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(54) Title: METHODS AND COMPOSITONS FOR TREATING CANCER

(57) Abstract: Described are compositions and methods relating to immune cells which express both a chimeric antigen receptor which binds to the IL13 receptor α-2 (IL13Rα2) and a O^6- methylguanine DNA methyltransferase (MGMT) protein. Viral particles containing an IL13 chimeric antigen receptor (IL13CAR) or variant thereof and an MGMT protein or variant thereof are used to transfect immune cells such as T cells, imparting to the transfected cells both IL13Rα2-targeting activity and resistance to the chemotherapeutic agent temozolomide (TMZ). The compositions and methods described are useful for cancer therapy such as the treatment of a high-grade malignant glioma.

METHODS AND COMPOSITIONS FOR TREATING CANCER

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority to U.S. provisional application No. 62/086,346, filed December 2, 2014, which is hereby incorporated by reference in its entirety.

CROSS-REFERENCE TO A SEQUENCE LISTING

[0002] A "Sequence Listing" is submitted with this application in the form of a text file, created November 30, 2015, and named "0962018010WOseqlist.txt" (69892 bytes), the contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0003] Targeted immunotherapy has thus emerged as promising field of research in the treatment of malignancies and has received a great deal of interest in recent years (Carpentier and Meng, 2006, Curr Opin Oncol, 18(6):631-636; Wainwright et al., 2012, Exp Opin Emerging Drugs; 17(2):181-202). One of the most extensively studied targets is the interleukin-13 receptor alpha 2 (IL13Rα2) (Thaci et al., 2014, Neuro-Oncol, 16(10):1304-1324). IL13Rα2 is a decoy receptor for interleukin-13 (IL13), lacking the signaling chain that is present on the ubiquitous IL13Rα1, thus preventing any IL13-mediated downstream signaling pathway (Arima et al., 2005, J Biol Chem, 280(26):24915-24922). Increased expression of IL13Rα2 has been reported to promote tumor progression in glioma and other tumor models. IL13Rα2 expression is a prognostic marker for glioma malignancy grade and for poor patient survival (Brown et al., 2013, PLoS ONE, 8(10): Article ID e77769). Its selective expression on MG, discovered almost two decades ago, has been a target for therapy ever since (Debinski et al., 1999, Clin Canc Res, 5(5):985-990).

[0004] Glioblastoma is the most common primary brain tumor in adults. More than half of the 18,000 patients diagnosed with malignant primary brain tumors in US each year have glioblastoma multiforme. Glioblastoma multiforme is an anaplastic, highly cellular tumor, with high proliferation indices, microvascular proliferation and focal necrosis. Signs and symptoms depend on several factors (size, rate of growth, localization of the tumor within the brain) and are mainly represented by headache, seizures, neurological deficits, changes in mental status. Glioblastoma multiforme prognosis remains dismal. Survival time is less than 2 years for the majority of patients.

[0005] Despite incremental improvements in survival with the current standard of care for glioblastoma (GBM), which is a tripartite regimen of surgery, radiotherapy, and chemotherapy (Rolle et al., 2010, Neurosurgery Clin of North America, 21(1):201-214; Ashby and Ryken, 2006, Neurosurgical focus, 20(4):E3), the prognosis for most patients remains

dismal (Stupp et al, 2009, Lancet Oncol, 10(5):459-466; Omuro and DeAngelis, 2013, JAMA, 310(17):1842-1850). Major limitations in the treatment of GBM are the tumor's location within the brain that impedes delivery of cytotoxic agents across the blood-brain barrier (Ashby and Ryken, 2006, Neurosurgical Focus, 20(4):E3), compounded with a strong immunosuppressive environment (Rolle et al., 2012, Adv Exp Med Biol, 746:53-76) and chemo- and radioresistant glioma-initiating cells (Bao et al, 2006, Nature, 444(7120):756-760; Frosina, Mol Canc Res, 2009 7(7):989-999). As a result, novel strategies are continually being tested to improve patient survival, quality of life, and overall outcomes.

[0006] Accordingly, described herein are compositions and methods for more efficacious treatment of cancers in which malignant cells express or over-express IL13Rα2, including brain cancers.

SUMMARY OF THE INVENTION

[0007] In one aspect, a chimeric nucleic acid sequence is provided, wherein the chimeric nucleic acid sequence comprises a first nucleic acid which encodes an IL13 chimeric antigen receptor (IL13CAR) which binds the IL1Rα2 receptor (IL13Rα2) and a second nucleic acid which encodes a drug-resistance polypeptide which is a O^6-methylguanine DNA methyltransferase (MGMT) protein.

[0008] In one embodiment, the IL13CAR comprises a ligand to the IL13Rα2. In another embodiment, the ligand is IL13. In yet another embodiment, the ligand is a fragment of IL13 which binds the IL13Rα2. In still another embodiment, the ligand is an antibody variable domain or fragment thereof which selectively binds IL13Rα2.

[0009] In one embodiment, the MGMT protein comprises a P140K substitution.

[0010] In one embodiment, the chimeric nucleic acid sequence comprises a first nucleic acid sequence encoding the IL13CAR and a second nucleic acid sequence encoding the MGMT protein.

[0011] In one embodiment, the first nucleic acid sequence encoding the IL13CAR is 5' to the second nucleic acid sequence encoding the MGMT polypeptide. In an alternative embodiment, the first nucleic acid sequence encoding the IL13CAR is 3' to the second nucleic acid sequence encoding the MGMT polypeptide.

[0012] In one embodiment, the first nucleic acid sequence encoding the IL13CAR comprises in a 5' to 3' direction: a nucleic acid sequence encoding an IL13Rα2 ligand domain, a nucleic acid sequence encoding a transmembrane (TM) domain, and a nucleic acid sequence encoding a cytoplasmic domain which comprises a CD3 zeta signaling domain. In another embodiment, the first nucleic acid sequence further comprises a nucleic acid sequence which encodes a hinge region, wherein the hinge region positioned between the

IL13 ligand domain and the TM domain. In still another embodiment, the first nucleic acid sequence further comprises a nucleic acid sequence which encodes a CD28 co-stimulatory domain wherein the CD28 co-stimulatory domain is positioned between the TM domain and the CD3-zeta chain. In still another embodiment, the first nucleic acid sequence further comprises a nucleic acid which encodes a signal sequence wherein the signal sequence is positioned N-terminal to the IL13Rα2 ligand domain.

[0013] In one embodiment, the hinge domain is a CD8 hinge domain. In another embodiment, the CD8 hinge domain comprises SEQ ID NO:27.

[0014] In one embodiment, the cytoplasmic domain further comprises one or more co-stimulatory domains. In one embodiment, the co-stimulatory domain is a CD28 co-stimulatory domain. In another embodiment, the CD28 co-stimulatory domain is positioned between the TM domain and the CD3-zeta signaling domain.

[0015] In one embodiment, the cytoplasmic domain further comprises one or more co-stimulatory domains selected from the group consisting of an OX-40 costimulatory domain, an HVEM co-stimulatory domain, a 41BB co-stimulatory domain, an ICOS co-stimulatory domain, an OX40 co-stimulatory domain and a CD27 co-stimulatory domain. In one embodiment, the additional co-stimulatory domain is positioned between a CD28 co-stimulatory domain and a CD3-zeta signaling domain.

[0016] In one embodiment, the signal sequence is a heterologous signal sequence. In another embodiment, the signal sequence is an IL13 signal sequence or a variant thereof. In still another embodiment, the IL13 signal sequence comprises SEQ ID NO:25. In yet another embodiment, the nucleic acid sequence encoding the signal sequence comprises SEQ ID NO:9.

[0017] In one embodiment, the IL13 ligand binding domain comprises the mature IL13 protein (SEQ ID NO:26). In another embodiment, the IL13 ligand binding domain consists of a fragment of the mature IL13 wherein the fragment binds to the IL13Rα2 protein with approximately the same affinity as does the mature IL13 protein (SEQ ID NO:26).

[0018] In one embodiment, the nucleic acid sequence encoding the IL13 ligand encodes a polypeptide selected from the group consisting of SEQ ID NO:26, SEQ ID NO:36 and SEQ ID NO:37. In another embodiment, the nucleic acid sequence encoding the mature IL13 polypeptide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:10, SEQ ID NO:34, and SEQ ID NO:35.

[0019] In one embodiment, the first nucleic acid comprises in a 5' to 3' direction, a nucleic acid selected from the group consisting of SEQ ID NO:10, SEQ ID NO:34, or SEQ ID NO:35 which encodes the IL13 ligand domain, a nucleic acid comprising SEQ ID NO:14 or a variant thereof which encodes the TM domain, and a nucleic acid comprising SEQ ID NO:18

or a variant thereof which encodes the CD3-zeta signaling domain. In another embodiment, the first nucleic acid further comprises SEQ ID NO:9 or a variant thereof which encodes the IL13 signal sequence wherein the sequence of SEQ ID NO:9 is upstream of the nucleic acid sequence encoding the IL13 ligand domain. In another embodiment, the first nucleic acid further comprises SEQ ID NO:12 or a variant thereof which encodes the CD8 hinge domain. In still another embodiment, the first nucleic acid further comprises the nucleic acid of SEQ ID NO:16 or a variant thereof which encodes the CD28 co-stimulatory domain.

