embodiments, the method further comprises a step of introducing into cells an exogeneous nucleic acid comprising at least a sequence homologous to a portion of the target nucleic acid sequence, such that homologous recombination occurs between the target nucleic acid sequence and the exogeneous nucleic acid. In some embodimentss, said exogenous nucleic acid comprises first and second portions which are homologous to region 5′ and 3′ of the target nucleic acid sequence, respectively. The exogenous nucleic acid may also comprise a third portion positioned between the first and the second portion which comprises no homology with the regions 5′ and 3′ of the target nucleic acid sequence. Following cleavage of the target nucleic acid sequence, a homologous recombination event is stimulated between the target nucleic acid sequence and the exogenous nucleic acid. In some embodiments, homologous sequences of at least about 50 bp, greater than about 100 bp, or greater than about 200 bp can be used within the donor matrix. The exogenous nucleic acid can be, for example without limitation, from about 200 bp to about 6000 bp, more preferably from about 1000 bp to about 2000 bp. Shared nucleic acid homologies are located in regions flanking upstream and downstream the site of the break, and the nucleic acid sequence to be introduced is located between the two arms.

In some embodiments, a nucleic acid successively comprises a first region of homology to sequences upstream of said cleavage; a sequence to inactivate a targeted gene selected from the group consisting of TCRα, TCRβ, CD52, glucocorticoid receptor

(GR), deoxycytidine kinase (dCK), and an immune checkpoint protein such as for example programmed death-1 (PD-1); and a second region of homology to sequences downstream of the cleavage. The polynucleotide introduction step can be simultaneous, before or after the introduction or expression of the rare-cutting endonuclease. Depending on the location of the target nucleic acid sequence wherein break event has occurred, such exogenous nucleic acid can be used to knock-out a gene, e.g. when exogenous nucleic acid is located within the open reading frame of the gene, or to introduce new sequences or genes of interest. Sequence insertions by using such exogenous nucleic acid can be used to modify a targeted existing gene, by correction or replacement of the gene (allele swap as a non-limiting example), or to up- or down-regulate the expression of the targeted gene (promoter swap as non-limiting example), the targeted gene correction or replacement. In some embodiments, inactivation of a genes selected from the group consisting of TCRα, TCRβ, CD52, GR, dCK, and immune checkpoint proteins, can be done at a precise genomic location targeted by a specific TALE-nuclease, wherein said specific TALE-nuclease catalyzes a cleavage and wherein the exogenous nucleic acid successively comprising at least a region of homology and a sequence to inactivate one targeted gene selected from the group consisting of TCRα, TCRβ, CD52, GR, dCK, immune checkpoint proteins which is integrated by homologous recombination. In some embodiments, several genes can be, successively or at the same time, inactivated by using several TALE-nucleases respectively and specifically targeting one defined gene and several specific polynucleotides for specific gene inactivation.

In some embodiments, the method comprises inactivation of one or more additional genes selected from the group consisting of TCRα, TCRβ, CD52, GR, dCK, and immune checkpoint proteins. In some embodiments, inactivation of a gene can be accomplished by introducing into the cells at least one rare-cutting endonuclease such that the rare-cutting endonuclease specifically catalyzes cleavage in a targeted sequence of the cell genome; and optionally, introducing into the cells an exogenous nucleic acid successively comprising a first region of homology to sequences upstream of the cleavage, a sequence to be inserted in the genome of the cell, and a second region of homology to sequences downstream of the cleavage; wherein the introduced exogenous nucleic acid inactivates a gene and integrates at least one exogenous polynucleotide sequence encoding at least one recombinant protein of interest. In some

embodiments, the exogenous polynucleotide sequence is integrated within a gene encoding a protein selected from the group consisting of TCRα, TCRβ, CD52, GR, dCK, and immune checkpoint protein.

In another aspect, a step of genetically modifying cells can comprise: modifying T cells by inactivating at least one gene expressing a target for an immunosuppressive agent, and; expanding the cells, optionally in presence of the immunosuppressive agent. An immunosuppressive agent is an agent that suppresses immune function by one of several mechanisms of action. An immunosuppressive agent can diminish the extent and/or voracity of an immune response. Non-limiting examples of immunosuppressive agents include calcineurin inhibitors, targets of rapamycin, interleukin-2 α-chain blockers, inhibitors of inosine monophosphate dehydrogenase, inhibitors of dihydrofolic acid reductase, corticosteroids, and immunosuppressive antimetabolites. Some cytotoxic immunosuppressants act by inhibiting DNA synthesis. Others may act through activation of T cells or by inhibiting the activation of helper cells. The methods according to the invention allow conferring immunosuppressive resistance to T cells for immunotherapy by inactivating the target of the immunosuppressive agent in T cells. As non-limiting examples, targets for immunosuppressive agent can be a receptor for an immunosuppressive agent such as for example without limitation CD52, glucocorticoid receptor (GR), FKBP family gene members, and cyclophilin family gene members.

In some embodiments, the genetic modification of the method involves expression, in provided cells to engineer, of one rare-cutting endonuclease such that the rare-cutting endonuclease specifically catalyzes cleavage in one targeted gene thereby inactivating the targeted gene. In some embodiments, a method of engineering cells comprises at least one of the following steps: providing a T cell, such as from a cell culture or from a blood sample; selecting a gene in the T cell expressing a target for an immunosuppressive agent; introducing into the T cell a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break the gene encoding a target for the immunosuppressive agent, and expanding the cells, optionally in presence of the immunosuppressive agent.

In some embodiments, the method comprises: providing a T cell, such as from a cell culture or from a blood sample; selecting a gene in the T cell wherein the gene expresses a target for an immunosuppressive agent; transforming the T cell with

nucleic acid encoding a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break the gene encoding a target for the immunosuppressive agent, and expressing the rare-cutting endonucleases into the T cells; and expanding the cells, optionally in presence of the immunosuppressive agent.

In some embodiments, the rare-cutting endonuclease specifically targets CD52 or GR. In some embodiments, the gene selected for inactivation encodes CD52, and the immunosuppressive treatment comprises a humanized antibody targeting CD52 antigen. In some embodiments, the gene selected for inactivation encodes GR, and the immunosuppressive treatment comprises a corticosteroid such as dexamethasone. In some embodiments, the gene selected for inactivation is a FKBP family gene member or a variant thereof and the immunosuppressive treatment comprises FK506, also known as Tacrolimus or fujimycin. In some embodiments, the FKBP family gene member is FKBP12 or a variant thereof. In some embodiments, gene selected for inactivation is a cyclophilin family gene member or a variant thereof and the immunosuppressive treatment comprises cyclosporine.

In some embodiments, the rare-cutting endonuclease can be, for example, a meganuclease, a zinc finger nuclease, or a TALE-nuclease. In some embodiments, the rare-cutting endonuclease is a TALE-nuclease.

Also provided herein are methods of engineering T cells, suitable for immunotherapy, wherein the methods comprise: genetically modifying T cells by inactivating at least one immune checkpoint protein. In some embodiments the immune checkpoint protein is, for example, PD-1 and/or CTLA-4. In some embodiments, methods of genetically modifying a cell comprises: modifying T cells by inactivating at least one immune checkpoint protein; and expanding the cells. Immune checkpoint proteins include, but are not limited to Programmed Death 1 (PD-1, also known as PDCD1 or CD279, accession number: NM_005018), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4, also known as CD152, GenBank accession number AF414120.1), LAG3 (also known as CD223, accession number: NM_002286.5), Tim3 (also known as HAVCR2, GenBank accession number: JX049979.1), BTLA (also known as CD272, accession number: NM_181780.3), BY55 (also known as CD160, GenBank accession number: CR541888.1), TIGIT (also known as VSTM3, accession number: NM_173799), B7H5 (also known as C10orf54, homolog of mouse vista gene, accession number: NM_022153.1), LAIR1 (also known as CD305, GenBank accession number: CR542051.1),

SIGLEC10 (GeneBank accession number: AY358337.1), 2B4 (also known as CD244, accession number: NM_001166664.1), which directly inhibit immune cells. For example, CTLA-4 is a cell-surface protein expressed on certain CD4 and CD8 T cells; when engaged by its ligands (B7-1 and B7-2) on antigen presenting cells, T cell activation and effector function are inhibited.

In some embodiments, said method to engineer cells comprises at least one of the following steps: providing a T cell, such as from a cell culture or from a blood sample; introducing into the T cell a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break one gene encoding an immune checkpoint protein; and expanding the cells. In some embodiments, the method comprises: providing a T cell, such as from a cell culture or from a blood sample; transfecting said T cell with nucleic acid encoding a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break a gene encoding an immune checkpoint protein; expressing the rare-cutting endonucleases into the T cells; expanding the cells. In some embodiments, the rare-cutting endonuclease specifically targets a gene selected from the group consisting of: PD-1, CTLA-4, LAG3, Tim3, BTLA, BY55, TIGIT, B7H5, LAIR1, SIGLEC10, 2B4, TCRα, and TCRβ. In some embodiments, the rare-cutting endonuclease can be a meganuclease, a zinc finger nuclease or a TALE-nuclease. In some embodiments, the rare-cutting endonuclease is a TALE-nuclease.

In some embodiments, the present invention can be particularly suitable for allogeneic immunotherapy. In such embodiments, cells may be modified by a method comprising: inactivating at least one gene encoding a component of the T cell receptor (TCR) in T cells; and expanding the T cells. In some embodiments, the genetic modification of the method relies on the expression, in provided cells to engineer, of one rare-cutting endonuclease such that the rare-cutting endonuclease specifically catalyzes cleavage in one targeted gene thereby inactivating the targeted gene. In some embodiments, said method to engineer cells comprises at least one of the following steps: providing a T cell, such as from a cell culture or from a blood sample; introducing into the T cell a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break at least one gene encoding a component of the T cell receptor (TCR), and expanding the cells.

In some embodiments, the method comprises: providing a T cell, such as from a cell culture or from a blood sample; transfecting said T cell with nucleic acid encoding a rare-cutting endonuclease able to selectively inactivate by DNA cleavage, preferably by double-strand break at least one gene encoding a component of the T cell receptor (TCR); expressing the rare-cutting endonucleases into the T cells; sorting the transfected T cells, which do not express TCR on their cell surface; and expanding the cells.

In some embodiments, the rare-cutting endonuclease can be a meganuclease, a zinc finger nuclease or a TALE-nuclease. In some embodiments, the rare-cutting endonuclease is a TALE-nuclease. In some embodiments the TALE-nucleases recognize and cleave a sequence encoding TCRα or TCRβ. In some embodiments a TALE-nuclease comprises a polypeptide sequence selected from the amino acid sequence shown in SEQ ID NO: 218, 219, 220, 221, 222, 223, 224, or 225.

TALE-nuclease polypeptide sequences:

Repeat TRAC_T01-L

LTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQ
RLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHD
GGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTP
EQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPV
LCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQA
LETVQALLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVVA
IASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQA
HGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNGGGKQALETV
QRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN
GGGRPALE (SEQ ID NO: 218)

Repeat TRAC_T01-R

LTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQR
LLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGG
KQALETVQALLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQ
VVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVL
CQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQA

LETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVA
IASNIGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAH
GLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQALETVQ
RLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNG
GGRPALE (SEQ ID NO: 219)

Repeat TRBC_T01-L

LTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQ
RLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNG
GGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLT
PQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRL
LPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNNGG
KQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQ
VVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLC
QAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQAL
ETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIA
SNGGGRPALE (SEQ ID NO: 220)

Repeat TRBC_T01-R

NPQRSTVWYLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGG
GKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTP
QQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLP
VLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGK
QALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQ
VVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVL
CQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQA
LETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVA
IASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQA
HGLTPQQVVAIASNGGGRPALE (SEQ ID NO: 221)

Repeat TRBC_T02-L

LTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQR
LLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGG

KQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQ
VVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVL
CQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQAL
ETVQALLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAI
ASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQA
HGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETV
QRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN
GGGRPALE (SEQ ID NO: 222)

Repeat TRBC_T02-R

LTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQ
RLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIG
GKQALETVQALLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPE
QVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVL
CQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQA
LETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVA
IASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQA
HGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETV
QRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN
GGGRPALE (SEQ ID NO: 223)

Repeat CD52_T02-L

LTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQR
LLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGG
GKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPE
QVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPV
LCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQA
LETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVA
IASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAH
GLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQ
RLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNGG
GRPALE (SEQ ID NO: 224)

Repeat CD52_T02-R

LTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQ
RLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNG
GGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTP
QQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLL
PVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGK
QALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQV
VAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLC
QAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALE
TVQALLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIA
SNGGGRPALE (SEQ ID NO: 225)

In another aspect, another step of genetically modifying a cell can be a method of expanding TCRα deficient T cells comprising introducing into the T cell pTα (also known as preTCRα) or a functional variant thereof and expanding the cells, optionally through stimulation of the CD3 complex. In some embodiments, the method comprises: a) transforming the cells with a nucleic acid encoding at least a fragment of pTα to support CD3 surface expression; b) expressing said pTα into the cells; and c) expanding the cells, optionally through stimulation of the CD3 complex.