[0020] In one embodiment, the first nucleic acid comprises in a 5' to 3' direction, the nucleic acid sequence of SEQ ID NO:9 or variant thereof which encodes the signaling domain, a nucleic acid selected from the group consisting of SEQ ID NO:10, SEQ ID NO:34, or SEQ ID NO:35 which encodes the IL13 ligand domain, the nucleic acid sequence of SEQ ID NO:12 or a variant thereof which encodes the CD8 hinge domain, a nucleic acid comprising SEQ ID NO:14 or a variant thereof which encodes the TM domain, the nucleic acid of SEQ ID NO:16 or a variant thereof which encodes the CD28 co-stimulatory domain and the nucleic acid sequence of SEQ ID NO:18 or a variant thereof which encodes the CD3-zeta signaling domain.

[0021] In one embodiment, the first nucleic acid sequence encoding the CAR further comprises a nucleic acid sequence encoding a linker between the mature IL13 ligand and the CD8 hinge domain. In another embodiment, the nucleic acid sequence encoding the linker between the mature IL13 ligand and the CD8 hinge domain consists of SEQ ID NO:11.

[0022] In one embodiment, the first nucleic acid sequence encoding the CAR further comprises a nucleic acid sequence encoding a linker between SEQ ID NO:12 and SEQ ID NO:14. In another embodiment, the nucleic acid sequence encoding the linker between SEQ ID NO:12 and SEQ ID NO:14 consists of SEQ ID NO:13.

[0023] In one embodiment, the first nucleic acid sequence encoding the CAR further comprises a nucleic acid sequence encoding a linker between SEQ ID NO:14 and SEQ ID NO:16. In another embodiment, the nucleic acid sequence encoding the linker between SEQ ID NO:14 and SEQ ID NO:16 consists of SEQ ID NO:15.

[0024] In one embodiment, the first nucleic acid sequence encoding the CAR further comprises a nucleic acid sequence encoding a linker between SEQ ID NO:16 and SEQ ID NO:18. In another embodiment, the nucleic acid sequence encoding the linker between SEQ ID NO:16 and SEQ ID NO:18 consists of SEQ ID NO:17.

[0025] In one embodiment, the second nucleic acid sequence encoding the MGMT protein comprises P140KMGMT (SEQ ID NO:22). In another embodiment, the second nucleic acid sequence encoding the MGMT protein comprises a nucleic acid sequence which encodes a protein comprising SEQ ID NO:33.

[0026] In one embodiment, the second nucleic acid sequence encoding the MGMT protein comprises an amino acid sequence selected from the group consisting of G156A-MGMT (SEQ ID NO:38), MGMT-2 (SEQ ID NO:39), MGMT-3 (SEQ ID NO:40) and MGMT-5 (SEQ ID NO:41). In another embodiment, the second nucleic acid sequence encoding the MGMT protein comprises a nucleic acid sequence which encodes a protein comprising G156A-MGMT (SEQ ID NO:38), MGMT-2 (SEQ ID NO:39), MGMT-3 (SEQ ID NO:40) and MGMT-5 (SEQ ID NO:41).

[0027] In one embodiment, the second nucleic sequence encoding the MGMT protein does not comprise SEQ ID NO:48. In another embodiment, the second nucleic acid sequence encodes an MGMT protein that does not comprise SEQ ID NO:49.

[0028] In one embodiment, the chimeric nucleic acid sequence further comprises a nucleic acid sequence encoding a self-cleaving peptide. In another embodiment, the nucleic acid sequence encoding the self-cleaving peptide comprises SEQ ID NO:21. In still another embodiment, the self-cleaving peptide comprises the amino acid sequence of SEQ ID NO:32.

[0029] In one embodiment, the chimeric nucleic acid sequence further comprises a Kozak sequence. In another embodiment, the Kozak sequence comprises SEQ ID NO:8. In still another embodiment, the Kozak sequence is located upstream of the nucleic acid sequences which encode the IL13CAR and the MGMT proteins. In one embodiment, the chimeric nucleic acid sequence further comprises a first restriction endonuclease site which is upstream of the Kozak sequence. In another embodiment, the restriction endonuclease site upstream of the Kozak sequence consists of SEQ ID NO:7. In one embodiment, the chimeric nucleic acid sequence further comprises a second endonuclease site which is downstream of the nucleic acid sequences which encode the IL13CAR and the MGMT proteins.

[0030] In one embodiment, the chimeric nucleic acid sequence comprises a nucleotide sequence selected from the group consisting of nucleotides 109 to 1836 of SEQ ID NO:1, nucleotides 109 to 1836 of SEQ ID NO:2 and nucleotides 109 to 1836 of SEQ ID NO:3. In one embodiment, the chimeric nucleic acid sequence comprises a nucleotide sequence selected from the group consisting of nucleotides 13 to 1836 of SEQ ID NO:1, nucleotides 13 to 1836 of SEQ ID NO:2 and nucleotides 13 to 1836 of SEQ ID NO:3. In one embodiment, the chimeric nucleic acid sequence comprises a nucleotide sequence selected from the group consisting of nucleotides 7 to 1842 of SEQ ID NO:1, nucleotides 7 to 1842 of SEQ ID NO:2 and nucleotides 7 to 1842 of SEQ ID NO:3. In another embodiment, the chimeric nucleic acid sequence comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3.

[0031] In one embodiment, the chimeric nucleic acid sequence comprises a nucleotide sequence which encodes the protein of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47.

[0032] In one embodiment, the IL13Rα2 receptor ligand is a variant of IL-13 or fragment thereof which binds the IL13Rα2 with about 5-fold, 10-fold, or 100-fold less affinity than wild-type IL13 (SEQ ID NO:26). In an alternative embodiment, the IL13Rα2 receptor ligand is a variant of IL13 or fragment thereof which binds the IL13Rα2 with about 5-fold, 10-fold, or 100-fold higher affinity than wild-type IL13 (SEQ ID NO:26).

[0033] In one embodiment, the IL13Rα2 receptor ligand is not identical to wild-type IL-13. In another embodiment, IL13Rα2 receptor ligand is not identical to SEQ ID NO:26.

[0034] In one embodiment, P140KMGMT protein is effective in increasing in vitro and/or in vivo viability of a cell expressing the drug-resistance polypeptide when a cell transfected with an IL13-CAR-T is treated with a chemotherapeutic agent as compared to the cell treated with the chemotherapeutic but not expressing the drug-resistance polypeptide.

[0035] In one embodiment, the IL13Rα2 receptor ligand is not identical to wild-type IL-13. In another embodiment, IL13Rα2 receptor ligand is not identical to SEQ ID NO:26.

[0036] In another aspect, a vector comprising a nucleic acid sequence which encodes the IL13CAR and MGMT protein as described herein is provided.

[0037] In one embodiment, the vector comprises a monocistronic nucleic acid sequence which encodes an IL13CAR, a self-cleaving peptide, and a MGMT protein as described herein. In another embodiment, the self-cleaving peptide comprises a 2A peptide.

[0038] In an alternative embodiment, the vector comprises a polycistronic chimeric nucleic acid sequence which encodes an IL13CAR and an MGMT protein. In another embodiment the polycistronic chimeric nucleic acid sequence which encodes the IL13CAR and the MGMT protein further comprises an internal ribosome entry site (IRES) positioned between the nucleic acid sequence encoding the IL13CAR and the nucleic acid sequence encoding the MGMT protein. In still another embodiment, the polycistronic chimeric nucleic acid sequence which encodes the IL13CAR and the MGMT protein further comprises a promoter positioned between the nucleic acid sequence encoding the IL13CAR and the nucleic acid sequence encoding the MGMT protein.

[0039] In one embodiment, the vector is a bacterial plasmid vector. In another embodiment, the vector is an expression vector.

[0040] In one embodiment, the vector is a viral vector. In another embodiment, the viral vector is selected from the group consisting of a retroviral vector, a lentiviral vector, an adenoviral vector and an adeno-associated viral vector.

[0041] In another aspect, a cell transfected with a vector comprising a chimeric nucleic acid sequence which encodes a chimeric antigen receptor (CAR) and a drug-resistance polypeptide as described herein is provided.

[0042] In one embodiment, the cell is selected from the group consisting of a T-cell, an NK-cell, and NKT-cell.

[0043] In another, a recombinant polypeptide is provided comprising in an N-terminal to C-terminal direction, a ligand which binds to a tumor antigen, a transmembrane domain, and a cytoplasmic signaling domain as described herein.

[0044] In one embodiment, the recombinant polypeptide comprising, in an N-terminal to C-terminal direction, a ligand which binds to a tumor antigen, a transmembrane domain, and a cytoplasmic signaling domain, further comprises a self-cleaving peptide positioned between the CAR and drug resistance polypeptide. In another embodiment, the drug resistance polypeptide is N-terminal to the CAR. In still another embodiment, the drug resistance polypeptide is C-terminal to the CAR.

[0045] In another aspect, a recombinant polypeptide is provided comprising a modified MGMT polypeptide which increases viability of a cell exposed to TMZ, wherein the cell is genetically modified to express a CAR as described herein and wherein the cell is administered to a patient diagnosed with a brain cancer.

[0046] In another aspect, a composition comprising a first nucleic acid which encodes a CAR as described herein and a second nucleic acid which encodes an MGMT protein as described herein is provided.

[0047] In one embodiment, the first nucleic acid encodes a CAR protein which comprises an IL13 ligand domain as described herein, a TM domain as described herein, and a cytoplasmic domain comprising a CD3-zeta signaling domain as described herein. In another embodiment, the first nucleic acid further encodes a signal sequence which is upstream of the IL13 ligand binding domain of the CAR protein. In still another embodiment, the first nucleic acid further encodes a hinge region as described herein wherein the hinge region is positioned between the IL13 ligand domain and the TM domain of the CAR protein. In yet another embodiment, the first nucleic acid further encodes a CD28 co-stimulatory domain which is positioned between the TM domain and the CD3-zeta signaling domain. In still another embodiment, the first nucleic acid further encodes an additional co-stimulatory domain. In another embodiment, the first nucleic acid further comprises a Kozak sequence upstream of the nucleic acid encoding the CAR protein.

[0048] In one embodiment, the second nucleic acid encodes an MGMT protein which has an amino acid sequence selected from the group consisting of SEQ ID NO:33, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41 and SEQ ID NO:43.

[0049] In one embodiment, the MGMT protein does not comprise SEQ ID NO:49.

[0050] In one embodiment, the composition comprises a chimeric nucleic acid which comprises the first nucleic acid and the second nucleic acid.

[0051] In one embodiment, the chimeric nucleic acid further comprises a nucleic acid which encodes a self-cleaving linker peptide as described herein, wherein the nucleic acid encoding the self-cleaving linker peptide is positioned between the first nucleic acid and the second nucleic acid.

[0052] In one embodiment, the chimeric nucleic acid further comprises an internal ribosome entry site (IRES) as described herein, wherein the IRES is positioned between the first nucleic acid and the second nucleic acid.

[0053] In one embodiment, the chimeric nucleic acid is a bicistronic construct which comprises a first promoter upstream of the first nucleic acid encoding the CAR protein and a second promoter upstream of the second nucleic acid encoding the MGMT protein.

[0054] In one embodiment, the composition comprises a first vector which comprises the first nucleic acid which encodes the CAR protein and a second vector which comprises the second nucleic acid which encodes the MGMT protein. In another embodiment, the first and second vectors are each a plasmid or expression vector. In yet another embodiment, the first and second vectors are each a retroviral particle.

[0055] In another aspect, a host cell comprising a first nucleic acid which encodes a CAR as described herein and a second nucleic acid which encodes an MGMT protein as described herein. In one embodiment, the first nucleic acid encodes a CAR protein which comprises an IL13 ligand domain as described herein, a TM domain as described herein, and a cytoplasmic domain comprising a CD3-zeta signaling domain as described herein. In another embodiment, the first nucleic acid further encodes a signal sequence which is upstream of the IL13 ligand binding domain of the CAR protein. In still another embodiment, the first nucleic acid further encodes a hinge region as described herein wherein the hinge region is positioned between the IL13 ligand domain and the TM domain of the CAR protein. In yet another embodiment, the first nucleic acid further encodes a CD28 co-stimulatory domain which is positioned between the TM domain and the CD3-zeta signaling domain. In still another embodiment, the first nucleic acid further encodes an additional co-stimulatory domain. In another embodiment, the first nucleic acid further comprises a Kozak sequence upstream of the nucleic acid encoding the CAR protein.

[0056] In one embodiment, the second nucleic acid encodes an MGMT protein which has an amino acid sequence selected from the group consisting of SEQ ID NO:33, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41 and SEQ ID NO:43. In another embodiment, the MGMT protein is not SEQ ID NO:49.

[0057] In one embodiment, the host cell comprises a chimeric nucleic acid which comprises the first nucleic acid and the second nucleic acid.

[0058] In one embodiment, the chimeric nucleic acid further comprises a nucleic acid which encodes a self-cleaving linker peptide as described herein, wherein the nucleic acid encoding the self-cleaving linker peptide is positioned between the first nucleic acid and the second nucleic acid.

[0059] In one embodiment, the chimeric nucleic acid further comprises an internal ribosome entry site (IRES) as described herein, wherein the IRES is positioned between the first nucleic acid and the second nucleic acid.

[0060] In one embodiment, the chimeric nucleic acid is a dicistronic construct which comprises a first promoter upstream of the first nucleic acid encoding the CAR protein and a second promoter upstream of the second nucleic acid encoding the MGMT protein.

[0061] In one embodiment, the host cell comprises a first vector which comprises the first nucleic acid which encodes the CAR protein and a second vector which comprises the second nucleic acid which encodes the MGMT protein. In another embodiment, the first and second vectors are each a plasmid or expression vector as described herein. In yet another embodiment, the first and second vectors are each a retroviral particle as described herein.

[0062] In another aspect, a composition comprising a recombinant polypeptide is provided, wherein the recombinant polypeptide comprises in an N-terminal to C-terminal direction, a signal sequence as described herein, an IL13 ligand as described herein, a transmembrane domain as described herein, a cytoplasmic signaling domain as described herein, an MGMT protein as described herein and a pharmaceutically acceptable excipient. In another embodiment, the recombinant polypeptide further comprises a hinge domain as described herein wherein the hinge domain is positioned between the IL13 ligand domain and the transmembrane domain. In still another embodiment, the recombinant polypeptide further comprises a self-cleaving peptide as described herein wherein the self-cleaving peptide is positioned between the cytoplasmic signaling domain and the MGMT protein.

[0063] In one embodiment, the composition is a pharmaceutical composition.

[0064] In another aspect, a method for treating a subject diagnosed with a cancer is provided.

[0065] In one embodiment, the method comprises obtaining a cell from the subject, transducing the cell with one or more nucleic acids which encode an IL13CAR as described herein and an MGMT protein as described herein, maintaining the cell under conditions in which the nucleic acids are expressed by the cell, and administering to the patient a therapeutically effective number of the cells expressing the IL13CAR and MGMT proteins.

[0066] In one embodiment, the introducing into the cell comprises using a first vector comprising a nucleic acid encoding the IL13CAR and a second vector comprising a nucleic acid encoding the MGMT protein. In another embodiment, the introducing into the cell comprises using a vector that comprises a nucleic acid encoding the IL13CAR and the MGMT protein.

[0067] In one embodiment, the cell is transduced with a vector comprising an IL13CAR-P140KMGMT chimeric construct as described herein.

[0068] In one embodiment the subject is a mammal. In another embodiment, the mammal is a primate, a human, or a mouse.

[0069] In one embodiment, the one or more nucleic acids are introduced into the cell using a viral vector selected from the group consisting of a retroviral vector, a lentiviral vector, an adenoviral vector or a combination thereof.

[0070] In one embodiment, the cell is a T cell.

[0071] In one embodiment, the T cells are obtained using plasmapheresis.

[0072] In one embodiment, the subject has been diagnosed with a cancer selected from the group consisting of brain, breast, pancreatic, head and neck, ovarian and colorectal. In another embodiment, the cancer has metastasized.

[0073] In one embodiment, the subject has been diagnosed with a high-grade malignant glioma. In another embodiment, the subject has been diagnosed with glioblastoma multiforme (GMB), an anaplastic astrocytoma or a pediatric glioma.

[0074] In one embodiment, the brain cancer is a glioblastoma. In another embodiment, the brain cancer is a high-grade astrocytoma.

[0075] In one embodiment, the breast cancer is a basal-like breast cancer.

[0076] In one embodiment, the method further comprises treating the subject with one or more chemotherapeutic agents. In another embodiment, the method comprises treating the subject with temozolomide (TMZ).

[0077] In one embodiment, the one or more chemotherapeutic agents is administered to the subject before, during, and/or after administration of a dose of the modified cells.

[0078] In one embodiment, the administering is intracranial, intramedullary, intradermally, subcutaneously, topically, or intravenously.

[0079] In another aspect, a method for producing a cell which expresses an IL13CAR-P140KMGMT construct as described herein is provided comprising introducing into the cell a nucleic acid sequence encoding the IL13CAR-P140KMGMT chimeric protein, maintaining the cell under conditions in which the IL13CAR-P140KMGMT chimeric protein is expressed by the cell.

[0080] In one embodiment the cell is a mammalian cell. In another embodiment, the mammalian cell is a human cell, a primate cell or a mouse cell.

[0081] In one embodiment, the cell is a T cell. In another embodiment, the cell is an autologous cell or a human leukocyte antigen (HLA)-matched cell.

[0082] In one embodiment, the cell is obtained from one or more subjects diagnosed with a brain cancer or malignancy.

[0083] In another aspect, a population of cells comprising the IL13CAR-P140KMGMT construct is provided. In another embodiment, at least about 50%, 60%, 70%, 80T, 90% or 95% of cells in the population of cells express the IL13CAR-P140KMGMT construct is provided.

[0084] In one aspect, the invention is directed to an (one or more) isolated nucleic acid sequence encoding (having; comprising; consisting essentially of; consisting of) a chimeric antigen receptor (CAR) comprising (consisting essentially of; consisting of) a T cell receptor that expresses one or more ligands (*e.g.*, an antibody) to one or tumor antigens of a brain cancer. In some aspects, the CAR further expresses one or more additional agents useful for treating a brain cancer.

[0085] In another aspect, the invention is directed to an expression construct comprising (consisting essentially of; consisting of) one or more nucleic acid sequences encoding a CAR comprising a T cell receptor that expresses one or more ligands (*e.g.*, an antibody) of one or tumor antigens of a brain cancer (*e.g.*, a cancer antigen-binding domain). In some aspects, the CAR further expresses one or more additional agents useful for treating a brain cancer.