Also provided are methods of preparing T cells for immunotherapy comprising steps of a method provided herein for expansion of T cells. In some embodiments, the pTα polynucleotide sequence can be introduced randomly or by homologous recombination. In some embodiments, the insertion can be associated with the inactivation of the TCRα gene.

Different functional variants of pTα can be used. A “functional variant” of the peptide refers to a molecule substantially similar to either the entire peptide or a fragment thereof. A “fragment” of the pTα or functional variant thereof refers to any subset of the molecule, that is, a shorter peptide than the full-length pTα. In some embodiments, pTα or functional variants can be, for example, full-length pTα or a C-terminal truncated pTα version. C-terminal truncated pTα lacks in C-terminal end one or more residues. As non limiting examples, C-terminal truncated pTα version lacks 18, 48, 62, 78, 92, 110 or 114 residues from the C-terminus of the protein. Amino acid sequence variants of the peptide can be prepared by mutations in the DNA which encodes the peptide. Such functional variants include, for example, deletions from, or

insertions or substitutions of, residues within the amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity, in particular the restoration of a functional CD3 complex. In a preferred embodiment, at least one mutation is introduced in the different pTα versions as described above to affect dimerization. As a non limiting example, a mutated residue can be at least W46R, D22A, K24A, R102A or R117A of the human pTα protein or aligned positions using CLUSTALW method on pTα family or homologue member. Preferably pTα or a variant thereof as described above comprise the mutated residue W46R or the mutated residues D22A, K24A, R102A and R117A. In some embodiments, said pTα or variants are also fused to a signal-transducing domain such as CD28, OX40, ICOS, CD27, CD137 (4-1BB) and CD8 as non limiting examples. The extracellular domain of pTα or variants as described above can be fused to a fragment of the TCRα protein, particularly the transmembrane and intracellular domain of TCRα. pTα variants can also be fused to the intracellular domain of TCRα.

In some embodiments, pTα versions can be fused to an extracellular ligand-binding domain. In some embodiments, pTα or a functional variant thereof is fused to a single chain antibody fragment (scFv) comprising the light and the heavy variable fragment of a target antigen specific monoclonal antibody joined by a flexible linker.

The term “TCRα deficient T cell” refers to an isolated T cell that lacks expression of a functional TCRα chain. This may be accomplished by different means, as non limiting examples, by engineering a T cell such that it does not express any functional TCRα on its cell surface or by engineering a T cell such that it produces very little functional TCRα chain on its surface or by engineering a T cell to express mutated or truncated form of TCRα chain. TCRα deficient cells can no longer be expanded through CD3 complex. Thus, to overcome this problem and to allow proliferation of TCRα deficient cells, pTα or functional variant thereof is introduced into the cells, thus restoring a functional CD3 complex. In some embodiments, the method further comprises introducing into said T cells rare-cutting endonucleases able to selectively inactivate by DNA cleavage one gene encoding one component of the T cell receptor (TCR). In some embodiments, the rare-cutting endonuclease is a TALE-nuclease.

In another aspect, engineered T cells obtained by the methods described herein can be contacted with bispecific antibodies. For example, the T cells can be contacted

with bispecific antibodies ex vivo prior to administration to a patient, or in vivo following administration to a patient. Bispecific antibodies comprise two variable regions with distinct antigen properties that facilitate bringing the engineered cells into proximity to a target antigen. As a non-limiting example, a bispecific antibody can be directed against a tumor marker and lymphocyte antigen, such as for example without limitation CD3, and has the potential to redirect and activate any circulating T cells against tumors.

In some embodiments, polynucleotides encoding polypeptides according to the present invention can be mRNA which is introduced directly into the cells, for example by electroporation. In some embodiments, cytoPulse technology can be used to transiently permeabilize living cells for delivery of material into the cells. Parameters can be modified in order to determine conditions for high transfection efficiency with minimal mortality.

Also provided herein are methods of transforming T cells. In some embodiments, the method comprises: contacting a T cell with RNA and applying to the T cell an agile pulse sequence consisting of: (a) an electrical pulse with a voltage range from about 2250 to 3000 V per centimeter; (b) a pulse width of 0.1 ms; (c) a pulse interval of about 0.2 to 10 ms between the electrical pulses of step (a) and (b); (d) an electrical pulse with a voltage range from about 2250 to 3000 V with a pulse width of about 100 ms and a pulse interval of about 100 ms between the electrical pulse of step (b) and the first electrical pulse of step (c); and (e) four electrical pulses with a voltage of about 325 V with a pulse width of about 0.2 ms and a pulse interval of 2 ms between each of 4 electrical pulses. In some embodiments, a method of transforming T cell comprising contacting said T cell with RNA and applying to T cell an agile pulse sequence comprising: (a) an electrical pulse with a voltage of about 2250, 2300, 2350, 2400, 2450, 2500, 2550, 2400, 2450, 2500, 2600, 2700, 2800, 2900 or 3000V per centimeter; (b) a pulse width of 0.1 ms; (c) and a pulse interval of about 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 ms between the electrical pulses of step (a) and (b); (d) one electrical pulse with a voltage range from about 2250, of 2250, 2300, 2350, 2400, 2450, 2500, 2550, 2400, 2450, 2500, 2600, 2700, 2800, 2900 or 3000V with a pulse width of 100 ms and a pulse interval of 100 ms between the electrical pulse of step (b) and the first electrical pulse of step (c); and (e) 4 electrical pulses with a voltage of about 325 V with a pulse width of about 0.2 ms and a pulse interval of about 2 ms between each of 4 electrical pulses. Any values included in the value range described above are disclosed

in the present application. Electroporation medium can be any suitable medium known in the art. In some embodiments, the electroporation medium has conductivity in a range spanning about 0.01 to about 1.0 milliSiemens.

In some embodiments, as non limiting examples, an RNA encodes a rare-cutting endonuclease, one monomer of a rare-cutting endonuclease such as half-TALE-nuclease, a CAR, at least one component of a multi-chain chimeric antigen receptor, a pTα or functional variant thereof, an exogenous nucleic acid, and/or one additional catalytic domain.

Engineered immune cells

The invention also provides engineered immune cells comprising any of the CAR polynucleotides described herein. In some embodiments, a CAR can be introduced into an immune cell as a transgene via a plasmid vector. In some embodiments, the plasmid vector can also contain, for example, a selection marker which provides for identification and/or selection of cells which received the vector.

CAR polypeptides may be synthesized in situ in the cell after introduction of polynucleotides encoding the CAR polypeptides into the cell. Alternatively, CAR polypeptides may be produced outside of cells, and then introduced into cells. Methods for introducing a polynucleotide construct into cells are known in the art. In some embodiments, stable transfection methods can be used to integrate the polynucleotide construct into the genome of the cell. In other embodiments, transient transfection methods can be used to transiently express the polynucleotide construct, and the polynucleotide construct is not integrated into the genome of the cell. In other embodiments, virus-mediated methods can be used. The polynucleotides may be introduced into a cell by any suitable means such as for example, recombinant viral vectors (e.g. retroviruses, adenoviruses), liposomes, and the like. Transient transfection methods include, for example without limitation, microinjection, electroporation or particle bombardment. Polynucleotides may be included in vectors, such as for example plasmid vectors or viral vectors.

Also provided herein are isolated cells and cell lines obtained by the above-described methods of engineering cells provided herein. In some embodiments, an isolated cell comprises at least one CAR as described above. In some embodiments,

an isolated cell comprises a population of CARs, each CAR comprising different extracellular ligand-binding domains.

Also provided herein are isolated immune cells obtained according to any one of the methods described above. Any immune cell capable of expressing heterologous DNAs can be used for the purpose of expressing the CAR of interest. In some embodiments, the immune cell is a T cell. In some embodiments, an immune cell can be derived from, for example without limitation, a stem cell. The stem cells can be adult stem cells, non-human embryonic stem cells, more particularly non-human stem cells, cord blood stem cells, progenitor cells, bone marrow stem cells, induced pluripotent stem cells, totipotent stem cells or hematopoietic stem cells. Representative human cells are CD34+ cells. The isolated cell can also be a dendritic cell, killer dendritic cell, a mast cell, a NK-cell, a B-cell or a T cell selected from the group consisting of inflammatory T-lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes, memory T-lymphocytes, or helper T-lymphocytes. In some embodiments, the cell can be derived from the group consisting of CD4+ T-lymphocytes and CD8+ T-lymphocytes.

Prior to expansion and genetic modification, a source of cells can be obtained from a subject through a variety of non-limiting methods. Cells can be obtained from a number of non-limiting sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In some embodiments, any number of T cell lines available and known to those skilled in the art, may be used. In some embodiments, cells can be derived from a healthy donor, from a patient diagnosed with cancer or from a patient diagnosed with an infection. In some embodiments, cells can be part of a mixed population of cells which present different phenotypic characteristics.

Also provided herein are cell lines obtained from a transfected T cell according to any of the above-described methods. Also provided herein are modified cells resistant to an immunosuppressive treatment. In some embodiments, an isolated cell according to the invention comprises a polynucleotide encoding a CAR.

The immune cells of the invention can be activated and expanded, either prior to or after genetic modification of the T cells, using methods as generally described, for example without limitation, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application

Publication No. 20060121005. T cells can be expanded in vitro or in vivo. Generally, the T cells of the invention can be expanded, for example, by contact with an agent that stimulates a CD3 TCR complex and a co-stimulatory molecule on the surface of the T cells to create an activation signal for the T cell. For example, chemicals such as calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), or mitogenic lectins like phytohemagglutinin (PHA) can be used to create an activation signal for the T cell.

In some embodiments, T cell populations may be stimulated in vitro by contact with, for example, an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. Conditions appropriate for T cell culture include an appropriate media (e.g., Minimal Essential Media or RPMI Media 1640 or, X-Vivo 15, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-γ, IL-4, IL-7, GM-CSF, IL-10, IL-2, IL-15, TGFp, and TNF, or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl- cysteine and 2-mercaptoethanoi. Media can include RPMI 1640, AIM-V, DMEM, MEM, a- MEM, F-12, X-Vivo 10, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, e.g., penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (e.g., 37° C) and atmosphere (e.g., air plus 5% CO_2). T cells that have been exposed to varied stimulation times may exhibit different characteristics

In some embodiments, the cells of the invention can be expanded by co-culturing with tissue or cells. The cells can also be expanded in vivo, for example in the subject's blood after administering the cell into the subject.

In some embodiments, an isolated cell according to the present invention comprises one inactivated gene selected from the group consisting of CD52, dCK, GR, PD-1, CTLA-4, LAG3, Tim3, BTLA, BY55, TIGIT, B7H5, LAIR1, SIGLEC10, 2B4, HLA, TCRα and TCRβ and/or expresses a CAR, a multi-chain CAR and/or a pTα transgene. In some embodiments, an isolated cell comprises polynucleotides encoding polypeptides comprising a multi-chain CAR. In some embodiments, the isolated cell according to the present invention comprises two inactivated genes selected from the group consisting of: CD52 and GR, CD52 and TCRα, CDR52 and TCRβ, GR and TCRα, GR and TCRβ, TCRα and TCRβ, PD-1 and TCRα, PD-1 and TCRβ, CTLA-4 and TCRα, CTLA-4 and TCRβ, LAG3 and TCRα, LAG3 and TCRβ, Tim3 and TCRα, Tim3 and TCRβ, BTLA and TCRα, BTLA and TCRβ, BY55 and TCRα, BY55 and TCRβ, TIGIT and TCRα, TIGIT and TCRβ, B7H5 and TCRα, B7H5 and TCRβ, LAIR1 and TCRα, LAIR1 and TCRβ, SIGLEC10 and TCRα, SIGLEC10 and TCRβ, 2B4 and TCRα, 2B4 and TCRβ and/or expresses a CAR, a multi-chain CAR and a pTα transgene.