[0086] In another aspect, the invention is directed to a host cell comprising (consisting essentially of; consisting of) an expression construct comprising one or more nucleic acid sequences encoding a CAR comprising a T cell receptor that expresses one or more ligands (*e.g.*, an antibody) of one or tumor antigens of a brain cancer. In some aspects, the CAR further expresses one or more additional agents useful for treating a brain cancer.

[0087] In another aspect, the invention is directed to a method of producing a cell which expresses a CAR comprising (consisting essentially of; consisting of) a T cell receptor comprising one or more ligands (*e.g.*, an antibody) of one or tumor antigens of a brain cancer. In a particular aspect, the CAR further comprises one or more additional agents useful for treating a brain cancer.

[0088] In another aspect, the invention is directed to a CAR polypeptide comprising (having; consisting essentially of; consisting of) a T cell receptor comprising one or more ligands (*e.g.*, an antibody) of one or tumor antigens of a brain cancer. In a particular aspect, the CAR polypeptide further comprises one or more additional agents useful for treating a brain cancer.

[0089] In another aspect, the invention is directed to a method of treating brain cancer in an individual in need thereof comprising (consisting essentially of; consisting of) administering one or more T cells that express a CAR comprising a T cell receptor that expresses one or more ligands (*e.g.*, an antibody) of one or tumor antigens of a brain cancer. In some aspects, the CAR further expresses one or more additional agents useful for treating a brain cancer.

[0090] The invention is also directed to pharmaceutical compositions comprising compositions provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0091] The foregoing will be apparent from the following more particular description of example embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating embodiments of the present invention.

[0092] FIG. 1A provides a schematic of an IL13E13K.R109K CAR nucleic acid construct.

[0093] FIG. 1B provides a plasmid map of a pMFG host plasmid comprising an IL13E13K.R109K CAR.

[0094] FIG. 2A provides a schematic of an IL13.E13KR109K CAR-2A-P140KMGMT.

[0095] FIG. 2B: provides a plasmid map of a pMFG host plasmid comprising an IL-13-CAR-2A-P140KMGMT.

[0096] FIGS. 3A-3C illustrate FACS analysis of PG13 cells transduced with a virus containing an IL13CAR construct and a IL13CAR-2A-P140KMGMT construct, prior to enrichment (FIG. 3A) and after enrichment (FIG. 3B). FIG. 3C illustrates western blot analysis of cell lysates.

[0097] FIG. 4 provides a graph showing viability of T cells transduced with a retrovirus comprising an IL13CAR-2A-P140KMGMT construct and exposed to TMZ.

[0098] FIGS. 5A and 5B illustrate section of IL2 (FIG. 5A) and IFNγ (FIG. 5B) in cells transfected with an IL13CAR-2A-MGMT construct as described herein.

[0099] FIG. 6 illustrates viability of mice harboring a tumor and administered chimeric constructs and/or a chemotherapeutic agent as described herein.

[0100] FIGS. 7A-7B provide the chimeric nucleic acid sequence (FIG. 7A; SEQ ID NO:1) and amino acid sequence (FIG. 7B; SEQ ID NO:4) of an IL13CAR-P140KMGMT construct.

[0101] FIGS. 8A-8B provide the chimeric nucleic acid sequence (FIG. 8A; SEQ ID NO:2) and amino acid sequence (FIG. 8B; SEQ ID NO:5) of an IL13(E13Y)CAR-P140KMGMT construct.

[0102] FIGS. 9A-9B provide the chimeric nucleic acid sequence (FIG. 9A; SEQ ID NO:3) and amino acid sequence (FIG. 9B; SEQ ID NO:6) of an IL13(E13K.R109K)CAR-P140KMGMT construct.

DETAILED DESCRIPTION OF THE INVENTION

[0103] Described herein is the generation of a (one or more) chimeric antigen receptor (CAR) comprising a T cell receptor modified by genetic engineering to express one or more ligands (e.g., an antibody or other ligand to a cell surface protein) to one or more tumor antigens of a cancer. Specifically, the CAR proteins described herein include a ligand binding domain which binds to a protein which is expressed on the surface of a cancer cell. Preferably, the tumor antigen is not expressed on the surface of non-diseased or normal cells, or is expressed on non-diseased or normal cells at a level which is much lower than the level at which it is expressed on a cancer or other diseased cell. T cells modified to express the resulting CAR are redirected by the neo-specificity of the CAR to attack tumors expressing the surface antigen (e.g., a receptor) recognized by the CAR. Also shown herein is that the CAR can further comprise one or more additional agents useful for treating a brain cancer (e.g., an agent that overcomes the resistance of brain cancer cells to treatment). Specifically, the compositions and methods described herein are designed to treat brain cancers in which the malignant cells express the IL13α2 receptor (IL13Rα2). Accordingly, the disclosure is exemplified herein using a CAR that expresses and displays a ligand of the IL13Rα2, such as the cytokine interleukin 13 (IL13) or a variable domain of an antibody which selectively binds IL13Rα2. The IL13CAR is expressed with an O(6)-methylguanine-DNA-methyltransferase (MGMT) gene. In one embodiment, the MGMT gene is modified to encode a protein which imparts to or enhances a host cell's (e.g. T cell) resistance to temozolomide (TMZ), a chemotherapeutic agent used to treat brain cancer.

[0104] In one preferred embodiment, the IL13CAR comprises an IL13 which is mutated at position 13 (numbering relative to SEQ ID NO:26) to change glutamate to tyrosine. In an alternate preferred embodiment, the IL13 is mutated at position 13 to change glutamate to lysine and at position 109 to change arginine to lysine (amino acid positions 13 and 109 are with respect, e.g., to SEQ ID NO:26). In one embodiment, the IL13 is mutated so that the amino acid at position 109 is changed from arginine to lysine.

[0105] In one preferred embodiment, the modified MGMT gene encodes a MGMT variant referred to herein as P140KMGMT (SEQ ID NO:33), which protects IL13CAR-P140KMGMT-expressing T cells from cytotoxicity caused by treatment with a methylating agent such as TMZ.

[0106] In one embodiment, the IL13CAR and P140KMGMT proteins are expressed from a monocistronic construct which is transcribed to produce a single transcript which encodes a single protein comprising in an N-terminal to C-terminal direction: IL13CAR, a self-cleaving peptide (2A), and P140KMGMT. After translation of this fusion protein, cleavage of the self-cleaving peptide results in a P140KMGMT protein localized primarily to the nucleus and the IL13CAR, wherein the IL13 ligand domain is displayed on the surface of the host cell. However, it is understood that the IL13CAR and P140KMGMT proteins can be expressed from individual nucleic acids. For example, the nucleic acids encoding the IL12CAR and P140KMGMT proteins may be in separate vectors which are then introduced into the same cell, or they can be cloned into a single vector as individual monocistronic constructs (e.g., each having their own promoter).

[0107] T cells transduced with an IL13CAR-2A-P140KMGMT construct survived better when compared to IL13CAR-transduced T cells which did not express P140KMGMT, in the presence of TMZ (e.g., Example 6; FIG. 6). Accordingly, also disclosed herein are nucleic acid sequences encoding the CAR and drug resistance polypeptides (MGMT proteins), one or more nucleic acid or retroviral vectors comprising the nucleic acids, cells transfected or transduced with the one or more vectors, and methods for enhancing viability of genetically modified T cells which are exposed to a chemotherapeutic such as TMZ by co-expressing a modified MGMT gene in the modified T cell.

[0108] Also envisioned is a method for treating a subject diagnosed with a cancer, wherein the cancer includes cells which express IL13Rα2. For example, a brain cancer such as a high-grade malignant glioma and basal-like breast cancer cells overexpress the IL13Rα2 protein relative to non-diseased or non-cancerous cells of the same tissue. The claimed compositions are particularly useful when in which the subject is administered both a methylating chemotherapeutic agent and genetically modified immune cell (e.g., T cell) which expresses both an IL13CAR and a modified MGMT gene. This method can reduce treatment time and achieve glioma abolition faster and more effectively than if the subject were treated with only the chemotherapeutic or with the genetically modified T cell. Accordingly, in one aspect, the invention is directed to an (one or more) isolated nucleic acid sequence which encode a chimeric antigen receptor (CAR) for use in a T cell that expresses one or more ligands (*e.g.*, an antibody) to one or tumor antigens of a cancer. In some aspects, the T cell further expresses one or more additional agents useful for treating a brain cancer.

Definitions

[0109] As used herein a “chimeric antigen receptor (CAR)” refers to a molecule comprising one or more extracellular cancer antigen-binding domains, one or more transmembrane domains and one or more cytoplasmic signaling domains for T-cell activation,

and which has specificity for cells expressing the cancer ligand (*e.g.*, the cancer antigen). When introduced into a T cell, the CAR redirects the specificity of the T cell. In a particular aspect, the CAR is expressed as a single molecule.

[0110] As used herein, a “cancer antigen-binding domain” refers to a domain that binds one or more antigens expressed by a cancer cell (one or more cancer antigens). In a particular aspect, the cancer antigen-binding domain is a binding domain that specifically (selectively) binds a cancer antigen and does not bind a non-specific target not expressed by a non-cancer cell (*e.g.*, a normal cell, a healthy cell, a wild type cell).

[0111] A variety of cancer antigen-binding domains can be used and can be produced using known methods or obtained from commercial sources. The cancer antigen-binding domain can be, for example, a nucleic acid, a peptide (protein), an antibody, an organic molecule, a synthetic molecule and the like. Such cancer antigen-binding domains can be, for example, derived from libraries and/or obtained from natural sources.

[0112] The term “IL13CAR” as used herein encompasses a CAR which comprises an IL13 ligand as described herein or as known in the art, including but not limited to variants of IL13 (including, but not limited to functional fragments of IL13 (*e.g.,* fragments of IL13 that can bind to IL13Rα2)), and other IL13 ligands such as an immunoglobulin domain which selectively binds IL13Rα2. Similarly, the term “MGMT” as used herein encompasses a wildtype MGMT and any MGMT variant as described herein or as known in the art.

[0113] As used herein the term "therapeutically effective" applied to dose or amount refers to that quantity of a compound or pharmaceutical composition (e.g., a composition comprising immune cells such as T lymphocytes and/or NK cells) comprising a chimeric receptor of the disclosure, and further comprising a drug resistance polypeptide that is sufficient to result in a desired activity upon administration to a subject in need thereof. Within the context of the present disclosure, the term "therapeutically effective" refers to that quantity of a compound or pharmaceutical composition that is sufficient to delay the manifestation, arrest the progression, relieve or alleviate at least one symptom of a disorder treated by the methods of the present disclosure. Note that when a combination of active ingredients is administered the effective amount of the combination may or may not include amounts of each ingredient that would have been effective if administered individually.

[0114] The phrase "pharmaceutically acceptable" as used in connection with compositions of the present disclosure refers to molecular entities and other ingredients of such compositions that are physiologically tolerable and do not typically produce untoward reactions when administered to a mammal (e.g., a human). Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or

a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in mammals, and more particularly in humans.

[0115] As used herein, the term "subject" refers to any mammal. In a preferred embodiment, the subject is human.

[0116] In other aspects, the cancer antigen-binding domain is all or a biologically active portion of an antibody. The term “antibody” as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that selectively binds an antigen. As used herein, “selectively binds” refers to the ability of the antibody to bind to an antigen or a fragment thereof and the inability to substantially bind to other molecules (*e.g.*, antigens) in a sample. Examples of immunologically active portions of immunoglobulin molecules include Fab fragments (*e.g.*, F(ab), F(ab')$_2$), variable fragments (*e.g.*, single chain variable (scFv), di-scFv, single domain antibody fragment (sdAb), bi-specific fragments (*e.g.*, bi-specific T cell engagers (BiTE)). Such fragments can be obtained from commercial sources and/or generated by, for example, treating the antibody with an enzyme such as pepsin.

[0117] The antibody can be a polyclonal or monoclonal antibody that binds (*e.g.*, selectively binds) to one or more antigens expressed by a cancer cell. As used herein, a “polyclonal antibody” is an antibody from a collection of antibodies that bind to a specific antigen, each identifying a different epitope. As used herein, a “monoclonal antibody” or “monoclonal antibody composition” refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of one or more antigens. A monoclonal antibody composition thus typically displays a single binding affinity for a particular antigen with which it immunoreacts.

[0118] Polyclonal antibodies can be prepared as described above by immunizing a suitable subject with one or more desired cancer antigens such as the extracellular domain of the IL13Rα2 protein. The antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized polypeptide. If desired, the antibody molecules directed against the cancer antigen can be isolated from the mammal (e.g., from tissue, blood) and further purified by well-known techniques, such as protein A chromatography to obtain the IgG fraction. A person having ordinary skill in the art would be able to make a polyclonal antibody that selectively binds to the extracellular domain of the IL13Rα2 protein.

[0119] At an appropriate time after immunization, e.g., when the antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein, Nature 256:495-497 (1975), the human B cell hybridoma

technique (Kozbor et al., Immunol. Today 4:72 (1983)), the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 (1985)) or trioma techniques. The technology for producing hybridomas is well known (see generally Current Protocols in Immunology, Coligan et al., (eds.) John Wiley & Sons, Inc., New York, NY (1994)). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with an immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds a polypeptide of the invention.

[0120] Any of the many well-known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating a monoclonal antibody to a cancer antigen (see, e.g., Current Protocols in Immunology, *supra*; Galfre et al., Nature, 266:55052 (1977); R.H. Kenneth, in Monoclonal Antibodies: A New Dimension In Biological Analyses, Plenum Publishing Corp., New York, New York (1980); and Lerner, Yale J. Biol. Med. 54:387-402 (1981)). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods that also would be useful.

[0121] In one alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal antibody to a cancer antigen can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with the polypeptide to thereby isolate immunoglobulin library members that bind the cancer antigen. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia Recombinant Phage Antibody System, Catalog No. 27-9400-01; and the Stratagene SurfZAP^{TM} Phage Display Kit, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al., Bio/Technology 9:1370-1372 (1991); Hay et al., Hum. Antibod. Hybridomas 3:81-85 (1992); Huse et al., Science 246:1275-1281 (1989); and Griffiths et al., EMBO J. 12:725-734 (1993).

[0122] Additionally, recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art.

[0123] Any of the above routine methods for generating polyclonal antibodies or monoclonal antibodies can be readily applied to a method for generating a monoclonal antibody which selectively binds to the extracellular domain of the IL13Rα2 protein. The variable domain of the resultant antibody or fragment thereof can then be used to generate an IL13 ligand domain which binds to the IL13Rα2 protein with an affinity about the same as the affinity with which the IL13 ligand domain (SEQ ID NO:26) binds the IL13Rα2 protein.

Constructs of the Invention

[0124] The invention is based, at least in part, on an immune cell expressing a CAR which is specific for IL13Rα2, where the cell is resistant to a chemotherapeutic agent, such as TMZ.

[0125] In certain embodiments, the invention provides a nucleic acid (also referred to herein as a chimeric nucleic acid sequence) encoding both a CAR protein that is selective for a brain cancer cell, and a drug-resistance polypeptide. In a preferred embodiment, the CAR protein is an IL13CAR and the drug resistant polypeptide is an MGMT protein which is capable of conferring TMZ-resistance to cells that express it. A chimeric nucleic acid sequence can be constructed, as described herein, to encode both an IL13CAR protein and an MGMT drug resistance protein. Provided below is a description of the domains or sequence regions of the IL13CAR protein and of the MGMT protein and non-limiting examples of each domain or region. The IL13CAR protein is a linear chimeric (fusion) protein which comprises, in an N-terminal to C-terminal direction, an IL13 ligand domain which selectively binds IL13R2α on a diseased cell such as a brain cancer cell, a transmembrane domain, and an intracellular signaling domain. In certain embodiments, the IL13CAR protein described herein may comprises a signal domain positioned N-terminal to the IL13 ligand domain and/or a hinge region positioned between the ligand domain and the transmembrane domain.

[0126] In certain embodiments, a short peptide linker comprising 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid residues is included in the IL13CAR in order to separate regions or domains of the CAR protein (e.g. the signal sequence, IL13 ligand domain, hinge region, transmembrane domain, CD28 co-stimulatory domain, CD3-zeta signaling domain, or an additional co-stimulatory domain). It is understood that the short peptide linker can be present between any two regions (domains) independent of the presence or absence of a short peptide linker between any other two regions (domains). For example, a small peptide linker can be present in the CAR wherein the linker separates the signal sequence (if present) and the IL13 ligand domain, the IL13 ligand domain and the transmembrane domain, the IL13 ligand domain and the hinge region (if present), the hinge region (if present) and transmembrane domain, the transmembrane domain and the CD28 co-signaling domain (if

present), the CD28 co-signaling domain (if present) and the CD3-zeta signaling domain, the transmembrane domain and the CD3 zeta signaling domain, and/or the CD3-zeta signaling domain and the additional co-stimulatory domain (if present). The peptide linkers can be amino acids which are encoded by a nucleic acid sequence that contains a restriction endonuclease site or other feature which allows ligation of the nucleic acids encoding each of the regions or, in the case of a monocistronic construct, domains of the IL13CAR-self-cleaving peptide-MGMT chimeric protein. Each of these domains and linkers are described in greater detail below.

[0127] In one aspect, the cancer antigen-binding domain is a peptide or protein (*e.g.*, a ligand that binds an antigen expressed on the surface of a cancer cell; a ligand that binds a receptor expressed on the surface of a cancer cell). In a preferred embodiment, the receptor is an IL132α receptor and the CAR is constructed to comprise a ligand which selectively binds the IL132α receptor. In a particular aspect, the cancer antigen-binding domain is an interleukin 13 (IL13) or a variant of IL-13 having one or more insertions, deletions, or point mutations, (e.g., E13K IL13; R109K IL13; and/or E13Y IL13). The cancer antigen-binding domain can alternatively be a variable domain of an antibody or fragment thereof that selectively binds IL13Rα2, such as a scFv fragment.

[0128] Tables 1 and 2 below provide a summary of the nucleic acids and polypeptides, respectively, described herein that can be used in the compositions, *e.g.,* chimeric nucleic acid sequences, and methods of the invention. In one embodiment, the invention includes a chimeric nucleic acid sequence comprising any combination of nucleic acid sequences referred to in Table 1. In one embodiment, the invention includes a chimeric nucleic acid sequence encoding any combination of amino acid sequences referred to in Table 2.