In some embodiments, TCR is rendered not functional in the cells according to the invention by inactivating TCRα gene and/or TCRβ gene(s). In some embodiments, a method to obtain modified cells derived from an individual is provided, wherein the cells can proliferate independently of the major histocompatibility complex (MHC) signaling pathway. Modified cells, which can proliferate independently of the MHC signaling pathway, and which may be obtained by this method are encompassed in the scope of the present invention. Modified cells disclosed herein can be used for treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD); therefore in the scope of the present invention is a method of treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD) comprising treating said patient by administering to said patient an effective amount of modified cells comprising inactivated TCRα and/or TCRβ genes.

In some embodiments, the immune cells are engineered to be resistant to one or more chemotherapy drugs. The chemotherapy drug can be, for example, a purine nucleotide analogue (PNA), thus making the immune cell suitable for cancer treatment combining adoptive immunotherapy and chemotherapy. Exemplary PNAs include, for example, clofarabine, fludarabine, and cytarabine, alone or in combination. PNAs are

metabolized by deoxycytidine kinase (dCK) into mono-, di-, and tri-phosphate PNA. Their tri-phosphate forms compete with ATP for DNA synthesis, act as pro-apoptotic agents, and are potent inhibitors of ribonucleotide reductase (RNR), which is involved in trinucleotide production. Provided herein are EGFRvIII specific CAR-T cells comprising an inactivated dCK gene. In some embodiments, the dCK knockout cells are made by transfection of T cells using polynucleotides encoding specific TAL-nuclease directed against dCK genes by, for example, electroporation of mRNA. The dCK knockout EGFRvIII specific CAR-T cells are resistant to PNAs, including for example clorofarabine and/or fludarabine, and maintain T cell cytotoxic activity toward EGFRvIII-expressing cells. In another example, the chemotherapy drug can be, for example, a CD52-targeting molecule, such as a monoclonal anti-CD52 antibody (e.g. alemtuzumab). CD52 is a protein present on the surface of lymphocytes, and anti-CD52 antibodies can induce apoptosis and lysis of immune cells through antibody-and complement-dependent cytotoxicity, leading to lymphodepletion. Provided herein are EGFRvIII specific CAR-T cells comprising an inactivated CD52 gene. In some embodiments, CD52 knockout cells are made by transfection of T cells using polynucleotides encoding a specific TAL-nuclease directed against the CD52 gene by, for example, electroporation of mRNA. The CD52 knockout EGFRvIII specific CAR-T cells are resistant to anti-CD52 molecules, including for example alemtuzumab, and maintain T cell cytotoxic activity toward EGFRvIII-expressing cells in the presence of anti-CD52 molecules.

In some embodiments, isolated cells or cell lines of the invention can comprise a pTα or a functional variant thereof. In some embodiments, an isolated cell or cell line can be further genetically modified by inactivating the TCRα gene.

In some embodiments, the CAR-T cell comprises a polynucleotide encoding a suicide polypeptide, such as for example RQR8. See, e.g., WO2013153391A, which is hereby incorporated by reference in its entirety. In CAR-T cells comprising the polynucleotide encoding a suicide polypeptide, the suicide polypeptide is expressed at the surface of a CAR-T cell. In some embodiments, the suicide polypeptide comprises the amino acid sequence shown in SEQ ID NO: 226.

CPYSNPSLCSGGGGSELPTQGTFSNVSTNVSPAKPTTTACPYSNPSLCSGGGGSP
APRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLS
LVITLYCNHRNRRRVCKCPRPW (SEQ ID NO. 226).

In some embodiments, the suicide polypeptide may also comprise a signal peptide at the amino terminus. When the suicide polypeptide is expressed at the surface of a CAR-T cell, binding of rituximab to the R epitopes [i.e. the epitope recognized by rituximab – CPYSNPSLC (SEQ ID NO: 256)]) of the polypeptide causes lysis of the cell. More than one molecule of rituximab may bind per polypeptide expressed at the cell surface. Each R epitope of the polypeptide may bind a separate molecule of rituximab. Deletion of EGFRvIII specific CAR-T cells may occur in vivo, for example by administering rituximab to a patient. The decision to delete the transferred cells may arise from undesirable effects being detected in the patient which are attributable to the transferred cells, such as for example, when unacceptable levels of toxicity are detected.

In some embodiments, a suicide polypeptide may contain one, two, three or more epitopes recognized by an antibody (e.g. rituximab).

In some embodiments, a suicide polypeptide may be provided in an EGFRvIII specific CAR T cell in a polypeptide that is separate from the CAR-containing polypeptide. In some embodiments, a suicide polypeptide may be provided in an EGFRvIII specific CAR T cell in the same polypeptide chain as the CAR polypeptide. In CARs containing a suicide polypeptide, typically the suicide polypeptide is provided in the extracellular portion of the CAR.

In a CAR containing a suicide polypeptide, the suicide polypeptide may contain, for example, one or more copies of the amino acid sequence of the epitope recognized by rituximab [CPYSNPSLC (SEQ ID NO: 256)]. The suicide polypeptide may be located at different positions in the CAR. For example, the suicide polypeptide may be N-terminal to the scFv or it may be C-terminal to the scFv in the CAR. In some embodiments, the suicide peptide may be between the scFv and the hinge region of the CAR. In some embodiments, a CAR may contain more than one suicide polypeptide. For example, a CAR may contain a suicide polypeptide N-terminal to the scFv and a suicide polypeptide C-terminal to the scFv. Each of these polypeptides may contain one or more copies of the epitope recognized by rituximab. For example, a CAR may contain a first suicide polypeptide at a position N-terminal to the scFv, wherein the first suicide polypeptide contains one copy of the epitope recognized by rituximab, and a second suicide polypeptide at a position C-terminal to the scFv, wherein the second suicide polypeptide contains two copies of the epitope recognized by rituximab.

Also provided herein are nucleic acids encoding EGFRvIII-specific CARs that contain a suicide polypeptide sequence in the CAR.

In an example, a suicide polypeptide in the same polypeptide chain as an EGFRvIII specific CAR may have the sequence provided described herein as the "R2 suicide sequence". The R2 suicide sequence contains two copies of the epitope recognized by rituximab [CPYSNPSLC (SEQ ID NO: 256)]. Such EGFRvIII-specific CARs may be referred to herein as "EGFRvIII-R2 CARs". Table 5D provides amino acid sequences of exemplary EGFRvIII-R2 CARs of the present invention. In Table 5D, the signal/leader peptide sequence is in bold, the GS linker [$(GGGGS)_4$ (SEQ ID NO: 202)] is underlined, and the R2 suicide sequence is in bold and underlined. Table 5E provides exemplary nucleic acid sequences encoding exemplary EGFRvIII-R2 CARs of the present invention.

Table 5D: Amino acid sequences of exemplary EGFRvIII specific CARs with R2 suicide sequence

CAR	CAR Amino Acid Sequence	Components (in order, N-terminus to C-terminus)
14C11-R2	**MALPVTALLLPLALLLHAARP**QVTLKESGPVLVKPTETLTLTCTVSGFSLNNARMGVSWIRQPPGKALEWFAHIFSTDEKSFRTSLRSRLTLSKDTSKSQVVLTMTNMDPVDTATYYCARDSSNYEGYFDYWGQGILVTVSSGGGGSGGGGSGGGGSGGGGSEIVMTQSPATLSVSPGERATLSCRASQSVSNNLAWYQQKPGQAPRLLIYGASTRATGVPARFSGSDSGTEFSLTISSLQSEDFAVYFCQQYKDWPFTFGPGTKVEIK**GSGGGGGSCPYSNPSLCSGGGGSCPYSNPSLCSGGGGS**TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 248)	CD8α signal peptide; 14C11 VH (Table 1, SEQ ID NO: 15); GS linker; 14C11 VL (Table 1, SEQ ID NO: 16); R2 suicide sequence; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
32A10-	**MALPVTALLLPLALLLHAARP**QVTLKESGPVLVKPTETLTLTCTVSGFSLSNARMGVSWIRQPPGKALEWLAHIFSTD	CD8α signal peptide;

CAR	CAR Amino Acid Sequence	Components (in order, N-terminus to C-terminus)
R2	EKSIRRSLRSRLTLSKDTSKSQVVLTMTNMDPVDTATYF CARDSSNYEGYFDYWGQGTLVTVSSGGGGSGGGGSGGGG SGGGGSEVVMTQSPATLSVSPGERVTLSCRASQSVSSNF AWYQQRPGQAPRLLLYGATTRATGLPGRFSGSGSGTENI LTISSLQSEDFAIYFCQQYKDWPFTFGPGSKVDIK**GSGG GGSCPYSNPSLCSGGGGSCPYSNPSLCSGGGGS**TTTPAP RPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD IYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPF MRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPA YQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLY QGLSTATKDTYDALHMQALPPR(SEQ ID NO: 249)	32A10 VH (Table 1 SEQ ID NO: 11); GS linker; 32A10 VL (Table 1 SEQ ID NO: 12); R2 suicide sequence; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
26B9-R2	**MALPVTALLLPLALLLHAARP**EVQLVESWGVLVKPGGSL RLSCAASGFIFNNAWMSWVRQAPGKGLEWIGRIKSKSDG GTTDYAAPVKDRFTISRDDSKDTLYLQMNGLKTEDTAVY FCTTAPGGPFDYWGQGTLVTVSSGGGGSGGGGSGGGGSG GGGSDIVLTQSPLSLPVTPGEPASISCRSSQSLLHRDGF NYLDWFLQKPGQSPQLLIYLASSRASGVPDRFSGSDSGT DFTLKISRVEAEDVGVYYCMQALQTPITFGQGTRLEIK **GSGGGGSCPYSNPSLCSGGGGSCPYSNPSLCSGGGGS**TT TPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIF KQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSA DAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH DGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 250)	CD8α signal peptide; 26B9 VH (Table 1, SEQ ID NO: 41); GS linker; 26B9 VL (Table 1, SEQ ID NO: 42); R2 suicide sequence; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain
30D8-R2	**MALPVTALLLPLALLLHAARP**EVQLVESGGGLVKPGGSL RLSCEASGFTFSDAWMSWVRQAPGKGLEWVGRIKSKTDG GTTDYVVPLNGRFIISRDDSRNTLYLQLNNLKTEDTAVY YCTTVPGSYGYWGQGTLVTVSSGGGGSGGGGSGGGGSGG	CD8α signal peptide; 30D8 VH (Table 1, SEQ

CAR	CAR Amino Acid Sequence	Components (in order, N-terminus to C-terminus)
	GGSDIVMTQSPLSLPVTPGEPASISCRSSQSLLHNKRNN YLDWFLQKPGQSPQLLIYLASNRASGVPDRFSGGGSGTD FTLKISRVEAEDVGVYYCMQAQQTPITFGQGTRLEIK**GS GGGGSCPYSNPSLCSGGGGSCPYSNPSLCSGGGGS**TTTP APRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFA CDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQ PFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADA PAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG LYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 251)	ID NO: 37); GS linker; 30D8 VL (Table 1, SEQ ID NO: 38); R2 suicide sequence; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

Table 5E: Nucleic acid sequences of exemplary EGFR VIII specific CARs with R2 suicide sequence

CAR	CAR Nucleic Acid Sequence	Components
14C11-R2	ATGGCACTGCCAGTGACCGCCCTGCTGCTGCCTCTGGCCCTG CTGCTGCACGCAGCCAGACCCCAGGTGACACTGAAGGAGAG CGGCCCCGTGCTGGTGAAGCCTACAGAGACACTGACCCTGA CCTGCACAGTGAGCGGCTTCTCCCTGAACAATGCAAGGATG GGCGTGTCCTGGATCAGGCAGCCACCTGGCAAGGCCCTGGA GTGGTTCGCCCACATCTTTAGCACCGACGAGAAGTCCTTTCG CACATCTCTGAGAAGCAGGCTGACCCTGAGCAAGGATACAA GCAAGTCCCAGGTGGTGCTGACCATGACAAACATGGACCCT GTGGATACCGCCACATACTATTGTGCCCGGGACAGCTCCAAT TACGAGGGCTATTTCGATTACTGGGGCCAGGGCATCCTGGT GACCGTGTCTAGCGGCGGCGGCGGCTCTGGAGGAGGAGGA AGCGGAGGAGGAGGATCCGGCGGCGGCGGCTCTGAGATCG TGATGACCCAGTCCCCAGCCACACTGTCTGTGAGCCCAGGA GAGAGAGCCACCCTGTCTTGCAGGGCCTCCCAGTCTGTGAG CAACAATCTGGCCTGGTATCAGCAGAAGCCTGGCCAGGCCC CAAGGCTGCTGATCTACGGAGCAAGCACCAGAGCAACAGG AGTGCCTGCAAGGTTCTCCGGATCTGACAGCGGCACCGAGT TTTCTCTGACAATCTCCTCTCTGCAGAGCGAGGACTTCGCCG TGTATTTTTGTCAGCAGTACAAGGATTGGCCATTCACCTTTG	CD8α signal peptide; 14C11 VH (Table 1, SEQ ID NO: 15); GS linker; 14C11 VL (Table 1, SEQ ID NO: 16); R2 suicide sequence; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling

CAR	CAR Nucleic Acid Sequence	Components
	GCCCCGGCACAAAGGTGGAGATCAAGGGCTCCGGAGGAGG AGGATCCTGCCCCTATTCCAACCCTTCTCTGTGCAGCGGAGG AGGAGGAAGCTGTCCATACTCCAATCCCTCCCTGTGCTCCGG CGGCGGAGGATCCACCACAACCCCAGCACCTAGACCACCAA CCCCAGCACCAACAATCGCATCCCAGCCTCTGTCTCTGCGGC CCGAGGCATGCAGGCCAGCAGCAGGCGGCGCCGTGCACAC CAGGGGCCTGGACTTTGCCTGCGATATCTATATCTGGGCACC ACTGGCAGGAACCTGTGGCGTGCTGCTGCTGAGCCTGGTCA TCACCCTGTATTGCAAGCGCGGCCGGAAGAAGCTGCTGTAC ATCTTCAAGCAGCCTTTTATGCGCCCAGTGCAGACAACCCAG GAGGAGGACGGCTGCTCCTGTCGGTTCCCTGAAGAGGAGG AGGGAGGATGTGAGCTGCGCGTGAAGTTTTCCCGGTCTGCC GATGCCCCAGCCTATCAGCAGGGCCAGAACCAGCTGTACAA CGAGCTGAATCTGGGCCGGAGAGAGGAGTACGACGTGCTG GATAAGAGGAGGGGAAGAGATCCCGAGATGGGAGGCAAG CCTCGGAGAAAGAACCCACAGGAGGGCCTGTATAATGAGCT GCAGAAGGACAAGATGGCCGAGGCCTACTCTGAGATCGGC ATGAAGGGAGAGAGGCGCCGGGGCAAGGGACACGATGGC CTGTATCAGGGCCTGTCCACCGCCACAAAGGACACCTACGAT GCCCTGCACATGCAGGCCCTGCCTCCAAGGTGA (SEQ ID NO: 252)	domain
32A10-R2	ATGGCACTGCCAGTGACCGCCCTGCTGCTGCCTCTGGCCCTG CTGCTGCACGCAGCCAGACCCCAGGTGACACTGAAGGAGTC CGGCCCCGTGCTGGTGAAGCCTACAGAGACACTGACCCTGA CCTGCACAGTGAGCGGCTTCTCTCTGAGCAACGCAAGGATG GGCGTGTCCTGGATCAGGCAGCCACCTGGCAAGGCCCTGGA GTGGCTGGCCCACATCTTTTCCACCGACGAGAAGTCTATCCG GAGAAGCCTGCGCTCCCGGCTGACCCTGAGCAAGGATACAT CCAAGTCTCAGGTGGTGCTGACCATGACAAACATGGACCCT GTGGATACCGCCACATACTTCTGTGCCCGGGACAGCTCCAAT TACGAGGGCTATTTTGATTACTGGGGCCAGGGCACCCTGGT GACAGTGTCTAGCGGAGGAGGAGGAAGCGGAGGAGGAGG ATCAGGCGGCGGCGGCTCTGGCGGCGGCGGCAGCGAGGTG GTCATGACCCAGTCTCCAGCCACACTGAGCGTGTCCCCAGGA GAGCGCGTGACCCTGAGCTGCCGGGCCTCTCAGAGCGTGTC CTCTAACTTCGCCTGGTATCAGCAGCGGCCCGGACAGGCAC CAAGGCTGCTGCTGTACGGAGCAACCACAAGAGCAACAGGC CTGCCTGGCAGGTTTTCCGGCTCTGGCAGCGGCACCGAGAA TATCCTGACAATCAGCTCCCTGCAGAGCGAGGACTTCGCCAT CTATTTTTGTCAGCAGTACAAGGATTGGCCATTCACCTTTGG CCCCGGCTCCAAGGTGGACATCAAGGGATCCGGAGGAGGA GGATCTTGCCCCTATTCTAACCCTAGCCTGTGCTCCGGAGGA GGAGGATCCTGTCCATACTCTAATCCATCCCTGTGCAGCGGA	CD8α signal peptide; 32A10 VH (Table 1 SEQ ID NO: 11) ; GS linker; 32A10 VL (Table 1 SEQ ID NO: 12); R2 suicide sequence; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

CAR	CAR Nucleic Acid Sequence	Components
	GGAGGAGGATCTACCACAACCCCAGCACCTAGACCACCAAC CCCAGCACCCACAATCGCCTCTCAGCCTCTGAGCCTGCGCCC AGAGGCATGCAGGCCAGCAGCAGGAGGAGCAGTGCACACC AGGGGCCTGGACTTCGCCTGCGATATCTATATCTGGGCACCA CTGGCAGGAACCTGTGGCGTGCTGCTGCTGTCCCTGGTCATC ACCCTGTATTGCAAGAGAGGCAGGAAGAAGCTGCTGTACAT CTTCAAGCAGCCTTTTATGCGCCCAGTGCAGACAACCCAGGA GGAGGACGGCTGCAGCTGTCGGTTCCCTGAAGAGGAGGAG GGCGGCTGTGAGCTGAGAGTGAAGTTTTCCAGGTCTGCCGA TGCCCCAGCCTATCAGCAGGGCCAGAATCAGCTGTACAACG AGCTGAATCTGGGCAGGCGCGAGGAGTACGACGTGCTGGA TAAGAGGAGAGGACGCGATCCCGAGATGGGAGGCAAGCCT AGGCGCAAGAACCCACAGGAGGGCCTGTATAATGAGCTGC AGAAGGACAAGATGGCCGAGGCCTACTCTGAGATCGGCAT GAAGGGAGAGCGGAGAAGGGGCAAGGGACACGATGGCCT GTATCAGGGCCTGAGCACCGCCACAAAGGACACCTACGATG CCCTGCACATGCAGGCCCTGCCTCCAAGGTGA (SEQ ID NO: 253)	
26B9-R2	ATGGCCCTGCCAGTGACCGCCCTGCTGCTGCCACTGGCCCTG CTGCTGCACGCCGCCAGACCTGAGGTGCAGCTGGTGGAGAG CTGGGGCGTGCTGGTGAAGCCAGGAGGCTCTCTGAGGCTG AGCTGCGCAGCATCCGGCTTCATCTTTAACAATGCCTGGATG TCCTGGGTGAGACAGGCACCAGGCAAGGGCCTGGAGTGGA TCGGCAGGATCAAGAGCAAGTCCGACGGAGGAACCACAGA TTACGCAGCACCCGTGAAGGACCGCTTCACAATCTCTCGGGA CGATAGCAAGGATACCCTGTATCTGCAGATGAACGGCCTGA AGACAGAGGACACCGCCGTGTACTTCTGCACCACAGCCCCA GGCGGCCCCTTTGATTATTGGGGCCAGGGCACACTGGTGAC CGTGAGCTCCGGAGGAGGAGGAAGCGGCGGAGGAGGCAG CGGCGGCGGCGGCTCTGGCGGCGGCGGCAGCGACATCGTG CTGACACAGAGCCCACTGTCCCTGCCTGTGACCCCAGGAGA GCCCGCCTCTATCAGCTGTCGCTCTAGCCAGAGCCTGCTGCA CCGGGACGGCTTCAATTACCTGGATTGGTTTCTGCAGAAGCC TGGCCAGAGCCCACAGCTGCTGATCTATCTGGCCTCCTCTAG AGCATCCGGAGTGCCTGACAGGTTCTCCGGATCTGACAGCG GCACAGACTTCACCCTGAAGATCTCCCGCGTGGAGGCAGAG GATGTGGGCGTGTACTATTGCATGCAGGCCCTGCAGACACC AATCACCTTCGGCCAGGGCACACGGCTGGAGATCAAGGGAT CCGGAGGAGGAGGATCTTGCCCCTACTCTAACCCTAGCCTGT GCAGCGGCGGAGGAGGATCTTGTCCATATTCTAATCCAAGC CTGTGCAGCGGGGGAGGAGGAAGCACCACAACCCCTGCAC CAAGACCCCCTACACCAGCACCTACCATCGCATCCCAGCCAC TGTCTCTGCGGCCCGAGGCATGTAGGCCAGCAGCAGGAGG	CD8α signal peptide; 26B9 VH (Table 1, SEQ ID NO: 41); GS linker; 26B9 VL (Table 1, SEQ ID NO: 42); R2 suicide sequence; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

CAR	CAR Nucleic Acid Sequence	Components
	AGCAGTGCACACCAGGGGCCTGGACTTTGCCTGCGATATCT ACATCTGGGCACCACTGGCAGGAACATGTGGCGTGCTGCTG CTGAGCCTGGTCATCACCCTGTACTGCAAGAGAGGCAGGAA GAAGCTGCTGTATATCTTCAAGCAGCCTTTTATGCGCCCAGT GCAGACAACCCAGGAGGAGGACGGCTGCTCCTGTAGGTTCC CAGAAGAGGAGGAGGGAGGATGTGAGCTGCGCGTGAAGTT TTCCCGGTCTGCCGATGCACCTGCATACCAGCAGGGACAGA ACCAGCTGTATAACGAGCTGAATCTGGGCCGGAGAGAGGA GTACGACGTGCTGGATAAGAGGAGGGGACGCGATCCTGAG ATGGGAGGCAAGCCCCGGAGAAAGAACCCTCAGGAGGGCC TGTACAATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTAT TCCGAGATCGGCATGAAGGGAGAGAGGCGCCGGGGCAAG GGACACGATGGCCTGTACCAGGGCCTGTCTACAGCCACCAA GGACACCTATGATGCCCTGCACATGCAGGCCCTGCCACCAA GGTGA (SEQ ID NO: 254)	
30D8-R2	ATGGCACTGCCAGTGACAGCCCTGCTGCTGCCTCTGGCCCTG CTGCTGCACGCAGCCAGACCAGAGGTGCAGCTGGTGGAGTC CGGAGGAGGCCTGGTGAAGCCAGGAGGCTCCCTGAGGCTG TCTTGCGAGGCCAGCGGCTTCACCTTTAGCGACGCCTGGAT GTCCTGGGTGAGACAGGCACCAGGCAAGGGCCTGGAGTGG GTGGGCAGGATCAAGAGCAAGACAGACGGCGGCACCACAG ATTACGTGGTGCCTCTGAACGGCCGGTTCATCATCTCCCGCG ACGATTCTCGGAATACCCTGTATCTGCAGCTGAACAATCTGA AGACAGAGGATACCGCCGTGTACTATTGCACCACAGTGCCT GGCTCCTACGGCTATTGGGGCCAGGGCACACTGGTGACCGT GAGCTCCGGCGGCGGCGGCTCTGGAGGAGGAGGAAGCGG AGGAGGAGGAAGCGGGGGCGGCGGCTCTGACATCGTGATG ACACAGTCTCCACTGAGCCTGCCAGTGACCCCAGGAGAGCC TGCCTCCATCTCTTGTCGCTCTAGCCAGTCCCTGCTGCACAAC AAGCGGAACAATTACCTGGATTGGTTCCTGCAGAAGCCAGG CCAGTCTCCCCAGCTGCTGATCTATCTGGCCAGCAATAGAGC CTCCGGAGTGCCAGACAGGTTCTCTGGAGGAGGAAGCGGA ACAGACTTCACCCTGAAGATCAGCCGCGTGGAGGCAGAGGA CGTGGGCGTGTACTATTGCATGCAGGCCCAGCAGACACCCA TCACCTTTGGCCAGGGAACCCGGCTGGAGATCAAGGGCTCC GGAGGAGGAGGATCCTGCCCTTACTCCAACCCATCTCTGTGC AGCGGAGGAGGAGGATCTTGTCCATATTCCAATCCTTCCCTG TGCTCCGGAGGAGGAGGAAGCACCACAACCCCTGCACCAAG ACCCCCTACACCAGCACCTACCATCGCATCCCAGCCTCTGTCT CTGCGGCCCGAGGCATGTAGGCCAGCAGCAGGCGGCGCCG TGCACACCAGGGGCCTGGACTTTGCCTGCGATATCTACATCT GGGCACCACTGGCAGGAACATGTGGCGTGCTGCTGCTGTCT	CD8α signal peptide; 30D8 VH (Table 1, SEQ ID NO: 37); GS linker; 30D8 VL (Table 1, SEQ ID NO: 38); R2 suicide sequence; CD8α hinge; CD8α TM; 4-1BB signaling domain; CD3zeta signaling domain