Table 1

Sequence Identifiers for Nucleic Acid Sequences

SEQ ID NO	Description	Nucleotide Positions Relative to SEQ ID NO:1
1	IL13(WT)CAR-P140K Full Length (SEQ ID NO:1)	
2	IL13(E13Y)CAR-P140K Full Length (SEQ ID NO:2)	
3	IL13(E13K.R109K)CAR-P140K Full Length (SEQ ID NO:3)	
7	Restriction endonuclease site	1-6
8	Kozak sequence	7-12
9	IL13 Signal Sequence	13-108
10	Mature IL13	109-450
11	Dipeptide linker-1	451-456
12	CD8 Hinge region	457-591
13	Dipeptide linker-2	592-597
14	CD3 zeta transmembrane domain	598-666
15	Dipeptide linker-3	667-672
16	CD28 costimulatory domain	673-804
17	Dipeptide linker-4	805-810

18	CD3 zeta signaling domain	811-1140
19	Stop codon	1141-1143
20	5 amino acid linker/restriction site/reading frame adjust	1144-1158
21	Self-cleaving peptide	1159-1212
22	P140K MGMT drug resistance polypeptide	1213-1839
23	Stop codon	1840-1842
24	Restriction endonuclease site	1843-1846
34	Mature IL13 with E13Y mutation	109-450
35	Mature IL13 with E13KR109K	109-450
48	MGMT drug resistance polypeptide (P140)	

Table 2

Sequence Identifiers for Polypeptide Sequences

SEQ ID NO	**Description**	**Amino Acid Positions Relative to SEQ ID NO:4**
4	IL13(WT)CAR-P140K Full Length (SEQ ID NO:4)	
5	IL13(E13Y)CAR-P140K Full Length (SEQ ID NO:5)	
6	IL13(E13K.R109K)CAR-P140K Full Length (SEQ ID NO:6)	
25	IL13CAR-P140K – IL13 Signal Sequence	1-32
26	IL13CAR-P140K – wt mature IL13	33-146
	Dipeptide linker-1	147-148
27	IL13CAR-P140K – CD8 Hinge region	149-193
	Dipeptide linker-2	194-195
28	IL13CAR-P140K – CD3 zeta transmembrane domain	196-218
	Dipeptide linker-3	219-220
29	IL13CAR-P140K – CD28 costimulatory domain	221-264
	Dipeptide linker-4	265-266
30	IL13CAR-P140K – CD3 zeta signaling domain	267-376
31	5 amino acid linker/reading frame adjust/restriction site	377-381
32	IL13CAR-P140K – 2A self-cleaving peptide	382-399
33	IL13CAR-P140K – P140K MGMT drug resistance protein	400-606
36	Mature IL13 with E13Y mutation	33-146
37	Mature IL13 with E13KR109K	33-146
38	G156A-MGMT	
39	MGMT-2	
40	MGMT-3	
41	MGMT-5	
43	MGMT (GenBank NP_002403-(P140K))	
44	IL13Rα2	
45	IL13(WT)CAR-P140K Full Length (SEQ ID NO:45)	
46	IL13(E13Y)CAR-P140K Full Length (SEQ ID NO:46)	
47	IL13(E13K.R109K)CAR-P140K Full Length (SEQ ID NO:47)	
49	MGMT P140	

The ligand

[0129] The IL13CAR ligand (alternatively, ligand domain) is a peptide, polypeptide or protein which selectively binds to an IL13 receptor, *e.g.,* IL13Rα2, expressed by a diseased cell. The diseased cell may be a tumor cell or other cancerous or malignant cell and the

protein expressed by the diseased cell is alternatively referred to herein as a cancer antigen. In some embodiments, the cancer antigen is a protein which is expressed by none or few (less than 50%, 40%, 30% 20% or 10% as determined by mRNA expression profiling) healthy tissue cells. In one embodiment, the diseased cell is a brain cancer cell such as a glioblastoma cell. In a particular aspect, the cancer antigen-binding domain is all or a biologically active portion of an antibody that binds (*e.g.*, specifically or selectively binds) an IL-13R (*e.g.*, IL13Rα2, e.g., GenBank Acc. No. NP_000631; SEQ ID NO:44). In other aspects, the ligand (cancer antigen-binding domain) is a scFv directed against (binds to; specifically or selectively binds to) an IL-13R (*e.g.*, IL13Rα2).

[0130] A variety of antigens expressed by cancer (*e.g.*, tumor) cells are known in the art. In one aspect, the cancer antigen is a brain tumor antigen *e.g.*, that is expressed on the tumor surface. In a particular aspect, the brain tumor antigen is expressed by a high-grade malignant glioma (*e.g.*, a glioma/malignant brain tumor antigen). Specific examples of high-grade malignant gliomas include glioblastoma multiforme (GBM), anaplastic astrocytoma and pediatric glioma. Specific examples of antigens expressed by tumor cells of high-grade gliomas include EGFRvIII, EphA2, Her-2 and IL-13R (*e.g.*, IL13Rα2).

[0131] In one aspect, the cancer antigen targeted by the methods and compositions of the invention is an IL13 receptor α-2 (IL13Rα2), a glioblastoma multiforme (GBM)-associated protein that is overexpressed on GBM tumors but minimally, or not, expressed in normal brain tissue (Thaci *et al., Neuro-Onco, I6(10):*1304-1312 (2014); Sengupta *et al., Biomed Res Int, 2014:*952128 (2014)). In a one aspect, the IL13CAR expresses or contains a ligand domain which is IL13 and/or an IL13 mutant such as E13K IL13 (SEQ ID NO:36) and/or R109K IL13 (SEQ ID NO:37) that binds to an IL13α2 receptor (Kong *et al., Clin Cancer Res,18(21):*5949-5960 (2012)). The IL13Rα2 protein has also been found to be upregulated breast cancers including breast cancer metastasis (Papageorgis et al., 2015, Breast Canc Res, 17:98-112) in head and neck cancers (Joshi et al., 2000, Cancer Res, 60:1168-1172; Kawakami et al, 2003, 9:6381-6388) and were also shown to promote invasion and metastasis of pancreatic, ovarian, and colorectal cancers (Fujisawa et al., 2009, Int J Cancer, 69:8678-8695; Barderas et al., 2012, Cancer Res, 72:2780-2790). Accordingly, the compositions and methods described herein are useful in a method for treating a subject diagnosed with a malignancy wherein the type of malignancy includes but is not limited to a brain, head and neck, breast, pancreatic, ovarian and colorector cancer.

[0132] A person having ordinary skill in the art can generate ligands which specifically bind the IL13Rα2. For example, routine experimentation is done to generate antibodies which selectively bind IL13Rα2, such as though immunization of a mouse with the extracellular domain of IL13Rα2 or by phage display. The variable domain of the antibodies with desired

selective binding activity can then be used to create a single chain variable domain (scFv). In one embodiment, an scFv which selectively binds IL13Rα2 with the same affinity as wildtype IL13, IL13(E13Y), IL13(R109K) or IL13(E13K.R109K) can be used as a ligand in an IL13CAR construct as disclosed herein.

[0133] In one embodiment, the IL13CAR has an IL13 ligand domain which comprises an IL13 polypeptide which is at least 90%, 95%, 96%, 97%, 98%, 99% or 99.5% identical to SEQ ID NO:26, or functional fragments thereof, wherein: a) the amino acid at position 13 of SEQ ID NO:26 is a glutamate; b) the amino acid at position 13 of SEQ ID NO:26 is a tyrosine; or c) the amino acid at position 13 of SEQ ID NO:26 is a lysine and the amino acid at position 109 of SEQ ID NO:26 is an arginine. In one embodiment, the IL13CAR IL13 ligand domain comprises SEQ ID NO:26, SEQ ID NO:36 or SEQ ID NO:37, or functional fragments thereof.

[0134] A nucleic acid according to the present disclosure encodes the IL13 polypeptide as described above. In one embodiment, the nucleic acid which encodes the IL13 polypeptide is selected from the group consisting of SEQ ID NO:10, SEQ ID NO:34 and SEQ ID NO:35.

[0135] The IL13CAR optionally further comprises a signal (leader) peptide which allows the IL13CAR protein to be processed and displayed on the host cell surface. In one embodiment, the signal peptide is the naturally occurring signal peptide for the ligand protein. For example, the ligand domain comprises the full-length IL13 protein including its signal sequence (a signal sequence comprising or consisting of the amino acid sequence of SEQ ID NO:25). In one embodiment, the signal sequence comprises a sequence which is 95%, 96%, 97%, 98%, 99% or 99.5% identical to SEQ ID NO:25. A nucleic acid according to the present disclosure encodes the signal peptide. In one embodiment, the nucleic acid which encodes the signal peptide consists of SEQ ID NO:9.

[0136] A person having ordinary skill in the art understands that a heterologous signal sequence can be used—a signal peptide from a secreted or transmembrane protein that is not IL13. Signal peptides (signal sequences) are essential parts of membrane-bound or secreted polypeptides which are needed for membrane translocation of the polypeptide and which are processed after or during membrane translocation. A signal sequence has a length of about 13 and 36 amino acids and contains at least one positive residue at the amino-terminal end. The center of the signal sequence is a strongly hydrophobic part of 10 to 15 residues and is described, for example, by Nunnari, j., et al., Curr. Opin. Cell Biol. 4 (1992) 573-580 and by Gilmore, R., et al, Ann. N.Y. Acad. Sci. 674 (1992) 27-37. Some examples of signal peptides include but are not limited to the signal peptides of VHCAMP, CD40, CD40L or TNF-R. The signal peptide is cleaved off on integration into the membrane of the target cell. It is also contemplated that a heterologous signal sequence can be used, such as that of an IgG-like protein.