CAR	CAR Nucleic Acid Sequence	Components
	CTGGTCATCACCCTGTACTGCAAGAGAGGCAGGAAGAAGCT GCTGTATATCTTCAAGCAGCCCTTCATGCGGCCCGTGCAGAC AACCCAGGAGGAGGACGGCTGCAGCTGTCGGTTCCCTGAAG AGGAGGAGGGAGGATGTGAGCTGCGCGTGAAGTTTAGCCG GTCCGCCGATGCACCAGCATACCAGCAGGGCCAGAACCAGC TGTATAACGAGCTGAATCTGGGCCGGAGAGAGGAGTACGA CGTGCTGGATAAGAGGAGGGGACGCGATCCTGAGATGGGA GGCAAGCCTCGGAGAAAGAACCCACAGGAGGGCCTGTACA ATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTATAGCGA GATCGGCATGAAGGGAGAGAGGCGCCGGGGCAAGGGACA CGATGGCCTGTACCAGGGCCTGTCCACAGCCACCAAGGACA CCTATGATGCCCTGCACATGCAGGCCCTGCCACCAAGGTGA (SEQ ID NO: 255)	

Therapeutic applications

Isolated cells obtained by the methods described above, or cell lines derived from such isolated cells, can be used as a medicament. In some embodiments, such a medicament can be used for treating cancer, including solid tumors and liquid tumors. In some embodiments, the cancer is EGFRvIII related cancer (e.g., any EGFRvIII expressing cancer) including, but not limited to, glioblastoma (e.g., glioblastoma multiform), anaplastic astrocytoma, giant cell glioblastoma, gliosarcoma, anaplastic oligodendroglioma, anaplastic ependymoma, anaplastic oligoastrocytoma, choroid plexus carcinoma, anaplastic ganglioglioma, pineoblastoma, pineocytoma, meningioma, medulloepithelioma, ependymoblastoma, medulloblastoma, supraentorial primitive neuroectodermal tumor, atypical teratoid/rhabdoid tumor, mixed glioma, head and neck cancer, non-small cell lung cancer, breast cancer, ovarian cancer, prostate cancer, medullobastoma, colorectal cancer, anal cancer, cervical cancer, renal cancer, skin cancer, pancreatic cancer, liver cancer, bladder cancer, gastric cancer, thyroid cancer, mesothelioma, uterine cancer, lymphoma, or leukemia.

In some embodiments, the medicament as described herein can be used for 1) inhibiting tumor growth or progression in a subject who has malignant cells expressing EGFRvIII; 2) inhibiting metastasis of malignant cells expressing EGFRvIII in a subject; and 3) inducing tumor regression in a subject who has malignant cells expressing EGFRvIII.

In some embodiments, an isolated cell according to the invention, or cell line derived from the isolated cells, can be used in the manufacture of a medicament for treatment of a cancer in a patient in need thereof.

Also provided herein are methods for treating patients. In some embodiments the method comprises providing an immune cell of the invention to a patient in need thereof. In some embodiments, the method comprises a step of administering transformed immune cells of the invention to a patient in need thereof.

In some embodiments, T cells of the invention can undergo robust in vivo T cell expansion and can persist for an extended amount of time.

Methods of treatment of the invention can be ameliorating, curative or prophylactic. The method of the invention may be either part of an autologous immunotherapy or part of an allogenic immunotherapy treatment. The invention is particularly suitable for allogeneic immunotherapy. T cells from donors can be transformed into non-alloreactive cells using standard protocols and reproduced as needed, thereby producing CAR-T cells which may be administered to one or several patients. Such CAR-T cell therapy can be made available as an "off the shelf" therapeutic product.

Cells that can be used with the disclosed methods are described, for example, in the previous section. Treatment can be used to treat patients diagnosed with, for example, cancer. Cancers that may be treated include, for example, cancers that are related to EGFRvIII, including any of the above-listed cancers. Types of cancers to be treated with the CARs and CAR-T cells of the invention include, but are not limited to certain liquid and solid tumors, such as glioblastoma multiforme, head and neck cancer, non-small cell lung cancer, breast cancer, ovarian cancer and prostate cancer. Adult tumors/cancers and pediatric tumors/cancers are also included.

In some embodiments, the treatment can be in combination with one or more therapies against cancer selected from the group of antibodies therapy, antibody-drug conjugate therapy, chemotherapy, targeted therapy, cytokines therapy, vaccine therapy, oncolytic virus therapy, dendritic cell therapy, gene therapy, nanoparticle therapy, hormone therapy, surgical resection, laser light therapy, tumor treating fields, and radiation therapy. For example, CARs and CAR-T cells of the invention can be administered to a patient in conjunction with (e.g., before, simultaneously, or following) 1) standard of care, including radiation, surgical resection, chemotherapy (e.g.,

temozolomide, procarbazine, carmustine, lomustine, vincristine etc.), antibody therapy such as bevacizumab, anti-angiogenic therapy, and/or tumor treating fields; 2) vaccine, including EGFRvIII vaccine; 3) antibody-drug conjugate therapy, including but not limited to drug conjugates that target the HER family of receptors such as EGFR, HER2, HER3, and HER4; 4) targeted therapy, such as kinase inhibitors (e.g., everolimus); and 5) immunotherapies, including but not limited to anti-PD-1, anti-PD-L1, anti-PD-L2, anti-41BB, anti-TIM3, anti-LAG3, anti-TIGIT, anti-OX40, anti-HVEM, anti-BTLA, anti-CD40, anti-CD47, anti-CSF1R, anti-CSF1, anti-MARCO, anti-IL8, anti-CXCR4, and anti-CTLA4 antibodies.

In some embodiments, treatment can be administered to patients undergoing an immunosuppressive treatment. Indeed, the invention preferably relies on cells or population of cells, which have been made resistant to at least one immunosuppressive agent (e.g., cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506) due to the inactivation of a gene encoding a receptor for such immunosuppressive agent. In this aspect, the immunosuppressive treatment should help the selection and expansion of the T cells according to the invention within the patient. The administration of the cells or population of cells according to the invention may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermally, intratumorally, intracranially, intranodally, intramedullary, intramuscularly, by intravenous or intralymphatic injection, or intraperitoneally. In one embodiment, the cell compositions of the invention are preferably administered by intravenous injection.

In some embodiments, treatment can be administered to patients undergoing a lymphodepletion regimen. Depletion of the immune regulatory elements with cytotoxic agents or whole body irradiation can enhance the anti-tumor activity of the CARs and CAR-T cells of the present invention. For example, the cytotoxic agent in a lymphodepletion regimen includes, but is not limited to, fludarabine, cyclophosphamide, and/or alemtuzumab.

In some embodiments the administration of the cells or population of cells can comprise the administration of, for example, about 10^4 to about 10^9 cells per kg body weight including all integer values of cell numbers within those ranges. In some embodiments the administration of the cells or population of cells can comprise the

administration of about 10^5 to 10^6 cells per kg body weight including all integer values of cell numbers within those ranges. The cells or population of cells can be administered in one or more doses. In some embodiments, said effective amount of cells can be administered as a single dose. In some embodiments, said effective amount of cells can be administered as more than one dose over a period time. Timing of administration is within the judgment of a managing physician and depends on the clinical condition of the patient. The cells or population of cells may be obtained from any source, such as a blood bank or a donor. While individual needs vary, determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions within the skill of the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administered will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired. In some embodiments, an effective amount of cells or composition comprising those cells are administered parenterally. In some embodiments, administration can be an intravenous administration. In some embodiments, administration can be directly done by injection within a tumor.

Kits

The invention also provides kits for use in the instant methods. Kits of the invention include one or more containers comprising a polynucleotide encoding an EGFRvIII specific CAR, or an engineered immune cell comprising a polynucleotide encoding EGFRvIII specific CAR as described herein, and instructions for use in accordance with any of the methods of the invention described herein. Generally, these instructions comprise a description of administration of the engineered immune cell for the above described therapeutic treatments.

The instructions relating to the use of the engineered immune cells as described herein generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (e.g., multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (e.g., a paper sheet included in the kit), but machine-readable instructions (e.g., instructions carried on a magnetic or optical storage disk) are also acceptable.

The kits of this invention are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. Also contemplated are packages for use in combination with a specific device, such as an inhaler, nasal administration device (e.g., an atomizer) or an infusion device such as a minipump. A kit may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The container may also have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an EGFRvIII antibody. The container may further comprise a second pharmaceutically active agent.

Kits may optionally provide additional components such as buffers and interpretive information. Normally, the kit comprises a container and a label or package insert(s) on or associated with the container.

Incorporated by reference herein for all purposes is the content of U.S. Provisional Patent Application Nos. 62/281,533 (filed January 21, 2016) and 62/431,758 (Filed December 8, 2016).

Biological Deposit

Representative materials of the present invention were deposited in the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209, USA, on _____. Vector ____ having ATCC Accession No. ____ is a polynucleotide encoding the ____ light chain variable region, and vector ____ having ATCC Accession No._____ is a polynucleotide encoding the ____ heavy chain variable region. The deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Pfizer, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one

determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 U.S.C. § 122 and the Commissioner's rules pursuant thereto (including 37 C.F.R. § 1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the invention in any way. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

Examples

Example 1: Affinity Determination for Recombinant Anti-EGFRvIII Murine-Human Chimeric Antibody and Humanized Antibodies

This example determines the affinity of chimeric and humanized anti-EGFRvIII antibodies at 25°C and 37°C.

Anti-EGFRvIII mouse (m) antibody, m62G7, generated from hybridomas was sequenced and subcloned into suitable vectors for expression as murine-human chimeric antibodies. The CDRs of mouse antibody m62G7 were grafted onto human framework and expressed as human IgG1 recombinant antibody, h62G7. Affinity variants of h62G7 were made by introducing mutations in the CDRs of the heavy and light chains. The affinities of recombinant anti-EGFRvIII chimeric antibody m62G7 and humanized h62G7 antibodies were measured on a surface plasmon resonance Biacore™ T200 biosensor equipped with a research-grade anti-human Fc coupled CM4 sensor chip (GE Healthcare Inc., Piscataway, NJ). Anti-EGFRvIII antibodies were then captured by anti-human Fc. Monomeric 8-histidine tagged human EGFRvIII extracellular domain was then injected as the analyte at 10-fold dilution series with top concentration at 1000 nM. Affinity of anti-EGFRvIII antibodies towards human

EGFRvIII was measured at both 25°C and 37°C (Table 6). None of these antibodies showed detectable binding to 1000 nM 8-histidine tagged recombinant wild-type protein EGFRwt under the same assay condition.

In Table 6, variants of h62G7 are described with reference to the heavy chain variation then the light chain variation. For example, antibody clone "h62G7-EQ/L6" refers to the h62G7 clone containing the "EQ" variation in the heavy chain (also referred to herein as "h62G7-EQ") and the "L6" variation in the light chain (also referred to herein as "h62G7-L6"). These heavy chain and light chain amino acid sequences are provided in Table 2. Also, in the present application, a h62G7 variant may be referred to with either the heavy chain or the light chain variant written first – so, for example, "h62G7-EQ/L6" and "h62G7-L6/EQ" both refer to an antibody which contains a h62G7-EQ heavy chain and a h62G7-L6 light chain.

Table 6

Antibody	25°C			37°C		
	k_a(1/Ms)	k_d(1/s)	K_D(nM)	k_a(1/Ms)	k_d(1/s)	K_D(nM)
m62G7	7.30E+05	6.40E-02	88.7	8.00E+05	1.70E-01	207.0
h62G7-EQ/L6	2.40E+05	1.00E-02	43.8	6.60E+05	7.40E-02	112.8
h62G7-EQ/L1-DV	2.00E+05	1.20E-05	59.9	3.70E+05	6.90E-02	185.8
h62G7-H14/L1-DV	1.80E+04	2.00E-02	1087.9	6.60E+04	1.00E-01	1539.6
h62G7-H14/L6	1.30E+04	1.30E-02	992.2	4.30E+04	6.80E-02	1583.3

Example 2: Affinity Determination for Human Anti-EGFRvIII Antibodies

This example determines the affinity of various human anti-EGFRvIII antibodies at 37°C.

To generate human antibodies against EGFRvIII, transgenic AlivaMab mice (Ablexis LLC, San Francisco, CA) were immunized with alternating schedule of paraformaldehyde-fixed rat glioblastoma cell line expressing EGFRvIII, F98-npEGFRvIII (American Type Culture Collection, Manassas, VA) and peptides (SEQ ID NO: 227: CGSGSGLEEKKGNYVVTDH) directed to the junction region in EGFRvIII. Hybridomas were generated using standard techniques. To determine the binding affinity and specificity of these hybridomas to EGFRvIII, antibodies in culture supernatants were captured by anti-mouse Fc using Biacore™ T200 biosensor equipped with anti-mouse Fc coupled CM4 sensor chips (Biacore™ AB, Uppsala, Sweden – now GE Healthcare). Monomeric 8-histidine tagged human EGFRvIII extracellular domain was then injected

as the analyte at 10-fold dilution series starting with top concentration 1000 nM. Affinity of anti-EGFRvIII antibodies towards human EGFRvIII was measured at 37°C (Table 7). None of these hybridoma antibodies showed detectable binding to 1000 nM 8-histidine tagged recombinant wild-type protein EGFRwt under the same assay condition.

Table 7

Antibody	EGFRvIII binding at 37°C		
	k_a(1/Ms)	k_d(1/s)	K_D(nM)
42G9	6.88E+04	5.63E-04	8.2
32A10	6.54E+04	6.26E-04	9.6
21E11	6.66E+04	6.32E-04	9.5
49B11	7.64E+04	6.95E-04	9.1
46E10	5.97E+04	7.16E-04	12.0
12H6	5.93E+04	7.33E-04	12.4
19A9	5.58E+04	1.04E-03	18.6
11B11	5.21E+04	1.13E-03	21.7
21E7	6.52E+04	1.30E-03	19.9
20B9	4.67E+04	1.50E-03	32.1
12B2	7.38E+04	1.79E-03	24.3
11F10	6.63E+04	2.81E-03	42.4
17G11	5.61E+04	3.00E-03	53.5
29D5	1.02E+05	4.24E-03	41.6
14C11	7.55E+04	5.93E-03	78.5
20E12	3.99E+04	1.41E-02	353.4
20G5	1.25E+05	2.89E-02	231.2
26B9	1.31E+05	3.20E-02	244.3
30D8	1.61E+05	2.77E-02	172.0
32G8	6.82E+03	1.22E-02	1788.9
34E7	3.77E+04	1.28E-02	339.5

Example 3: Binding Specificity of Anti-EGFRvIII Antibodies to EGFRvIII Expressing Cell Lines by Flow Cytometry

This example demonstrates the cell binding specificity of anti-EGFRvIII antibodies to EGFRvIII expressing cells.

To assess the cell binding specificity of anti-EGFRvIII antibodies generated from the AlivaMab mice, three isogenic rat glioblastoma cell lines and a human cancer cell line were used: F98 (does not express any form of human EGFR), F98-EGFRwt (expresses wild-type EGFR), F98-npEGFRvIII (expresses EGFRvIII) and A431 (an epidermoid carcinoma cell line with wild-type EGFR over-expression), all obtained from

American Type Culture Collection (Manassas, VA). For cell staining, 500,000 cells were incubated with 50µl hybridoma supernatants for 45 min at 4°C, washed with binding buffer (PBS (Phosphate Buffered Saline) + 0.5% BSA (Bovine Serum Albumin)), followed by incubation with FITC-conjugated goat anti-mouse Fc specific secondary antibody from Jackson ImmunoResearch Laboratories (West Grove, PA). Tables 8A and 8B show mean fluorescent intensities (MFI) of EGFRvIII antibodies (except clone 20G5) on EGFRvIII expressing cell line were at least 10-fold higher than on non-expressing cell lines. FIG. 1A, FIG. 1B, and FIG. 1C show examples of the FACS binding histograms of three EGFRvIII specific clones which had been cloned and expressed as recombinant human IgG1 antibodies, 42G9 (FIG. 1A), 32A10 (FIG. 1B) and 32G8 (FIG. 1C), to the three F98 cell lines.

Table 8A

	F98		F98-EGFRwt		F98-EGFRvIII		A431	
Antibody	MFI	% positive	MFI	% positive	MFI	% positive	MFI	% positive
2nd Ab only	170	0.6	202	1.7	258	2.3	592	0.4
anti-EGFR(wt and vIII)	163	0.5	9608	98.3	5329	99.4	55240	100.0
42G9	159	0.4	185	1.6	3247	98.5	538	0.3
32A10	159	0.5	185	1.4	3349	98.3	531	0.2
21E11	159	0.3	184	1.3	3105	98.5	555	0.5
49B11	156	0.6	185	1.3	2980	98.5	599	0.8
46E10	158	0.4	187	1.6	2986	98.7	560	0.5
12H6	157	0.5	188	1.9	3445	98.3	569	0.8
19A9	158	0.5	168	1.6	3100	98.1	578	1.0
11B11	161	0.6	187	1.7	3391	98.2	589	1.2
21E7	159	0.3	184	1.3	3105	98.5	603	1.1
20B9	157	0.3	189	1.8	3418	98.3	558	0.7
12B2	156	0.4	185	1.5	2749	97.9	571	0.8
11F10	155	0.5	187	1.6	3283	98.0	582	1.1
17G11	157	0.6	184	1.5	3357	98.1	556	0.7
29D5	155	0.3	185	1.3	2829	97.9	531	0.4
14C11	157	0.4	185	1.3	3213	98.2	580	0.8

Table 8B

	F98		F98-EGFRwt		F98-EGFRvIII		A431	
Antibody	MFI	% positive	MFI	% positive	MFI	% positive	MFI	% positive
2nd Ab only	235	0.2	252	0.2	322	1.3	185	0.7

anti-EGFR(wt and vIII)	245	0.3	6857	97.2	5827	99.4	44493	100.0
20E12	381	6.0	348	3.4	3976	97.9	302	2.6
20G5	1248	16.8	1070	12.6	4639	98.5	391	2.0
26B9	310	4.1	298	2.3	5405	98.6	276	1.7
30D8	296	4.0	280	1.7	5165	98.6	269	1.3
32G8	329	4.9	301	1.6	3734	98.6	271	1.2
34E7	485	6.9	371	4.0	4128	98.5	294	1.1

These results demonstrate the binding specificity of the anti-EGFRvIII antibodies to cells that express EGFRvIII.

Example 4: Affinity Determination for Fully Human Anti-EGFRvIII Antibodies from Phage Library

This example determines the affinity of various human anti-EGFRvIII antibodies at 25°C.

Human anti-EGFRvIII antibodies were sequenced and subcloned into suitable vectors for expression as recombinant human IgG1 antibodies. The affinities of antibodies were measured at 25°C (Table 9) on a surface plasmon resonance Biacore™ T200 biosensor equipped with an anti-human Fc coupled CM4 sensor chip (GE Healthcare Inc., Piscataway, NJ). Anti-EGFRvIII antibodies were captured by anti-human Fc. Monomeric 8-histidine tagged human EGFRvIII extracellular domain was then injected as the analyte at 10-fold dilution series starting at 1000 nM. Among the two antibodies, only C6 showed very weak but detectable binding to 1000 nM 8-histidine tagged recombinant wild-type protein EGFRwt at 25°C.

Table 9

Antibody	EGFRvIII binding at 25°C		
	k_a(1/Ms)	k_d(1/s)	K_D(nM)
B5	2.08E+04	1.41E-02	677.9
C6	1.68E+04	8.94E-03	532.1

Example 5: Affinity Determination for Recombinant Single Chain Fv Formatted Anti-EGFRvIII Antibodies

This example determines the affinity of various recombinant single chain Fv formatted anti-EGFRvIII antibodies at 37°C.

To convert conventional antibody into single chain Fv (scFv) fragment, the heavy

chain variable domain and the light chain variable domain were joined together via a $(GGGGS)_4$ (SEQ ID NO: 202) linker. The scFv fragment was then fused to human IgG1 Fc moiety to facilitate recombinant expression and purification. Affinities of the scFv-Fc fusion proteins were measured at 37°C on a surface plasmon resonance Biacore™ T200 biosensor equipped with an anti-human Fc coupled CM4 sensor chip (GE Healthcare Inc., Piscataway, NJ) as described above. scFv-Fc proteins were captured by anti-human Fc. Monomeric 8-histidine tagged human EGFRvIII extracellular domain was then injected as the analyte at 10-fold dilution series starting at 1000 nM. Table 10 shows that scFv reformatted fusion proteins retain binding to EGFRvIII and that the affinities of the scFv-Fc proteins in both HL (with the heavy-chain variable domain at the N-terminus) and LH (with the light-chain variable domain at the N-terminus) orientations are comparable to their conventional antibody counterparts listed in Tables 6, 7 and 9. These scFv-Fc proteins were also tested for binding to 1000 nM 8-histidine tagged recombinant wild-type protein EGFRwt at 25°C, but none of them showed significant binding.

Table 10

scFv-Fc protein	EGFRvIII binding at 37°C		
	ka(1/Ms)	kd(1/s)	KD(nM)
h62G7-L6/EQ	7.5E+05	7.6E-02	100.9
C6.HL	1.9E+04	2.0E-02	1063.8
14C11.HL	4.2E+04	4.3E-03	104.3
14C11.LH	4.8E+04	6.5E-03	136.0
20B9.HL	2.4E+04	1.8E-03	73.8
20B9.LH	3.0E+04	2.5E-03	82.7
32A10.HL	4.7E+04	1.2E-03	24.5
32A10.LH	4.1E+04	1.2E-03	29.8
42G9.HL	5.6E+04	1.1E-03	18.8
42G9.LH	4.0E+04	8.8E-04	22.1
26B9.HL	5.7E+04	4.0E-02	704.9
26B9.LH	6.9E+04	3.4E-02	494.2
30D8.HL	2.3E+05	5.3E-02	224.6
30D8.LH	1.1E+05	3.8E-02	348.6
20E12.HL	2.1E+04	1.9E-02	893.7
20E12.LH	1.6E+04	1.5E-02	932.5
32G8.HL	8.7E+03	1.3E-02	1490.0

Example 6: Production and Detection of EGFRvIII Specific Chimeric Antigen Receptor (CAR) T Cells

This example determines the expression and antigen binding specificity of EGFRvIII specific CAR T cells.