Hinge region

[0137] The IL13CAR as disclosed herein can comprise a spacer region, also referred to as a hinge region or domain, which is positioned between the IL13 ligand domain (antigen binding domain) and the transmembrane domain. The IL13CAR encompassed by the present disclosure may or may not comprise a hinge region. A hinge region of the presently described CAR is a peptide sequence which is typically flexible enough to allow the antigen binding domain to orient in different directions to facilitate antigen recognition. As will be appreciated by those of skill in the art, other appropriate spacers can be determined. For example, a short oligo- or polypeptide linker, e.g., between 2 and 10 amino acids in length, may form the linkage between the transmembrane domain and the cytoplasmic region of the CAR. Examples of a hinge region is a hinge region from an immunoglobulin (*e.g.*, a hinge region of IgG1), include but are not limited to a hinge region from an immunoglobulin-like protein or domain, the CH2CH3 region of an immunoglobulin, and portions of CD3. In one embodiment, the hinge region comprises an Ig-like domain from the CD8 alpha chain.

[0138] In one embodiment of the IL13CAR, the hinge region comprises or consists of the amino acid sequence of SEQ ID NO:27. Alternatively, the hinge region comprises or consists of a sequence which is 95%, 96%, 97%, 98%, 99% or 99.5% identical to SEQ ID NO:27. A nucleic acid according to the present disclosure encodes the hinge region. In one embodiment, the nucleic acid which encodes the hinge region comprises SEQ ID NO:12. In one embodiment, the nucleic acid encoding the IL13CAR comprises a nucleic acid which is at least 90%, 95%, 96%, 97%, 98%, 99% or 99.5% identical to SEQ ID NO: 12.

Transmembrane domain

[0139] As described herein, the CAR comprises one or more transmembrane domains. Typically, the transmembrane domain is a hydrophobic region (*e.g.*, a hydrophobic alpha helix) that spans the membrane. Any of a variety of transmembrane domains can be used. Examples of suitable transmembrane domains include, but are not limited to, a CD3 (*e.g.*,, a CD3-zeta transmembrane domain) or a CD28 transmembrane domain. The transmembrane domain may be derived either from a natural or from a recombinant source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. In one aspect the transmembrane domain is capable of signaling to the intracellular domain(s) whenever the CAR has bound to a target. A transmembrane domain of particular use in this invention may include at least the transmembrane region(s) of e.g., the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154.

[0140] In one embodiment, the transmembrane domain of the IL13CAR comprises or consists of at least a portion of the human CD3 zeta chain, for example, SEQ ID NO:28. In one

embodiment, the transmembrane domain comprises or consists of a sequence which is 95%, 96%, 97%, 98%, 99% or 99.5% identical to SEQ ID NO:28. A nucleic acid according to the present disclosure encodes the transmembrane domain. In one embodiment, the nucleic acid which encodes the transmembrane domain comprises SEQ ID NO:14, or a nucleic acid which is at least 90%, 95%, 96%, 97%, 98%, 99% or 99.5% identical to SEQ ID NO: 14.

Cytoplasmic Signaling Domain(s)

[0141] As described herein, the IL13CAR has a cytoplasmic domain which transmits an activation and/or a stimulatory signal to the T cell after antigen is bound (*e.g.*, leading to the activation, initiation, expansion, persistence and/or amplification of the T cells and the T cell response (*e.g.*, the signal required for cytotoxicity) against tumor antigen). The cytoplasmic domain comprises one or more signaling domains (*e.g.*, co-stimulatory domains) and/or one or more activation domains of, or associated with, a T cell receptor. Examples of suitable cytoplasmic signaling domains include but are not limited to those of CD3-ζ (CD3-zeta) which contains an immunoreceptor tyrosine-based activation motif (ITAMs), FcεRIγ, CD28 (*e.g.*, chimeric CD28), 4-IBB (CD137), DAP10, OX40 (CD134), CD4, CD27, CD244, inducible T-cell co-stimulator (ICOS), leukocyte C-terminal SRC kinase (LCK), and CD137 (*e.g.*, Sadelain *et al., Cancer Discov, 3(4):*388-398 (2013); Lee *et al., Clin Cancer Res, 18(10):*2780-2790 (2012)). The presence of the one or more these cytoplasmic domains can initiate pathways by the association of ZAP70, TNF receptor-associated factor 1 (TRAF1), PI3K and growth factor receptor-bound protein 2 (GRB2) with elements in the cytoplasmic domain of the CARs, leading to the triggering of signaling intermediates and gene transcription.

[0142] "Co-stimulatory domain" or “co-stimulatory signaling domain” refers to the portion of the CAR comprising the intracellular domain of a co-stimulatory molecule. Co-stimulatory molecules are cell surface molecules other than antigen receptors or Fc receptors that provide a second signal required for efficient activation and function of T lymphocytes upon binding to antigen. A co-stimulatory domain undergoes a conformational change that leads to an activation signal to the cell through, for example, the CD3-zeta signaling domain. 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, M 1CB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. The inclusion of one or more co-stimulatory domains within the IL13CAR may enhance the efficacy and expansion of T cells expressing IL13CARs. In one embodiment, a co-stimulatory domain 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-IBB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated

antigen-1 (LFA-1), CD2, CD7, LTGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83.

[0143] As will be apparent to those of skill in the art, the IL13CAR can have any number of activation domains and/or stimulatory domains. Each of the signaling domains is linked via a peptide bond to form the cytoplasmic domain of the IL13CAR construct. In one aspect, the IL13CAR has an activation domain and a stimulatory domain. In another aspect, the IL13CAR has one, two three, four five, *etc.* activation domains and one two, three, four, five, *etc.* stimulatory domains.

[0144] In one embodiment, the IL13CAR of the present disclosure comprises the signaling domains of the human CD28 protein and/or of the human CD3-zeta chain. In one embodiment, IL13CAR comprises both a CD28 and a CD3-zeta chain signaling domain, wherein the CD28 co-stimulatory domain is N-terminal to the CD3-zeta signaling domain. Also contemplated is a CAR cytoplasmic domain in which the CD3-zeta domain is N-terminal to the CD28 co-stimulatory domain. As described herein, the CD28 co-stimulatory domain comprises the sequence of SEQ ID NO:29. The CD3-zeta signaling domain comprises or consists of the sequence of SEQ ID NO:30. A nucleic acid according to the present disclosure encodes the CD28 co-stimulatory domain. In one embodiment, the nucleic acid which encodes the CD28 co-stimulatory domain consists of SEQ ID NO:16. A nucleic acid according to the present disclosure encodes the CD3-zeta signaling domain. In one embodiment, the nucleic acid which encodes the CD3-zeta signaling domain consists of SEQ ID NO:18. Each of these signaling domains may contain one or more deletions, insertions, or point mutations (natural or artificial) wherein the signaling function of each domain is about the same as the signaling function of the protein that does not contain the one or more deletions, insertions, or point mutations.

[0145] In one embodiment, the cytoplasmic domain of the IL13CAR is encoded by a nucleic acid sequence comprising nucleotides 673-1140 of SEQ ID NO:1, or nucleotides 673-1143 of SEQ ID NO:1. In another embodiment, the IL13CAR comprises a cytoplasmic domain comprising amino acid residues 221-376 of SEQ ID NO:4.

[0146] FIG. 1A illustrates an embodiment of the IL13CAR construct in which the IL13 ligand is the variant containing the E13K and R109K substitutions.

Drug Resistant Polypeptide

[0147] The present disclosure is directed, at least in part, to a nucleic acid encoding both a CAR protein selective for a brain cancer cell as detailed above and a drug-resistance polypeptide, wherein expression of both the CAR and the drug resistance polypeptide is useful for treating a cancer. For example, the CAR and drug resistance polypeptide can be expressed in cells administered to a subject being treated with a chemotherapeutic agent

and/or an agent that enhances cytotoxicity of a chemotherapeutic agent. Examples of chemotherapeutic agents include 1, 3-bis(2-chloroethyl)-1-nitrosurea (BCNU or carmustine), fotemustine, lomustine and Temozolomide (TMZ) commonly used to treat malignant glioma (*e.g.*, glioblastoma multiforme (GBM)). The cytotoxic action of some chemotherapeutic agents involves formation of an O^6-methylguanine lesion that is capable of rearranging to form a lethal intrastrand crosslink. The effectiveness of these methylating agents is limited, however, by tumor overexpression of the DNA repair protein, O^6-methylguanine DNA methyltransferase (MGMT), a protein which removes cytotoxic O^6-alkylguanine adducts from DNA of treated cells. Tumor cells expressing high levels of MGMT are therefore partially or completely resistant to killing by TMZ chemotherapy. One means to prevent the reduced efficacy of a methylating agent is to treat a subject undergoing TMZ chemotherapy with an inhibitor of MGMT, specifically O^6-benzylguanine. However, dosing of O^6-benzylguanine is limited due to its toxic effect on hematopoietic cells.