PBMC from healthy donors provided by AllCells (Alameda, CA) were thawed according to the provider's specification and cultured overnight in X-Vivo™-15 medium (Lonza, Walkersville, MD) supplemented with 5% Human serum. T cells were activated for 3 days in X-Vivo™-15 medium (Lonza) supplemented with 20ng/mL Human IL-2, 5% Human serum, and Dynabeads Human T activator CD3/CD28 at a bead:cell ratio 1:1 (Life Technologies, Carlsbad, CA). T cells were then transduced with a bicistronic lentiviral vector harboring a BFP-T2A-EGFRvIII specific CAR expression cassette under the control of the EF1α promoter at a multiplicity of infection (MOI) of 5. In this construct, the EGFRvIII specific CAR is co-expressed with BFP (blue fluorescent protein), to facilitate detection of the EGFRvIII specific CAR. The EGFRvIII specific CARs contained VH and VL sequences of different anti-EGRRvIII clones (h62G7-L6/EQ, 14C11, 20B9, 32A10, 42G9, C6, 20E12, 26B9, 30D8, and 32G8; described elsewhere herein). CAR T cells were maintained in culture for up to 14 days post-transduction. Percentage of cells expressing the EGFRvIII specific CAR was monitored over time (on Day 4, 9, and 14 post-lentivirus transduction of primary T cells) by flow cytometry using BFP (blue fluorescent protein) for detection (FIG. 2A) (determined by percentage of BFP positive viable cells).

To determine target binding specificity of the CARs, recombinant proteins EGFRvIII-mFc and EGFR-mFc, which comprise of the extracellular domain of either human EGFRvIII or human wild-type EGFR, respectively, fused with mouse IgG1-Fc domain, were produced in HEK293 cells. The target binding specificity was determined by incubating the different EGFRvIII specific CAR T cells with either EGFRvIII-mFc or EGFR-mFc protein followed by a PE-labeled goat anti-mouse Fc secondary antibody (Jackson ImmunoResearch) and analysed by flow cytometry on Day 4 post transduction of the T cells with vectors encoding CARs containing different EGFRvIII specific clones (h62G7-L6/EQ, 14C11, 20B9, 32A10, 42G9, C6, 20E12, 26B9, 30D8, and 32G8). As shown in FIG. 2B, CAR T cells comprising the VH and VL sequences of clones h62G7-L6/EQ, 14C11, 20B9, 32A10, 42G9, 20E12, 26B9, and 30D8 bind to EGFRvIII-mFc but do not significantly bind to EGFR-mFc. On Day 14 post-transduction, the correlation of CAR expression as detected by BFP on the cells and recombinant EGFRvIII-mFc binding by the cells was determined. A representative set

of data is shown in FIG. 2C, which shows the expression of CARs containing ten different EGFRvIII specific clones (h62G7-L6/EQ, 14C11, 20B9, 32A10, 42G9, C6, 20E12, 26B9, 30D8, and 32G8) in CAR T cells on Day 14 post-transduction, as determined by BFP detection on the cells and recombinant EGFRvIII-mFc binding by the cells.

These results demonstrate that CAR T cells containing CARs comprising the VH and VL sequences of clones h62G7-L6/EQ, 14C11, 20B9, 32A10, 42G9, 20E12, 26B9, and 30D8 bind to EGFRvIII-mFc but do not significantly bind to EGFR-mFc.

Example 7: Target Dependent and Independent Degranulation of EGFRvIII specific CAR T Cells

This example determines the degranulation activity of EGFRvIII specific CAR T cells in the presence and absence of target-expression cancer cell lines.

Five cell lines were used to evaluate the degranulation activity of CAR T cells. Human lung cancer cell line NCI-H522 and glioblastoma cell line U87MG were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (Mediatech Inc., Manassas, VA). Since most cancer cell lines do not express EGFRvIII, NCI-H522 (which does not express detectable level of wild-type EGFR or EGFRvIII) was tranduced with a lentivirus vector encoding the full length EGFRvIII gene (SEQ ID NO: 201) to generate "NCI-H522-EGFRvIII", which expresses low level of EGFRvIII. To generate isogenic human glioblastoma cell lines that express various EGFR proteins, the endogenous wild-type EGFR gene of U87MG was first knocked out to generate an EGFR-null cell line "U87-KO". U87-KO cell was then transduced with a lentivirus vector encoding the full length wild-type EGFR gene (accession number NP_005219) to obtain EGFR overexpressing cell line, "U87-KO-EGFRwt". Similarly, "U87-KO-EGFRvIII" (which over-expresses EGFRvIII), was generated from U87-KO by lentivirus tranduction with a vector encoding the full length EGFRvIII gene (SEQ ID NO: 201).

For the degranulation assay, 100,000 T-cells expressing various EGFRvIII specific CARs (containing clones h62G7-L6/EQ, 14C11, 20B9, 32A10, 42G9, C6, 20E12, 26B9, 30D8, and 32G8) were incubated in 96-well plates with an equal number of cancer cells expressing various levels of the EGFRvIII protein or EGFRwt protein. Co-cultures were maintained in a final volume of 100µl of X-Vivo™-15 medium (Lonza,

Walkersville, MD) for 6 hours at 37°C with 5% CO_2. CD107a staining was done during cell stimulation, by the addition of a fluorescent anti-CD107a antibody (Miltenyi Biotec, San Diego, CA) at the beginning of the co-culture, together with a final concentration of 1µg/ml of anti-CD49d (BD Pharmingen, San Diego, CA) , 1µg/ml of anti-CD28 (Miltenyi Biotec), and 1x Monensin (eBioscience, San Diego, CA) solution. After the 6h incubation period, cells were stained with a fixable viability dye and fluorochrome-conjugated anti-CD8 antibody (Miltenyi Biotec) and analyzed by flow cytometry. The degranulation activity was determined as the % of viable/CD8+/BFP+/CD107a+ cells, and by determining the mean fluorescence intensity signal (MFI) for CD107a staining among CD8+/BFP+ cells. Degranulation assays were carried out on Day 14 after CAR transduction. FIG. 3 shows the results from two PBMC donors for each EGFRvIII specific CAR. Except for CAR T cells containing clones C6 and 32G8, an increase in degranulation activity (over CAR-T alone) was only observed when EGFRvIII specific CAR T cells were cocultured with cell lines that express EGFRvIII, such as NCI-H522-EGFRvIII and U87-KO-EGFRvIII.

These results demonstrate that EGFRvIII specific CAR T cells exhibit degranulation activity in the presence of EGFRvIII-expressing tumor cells, but do not exhibit degranulation activity in the presence of tumor cells that do not express EGFRvIII.

Example 8: Interferon Gamma (IFNγ) secretion of EGFRvIII specific CAR T Cells

This example shows the level of IFNγ secretion of EGFRvIII specific CAR T cells upon co-culture of EGFRvIII specific CAR T cells with target protein expressing cell lines.

EGFRvIII specific CAR T-cells expressing CARs containing different EGFRvIII specific clones (h62G7-L6/EQ, 14C11, 20B9, 32A10, 42G9, C6, 20E12, 26B9, 30D8, and 32G8) were incubated in 96-well plates (100,000 cells/well), together with an equal number of cells expressing various levels of EGFRvIII or EGFRwt proteins (cells NCI-H522, NCI-H522-EGFRvIII, U87-KO, U87-KO-EGFRwt, and U87-KO-EGFRvIII; each are described above in Example 7). Co-cultures were maintained in a final volume of 100µl of X-Vivo™-15 medium (Lonza) for 18 hours at 37°C with 5% CO_2. The supernatant was then collected and frozen. The IFNγ in the supernatant was measured with the Human IFN-gamma Quantikine ELISA Kit (R&D systems, Minneapolis, MN)

according to the manufacturer's specifications. As shown in FIG. 4, for the majority of CAR T cells, the level of IFNγ secretion correlates with the amount of EGFRvIII expressed by the co-cultured cells: low expressor NCI-H522-EGFRvIII induced small amount of IFNγ, high expressor U87-KO-EGFRvIII induced large amount of IFNγ. T cells expressing EGFRvIII specific clone 32G8 CAR did not secrete significant levels of IFNγ under all conditions tested. EGFRvIII specific clone C6 CAR T cells, on the other hand, secreted high levels of IFNγ upon coculture with either U87-KO-EGFRwt or U87-KO-EGFRvIII cells.

These results demonstrate that for the majority of EGFRvIII specific CAR T cells, the level of IFNγ secretion correlates with the amount of EGFRvIII expressed by co-cultured cells

Example 9: Cytotoxicity of EGFRvIII specific CAR T Cells

This example determines the cytotoxicity of EGFRvIII specific CAR T cells upon co-culture of EGFRvIII specific CAR T cells with target protein expressing cell lines.

EGFRvIII specific CAR T-cells expressing CARs containing different EGFRvIII specific clones (h62G7-L6/EQ, 14C11, 20B9, 32A10, 42G9, C6, 20E12, 26B9, 30D8, and 32G8) were seeded in 96-well plates (400,000 cells/well), together with 20,000 target cells expressing various levels of the EGFRvIII protein. The target cells were: U87-KO-EGFRwt, NCI-H522-EGFRvIII, and U87-KO-EGFRvIII, described above. Target (EGFRvIII positive: NCI-H522-EGFRvIII and U87-KO-EGFRvIII) and control (EGFRvIII negative: U87-KO-EGFRwt) cells were plated and labelled with the fluorescent intracellular dye CFSE before co-culturing them with the EGFRvIII specific CAR T-cells. The co-cultures were incubated for 4 hours at 37°C with 5% CO_2. After this incubation period, cells were labelled with a fixable viability dye and analyzed by flow cytometry. Viability of each cellular population (EGFRvIII positive target cells or EGFRvIII negative control cells) was determined and the % of specific cell lysis was calculated. Cytotoxicity assays were carried out 14 days post CAR transduction of the T cells. All EGFRvIII specific CAR T cells, except 32G8, were able to lyse both low-level target expressing cells (NCI-H522-EGFRvIII) and high-level target expressing cells (U87-KO-EGFRvIII) to various degrees. In addition, EGFRvIII specific clone C6 CAR T cells lysed both wild-type EGFR and EGFRvIII expressing cells (FIG. 5).

These results demonstrate that EGFRvIII-specific CAR T cells effectively kill cells

that express EGFRvIII.

Example 10: In vivo study of EGFRvIII specific CAR T Cells in U87-KO-EGFRvIII model

This example shows the anti-tumor activity of EGFRvIII specific CAR T cells in a subcutaneous U87-KO-EGFRvIII GBM xenograph model.

Three million U87-KO-EGFRvIII GBM tumor cells (described above) were implanted subcutaneously into 5-6 week old NSG mice (Jackson Laboratory, Sacramento, CA). Tumor volume was measured once a week by a caliper device and calculated with the following formula: Tumor volume = (length x width^2) / 2. On day 8 post tumor implantation, animals were randomized by tumor sizes into five animals per group and a single dose of 6 million CAR positive EGFRvIII specific CAR T cells (expressing the EGFRvIII specific clone 14C11, 32A10 or 26B9; the clones are described elsewhere herein) or the equivalent total number of non-transduced T cells were administered through bolus tail vein injection. FIG. 6 shows that the 14C11, 32A10 and 26B9 EGFRvIII specific CAR T cells, but not the non-transduced T cells, inhibited U87-KO-EGFRvIII xenograft tumor growth *in vivo*.

These results demonstrate that EGFRvIII-specific CAR T cells effectively inhibit tumor growth of EGFRvIII-expressing cells *in vivo*..

Example 11: Surface expression of EGFRvIII specific CARs containing the R2 suicide sequence

This example shows the expression of EGFRvIII specific CARs containing the R2 suicide sequence in T cells.

The R2 suicide sequence is a CD20 epitope based suicide sequence and contains two tandem copies of the rituximab recognition epitope. The R2 sequence was inserted between the scFv and the CD8α hinge sequences of various EGFRvIII specific CARs described elsewhere herein (14C11, 32A10, 30D8, and 26B9) to generate various EGFRvIII-R2 CARs (referred to herein as “14C11-R2”, “32A10-R2”, “30D8-R2”, and “26B9-R2”, respectively). T cells were transduced with a lentiviral vector harboring the various EGFRvIII-R2 CAR expression cassettes under the control of the EF1α promoter at a multiplicity of infection (MOI) of 5 or 25 (for clones 26B9-R2 and 30D8-R2). CAR T cells were maintained in culture for up to 14 or 15 days post-

transduction. CAR expression was monitored over time (on Day 4, Day 9/10, and Day 14/15 post-T cell transduction) by flow cytometry using biotinylated recombinant proteins: EGFRvIII-mFc or rituximab to the cells (conjugated with EZ-Link™ Sulfo-NHS-SS-Biotin; ThermoFisher, Waltham, MA), followed by PE conjugated streptavidin (BD Biosciences, San Diego, CA). The percentages of CAR positive cells as detected with biotinylated EGFRvIII-mFc and biotinylated rituximab are shown in FIGs. 7 and 8, respectively. Binding to non-transduced T cells (NTD) is provided as a control.

These results demonstrate the expression in T cells of EGFRvIII-specific CARs containing the R2 suicide sequence.

Example 12: Cytotoxicity of EGFRvIII-specific R2 CAR T Cells

This example shows the cytotoxicity of EGFRvIII-specific R2 CAR T cells upon co-culture with target expressing cell lines.

Cytotoxicity assays were carried out as described in Example 9, using the EGFRvIII-R2 CARs 14C11-R2, 32A10-R2, 30D8-R2, and 26B9-R2, which are described in Example 11. All EGFRvIII specific R2 CAR T cells tested were able to lyse both low-level target expressing cells (NCI-H522-EGFRvIII) and high-level target expressing cells (U87-KO-EGFRvIII) to various degrees (FIG. 9).

These results demonstrate that EGFRvIII-specific R2 CAR T cells effectively kill cells that express EGFRvIII.

Although the disclosed teachings have been described with reference to various applications, methods, kits, and compositions, it will be appreciated that various changes and modifications can be made without departing from the teachings herein and the claimed invention below. The foregoing examples are provided to better illustrate the disclosed teachings and are not intended to limit the scope of the teachings presented herein. While the present teachings have been described in terms of these exemplary embodiments, the skilled artisan will readily understand that numerous variations and modifications of these exemplary embodiments are possible without undue experimentation. All such variations and modifications are within the scope of the current teachings.

All references cited herein, including patents, patent applications, papers, text books, and the like, and the references cited therein, to the extent that they are not already, are hereby incorporated by reference in their entirety. In the event that one or

more of the incorporated literature and similar materials differs from or contradicts this application, including but not limited to defined terms, term usage, described techniques, or the like, this application controls.

The foregoing description and Examples detail certain specific embodiments of the invention and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the invention may be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof.

Claims

It is claimed:

1. An Epidermal Growth Factor Receptor Variant III (EGFRvIII) specific chimeric antigen receptor (CAR) comprising an extracellular ligand-binding domain, a first transmembrane domain, and an intracellular signaling domain, wherein the extracellular ligand-binding domain comprises a single chain Fv fragment (scFv) comprising a heavy chain variable (VH) region comprising three CDRs from the VH region comprising the sequence shown in SEQ ID NO: 5, 9, 11, 13, 15, 37, 39, 41, 43, or 48; and a light chain variable (VL) region comprising three CDRs from the VL region comprising the sequence shown in SEQ ID NO: 6, 10, 12, 14, 16, 38, 40, 42, or 49.

2. The EGFRvIII specific CAR of claim 1, wherein the VH region comprises the sequence shown in SEQ ID NO: 5, 9, 11, 13, 15, 37, 39, 41, 43, 48, or a variant thereof with one or several conservative amino acid substitutions in residues that are not within a CDR and/or the VL region comprises the amino acid sequence shown in SEQ ID NO: 6, 10, 12, 14, 16, 38, 40, 42, 49, or a variant thereof with one or several amino acid substitutions in amino acids that are not within a CDR.

3. The EGFRvIII specific CAR of claim 1 or 2, wherein the intracellular signaling domain comprises a CD3zeta signalling domain.

4. The EGFRvIII specific CAR of claim 1 or 2, wherein the intracellular signaling domain comprises a 4-1BB signaling domain.

5. The EGFRvIII specific CAR of claim of any one of claims 1 to 4, wherein the intracellular signaling domain is a first intracellular signaling domain and the the CAR further comprises a second intracellular signaling domain.

6. The EGFRvIII specific CAR of claim 5, wherein the first intracellular signaling domain comprises a CD3zeta signaling domain and the second intracellular signaling domain comprises a 4-1BB signaling domain.

7. The EGFRvIII specific CAR of any one of claims 1 to 6, further comprising a stalk domain between the extracellular ligand-binding domain and the first transmembrane domain.

8. The EGFRvIII specific CAR of claim 7, wherein the stalk domain is selected from the group consisting of: a human CD8α hinge, a human CD28 hinge, an IgG1 hinge, and an FcγRIIIα hinge.

9. The EGFRvIII specific CAR of any one of claims 1 to 8, wherein the first transmembrane domain comprises a CD8α chain transmembrane domain.

10. The EGFRvIII specific CAR of any one of claims 1 to 9, further comprising a second extracellular ligand-binding domain, wherein the second extracellular ligand-binding domain is not specific for EGFRvIII.

11. The EGFRvIII specific CAR of any one of claims 1 to 10, wherein the extracellular ligand-binding domain(s), the first transmembrane domain, and intracellular signaling domain(s) are on a single polypeptide.

12. The EGFRvIII specific CAR of any one of claims 1 to 6, further comprising a second transmembrane domain, wherein the first transmembrane domain and the extracellular ligand-binding domain(s) are on a first polypeptide, and wherein the second transmembrane domain and the intracellular signaling domain(s) are on a second polypeptide, wherein the first transmembrane domain comprises a transmembrane domain from the α chain of the high-affinity IgE receptor (FcεRI) and the second transmembrane domain comprises a transmembrane domain from the γ or β chain of FcεRI.

13. The EGFRvIII specific CAR of claim 12, further comprising a third polypeptide comprising a third transmembrane domain fused to an intracellular signaling domain from a co-stimulatory molecule, wherein the third transmembrane domain comprises a transmembrane domain from the γ or β chain of FcεRI.

14. A polynucleotide comprising a nucleic acid sequence encoding the EGFRvIII specific CAR of any one of claims 1 to 13.

15. An expression vector comprising the polynucleotide of claim 14.

16. An engineered immune cell expressing at its cell surface membrane an EGFRvIII specific CAR of any one of claims 1 to 13.

17. The engineered immune cell of claim 16, further comprising another CAR which is not specific for EGFRvIII.

18. The engineered immune cell of claim 16 or 17, further comprising a polynucleotide encoding a suicide polypeptide.

19. The engineered immune cell of claim 18, wherein the suicide polypeptide is RQR8.

20. The engineered immune cell of claim 18, wherein the polynucleotide encoding the suicide polypeptide is part of the same nucleic acid molecule as the polynucleotide comprising a nucleic acid sequence encoding the EGFRvIII specific CAR, and wherein the suicide polypeptide is in the same polypeptide chain as the CAR.

21. The engineered immune cell of claim 20, wherein the suicide polypeptide comprises an epitope that is recognized by rituximab.

22. The engineered immune cell of any one of claims 16 to 21, wherein the immune cell is derived from an inflammatory T-lymphocyte, a cytotoxic T-lymphocyte, a regulatory T- lymphocyte, a memory T-lymphocyte, a helper T-lymphocyte, a natural killer T-lymphocyte, or a natural killer cell.

23. The engineered immune cell of claim any one of claims 16 to 22, further comprising a disruption of one or more endogenous genes, wherein the endogenous gene encodes TCRα, TCRβ, CD52, glucocorticoid receptor (GR), deoxycytidine kinase (dCK), or programmed death-1 (PD-1).

24. The engineered immune cell of any one of claims 16 to 23, wherein the immune cell is obtained from a healthy donor.

25. The engineered immune cell of any one of claims 16 to 23, wherein the immune cell is obtained from a patient.

26. The engineered immune cell of any one of claims 16 to 25 for use as a medicament.

27. The engineered immune cell of claim 26, wherein the medicament is for use in treatment of an EGFRvIII related cancer selecting from the group consisting of glioblastoma multiform, anaplastic astrocytoma, giant cell glioblastoma, gliosarcoma, anaplastic oligodendroglioma, anaplastic ependymoma, anaplastic oligoastrocytoma, choroid plexus carcinoma, anaplastic ganglioglioma, pineoblastoma, pineocytoma, meningioma, medulloepithelioma, ependymoblastoma, medulloblastoma, supraentorial primitive neuroectodermal tumor, atypical teratoid/rhabdoid tumor, mixed glioma, head and neck cancer, non-small cell lung cancer, breast cancer, ovarian cancer, prostate cancer, medullobastoma, colorectal cancer, anal cancer, cervical cancer, renal cancer, skin cancer, pancreatic cancer, liver cancer, bladder cancer, gastric cancer, thyroid cancer, mesothelioma, uterine cancer, lymphoma, and leukemia.

28. The engineered immune cell of claim 26, wherein the cancer is glioblastoma multiforme, head and neck cancer, non-small cell lung cancer, breast cancer, ovarian cancer, or prostate cancer.

29. A method of engineering an immune cell comprising:

a. providing an immune cell; and

b. expressing at the surface of the cell at least one EGFRvIII specific CAR according to any one of claims 1 to 13.

30. The method of engineering an immune cell of claim 29 comprising:

a. providing an immune cell;

b. introducing into the cell at least one polynucleotide encoding said EGFRvIII specific CAR; and

c. expressing said polynucleotide into the cell.

31. The method of engineering an immune cell of claim 29 comprising:

a. providing an immune cell;

b. introducing into the cell at least one polynucleotide encoding said EGFRvIII specific CAR; and

c. introducing at least one other CAR which is not specific for EGFRvIII.

32. A method of treating a subject in need thereof comprising:

a. providing an immune cell expressing at the surface an EGFRvIII specific CAR according to any one of claims 1 to 13; and

b. administering said immune cells to said patient.

33. A pharmaceutical composition comprising the engineered immune cell of any one of claims 16 to 25.

34. A method of treating a condition associated with malignant cells expressing EGFRvIII in a subject comprising administering to a subject in need thereof an effective amount of the pharmaceutical composition of claim 33.

35. The method of claim 34, wherein the condition is a cancer.

36. The method of claim 35, wherein the cancer is an EGFRvIII related cancer selecting from the group consisting of glioblastoma multiform, anaplastic astrocytoma, giant cell glioblastoma, gliosarcoma, anaplastic oligodendroglioma, anaplastic ependymoma, anaplastic oligoastrocytoma, choroid plexus carcinoma, anaplastic ganglioglioma, pineoblastoma, pineocytoma, meningioma, medulloepithelioma, ependymoblastoma, medulloblastoma, supraentorial primitive neuroectodermal tumor, atypical teratoid/rhabdoid tumor, head and neck cancer, non-small cell lung cancer, breast cancer, ovarian cancer, prostate cancer, medullobastoma, colorectal cancer, anal cancer, cervical cancer, renal cancer, skin cancer, pancreatic cancer, liver cancer, bladder cancer, gastric cancer, thyroid cancer, mesothelioma, uterine cancer, lymphoma, and leukemia.

37. The method of claim 36, wherein the cancer is glioblastoma multiforme, head and neck cancer, non-small cell lung cancer, breast cancer, ovarian cancer, or prostate cancer.

38. A method of inhibiting tumor growth or progression in a subject who has malignant cells expressing EGFRvIII, comprising administering to the subject in need

thereof an effective amount of the pharmaceutical composition of claim 33 to the subject.

39. A method of inhibiting metastasis of malignant cells expressing EGFRvIII in a subject, comprising administering to the subject in need thereof an effective amount of the pharmaceutical composition of claim 33 to the subject.

40. A method of inducing tumor regression in a subject who has malignant cells expressing EGFRvIII, comprising administering to the subject in need thereof an effective amount of the pharmaceutical composition of claim 33 to the subject.