[0148] Temozolomide (TMZ) is an anti-glioma chemotherapeutic drug that has cytotoxic effects on hematopoietic cells including T cells. The standard dosage of TMZ as directed by the FDA, and hence an unavoidable step in anti-glioma treatment, kills the T cells by methylating their DNA, very much in the same fashion that TMZ destroys tumor cells (*Sengupta et al., Clin Dev Immunol*, 831090 (2012)). However, it has been shown that over-expression of wildtype MGMT or expression of one or more MGMT mutants (e.g., G156A; P140K) in a cell will confer protection to that cell against a methylating agent such as TMZ (Woolford *et al.*, *J Gene Med*, *8(1)*:29-31 (2006)). Accordingly, the drug resistance polypeptide encoded by the above-described nucleic acid is MGMT or an MGMT variant which confers resistance to TMZ. These TMZ resistant variants include but are not limited to P140K-MGMT (SEQ ID NO:33), P140K-MGMT (SEQ ID NO:43), G156A-MGMT (SEQ ID NO:38), MGMT-2 (SEQ ID NO:39), MGMT-3 (SEQ ID NO:40) and MGMT-5 (SEQ ID NO:41) (Fontes et al., Mol Cancer Ther, 5(1):121-128). The indicated positions (locations) of amino acid substitutions in the MGMT variants described throughout (e.g., P140K and G156A) are amino acid positions based on the sequence of SEQ ID NO:33. The MGMT-2 variant has the substitutions: S152H, A154G, Y158H, G160S and L162V. The MGMT-3 variant has the substitutions: C150Y, A154G, Y158F, L162P and K165R. The MGMT-5 variant has the substitutions: N157T, Y158H and A170S.

[0149] Particularly preferred for the constructs disclosed and used herein is the P140K-MGMT variant. A nucleic acid according to the present disclosure encodes the MGMT variant. In one embodiment, the nucleic acid which encodes P140K-MGMT consists of SEQ ID NO:22. Also contemplated is a nucleic acid which encodes G156A-MGMT (SEQ ID NO:38), MGMT-2 (SEQ ID NO:39), MGMT-3 (SEQ ID NO:40) or MGMT-5 (SEQ ID NO:41), each of which may

be present in an IL13CAR-MGMT construct. Also contemplated is an MGMT protein sequence comprising SEQ ID NO:43 (GenBank Acc. No. NP_002403 having the P140K point mutation) and any point mutations corresponding to the mutations of G156A-MGMT (SEQ ID NO:38), MGMT-2 (SEQ ID NO:39), MGMT-3 (SEQ ID NO:40) or MGMT-5 (SEQ ID NO:41).

[0150] The MGMT or variant thereof encoded by the chimeric nucleic acid sequence disclosed herein can be located downstream or upstream of the portion of the nucleic acid sequence encoding IL13CAR protein. In one embodiment, this chimeric nucleic acid sequence is monocistronic in which the construct comprises a single promoter sequence to drive transcription of a single transcript which in turn is translated into a single protein that is later cleaved. Use of this monocistronic construct requires a self-cleaving element positioned between the IL13CAR and MGMT proteins. For example, the chimeric nucleic acid sequence, when expressed in a host cell, initially gives rise to a single protein comprising, in an N-terminal to C-terminal direction, a CAR (e.g. the IL13 CAR described above), a self-cleaving peptide, and the MGMT protein or MGMT variant described herein. This protein is then cleaved to produce individual CAR and MGMT proteins. The CAR protein is processed and displayed on the cell surface while the MGMT protein can be retained within the cell nucleus. An alternative construct comprises in an N-terminal to C-terminal direction, an MGMT protein, a self-cleaving protein, and a CAR.

[0151] In one embodiment, the CAR and MGMT polypeptide portions are separated by a self-cleaving peptide. One example of a self-cleaving sequence is a 2A element which includes the 2A sequence from foot-and-mouth disease virus. In an exemplary embodiment, the self-cleaving sequence comprises or consists of the sequence of SEQ ID NO:32.

[0152] In an alternative embodiment, the nucleic acid sequence encoding both a CAR as described above and a MGMT or variant thereof as described above, is polycistronic, wherein it comprises a nucleic acid sequence encoding the CAR and a nucleic acid sequence encoding a MGMT or variant thereof, separated by a non-protein coding sequence such as an internal ribosome entry site (IRES). Examples of IRES sequences that can be used include, without limitation, the IRES elements of encephalomyelitis virus (EMCV), foot-and-mouth disease virus (FMDV), Theiler's murine encephalomyelitis virus (TMEV), human rhinovirus (HRV), coxsackievirus (CSV), poliovirus (POLIO), Hepatitis A virus (HAV), Hepatitis C virus (HCV), and Pestiviruses (e.g., hog cholera virus (HOCV) and bovine viral diarrhea virus (BVDV)) (see, e.g., Le et al., Virus Genes 12:135-147, 1996; and Le et al., Nuc. Acids Res. 25:362-369, 1997, each of which is incorporated by reference in their entirety).

[0153] An alternative embodiment of a polycistronic chimeric nucleic acid is one in which a second promoter is positioned between the nucleic acid sequence encoding the IL13CAR

and the nucleic acid sequence encoding the MGMT or variant thereof. In this embodiment, a nucleic acid sequence encoding a self-cleaving peptide is not present.

[0154] It is also contemplated that the nucleic acids encoding the IL13CAR and MGMT variants are each within an individual nucleic acid vector. I.e., also contemplated is a system or kit which comprises a first vector comprising a nucleic acid encoding the IL13CAR as described herein and a second vector encoding an MGMT protein as described herein.

[0155] As will be appreciated by those of skill in the art, the nucleic acid sequences encoding at least the IL13CAR and MGMT proteins or variants thereof can further comprise additional components to facilitate and/or enhance the expression and function of the CAR and/or MGMT in a host cell. For example, the CAR can further comprise a (one or more) sequence that initiates translation (*e.g.*, a Kozak sequence and/or a promoter sequence) as well as sequences for homologous recombination (retroviral 5'LTR; retrovial 3'LTR). A Kozak sequence may be used in the chimeric nucleic acid construct which comprises the nucleic acid sequence encoding the IL13CAR-2A-MGMT constructs as described herein.

[0156] A chimeric nucleic acid sequence for use according to the present disclosure is generated by ligating or linking together the following elements in a 5' to 3' direction: a Kozak sequence, a nucleic acid encoding a signal sequence, a nucleic acid encoding an IL13 ligand, a nucleic acid encoding a hinge region, a nucleic acid encoding a transmembrane domain, a nucleic acid encoding a CD28 costimulatory (signaling) domain, a nucleic acid encoding a CD3-zeta signaling domain, a nucleic acid encoding a self-cleaving peptide (e.g., the 2A peptide), and a nucleic acid encoding an MGMT protein, each of which was described above. The chimeric nucleic acid sequence can be generated by synthesizing a single sequence which includes and encodes the above elements. Alternatively, each of the elements above can be generated individually using methods known to the ordinarily skilled artisan. For example, each element was amplified using PCR wherein the PCR was designed to generate restriction endonuclease sites on the 5' and 3' ends of each element as needed, and the individual elements were digested with the appropriate endonucleases and ligated together to obtained the desired construct(s). As a result of this method for generating the IL13CAR-P140KMGMT constructs, short linker peptides are present between individual elements.

[0157] For example, a nucleic acid encoding 2, 3, 4, 5 or 6 amino acids can be positioned between the ligand domain and the hinge region, between the hinge region and the transmembrane domain, between the transmembrane domain and the CD28 co-stimulatory domain, between the CD28 co-stimulatory domain and the CD3-zeta signaling domain, between the CD3-zeta signaling domain and/or the self-cleaving peptide.

[0158] In an exemplary embodiment, the IL13CAR comprises a linker between the ligand and hinge domain consisting of 2 amino acids, proline-arginine. The linker between the

hinge region and the transmembrane domain is glutamine-lysine. The linker between the transmembrane domain and the CD28 co-stimulatory domain is valine-threonine. The linker between the CD28 co-stimulatory domain and the CD3-zeta signaling domain is threonine-arginine. The linker between the CD3-zeta signaling domain and the 2A peptide is glutamine-proline-alanine-alanine-alanine. It is contemplated that each of the linkers described above may or may not be present in the IL13 CAR protein independent of the others.

[0159] Accordingly, preferred embodiments of the IL13CAR-P140KMGMT constructs for use in transfecting a host cell such as a T-cell and for use in inhibiting or preventing growth of a cell expressing IL13Rα2 and which is exposed to TMZ, include but are not limited to IL13(WT)CAR-P140KMGMT (nucleotide sequence of SEQ ID NO:1; amino acid sequence of SEQ ID NO:4); IL13(E13Y)CAR-P140KMGMT (nucleotide sequence of SEQ ID NO:2; amino acid sequence of SEQ ID NO:5); and IL13(E13K.R109K)CAR-P140KMGMT (nucleotide sequence of SEQ ID NO:3; amino acid sequence of SEQ ID NO:6). FIG. 2A provides a schematic of the IL13(E13K.R109K)CAR-P140KMGMT construct. Additional embodiments of these IL13CAR-P140KMGMT constructs include but are not limited to those encoding the proteins IL13(WT)CAR-P140KMGMT (SEQ ID NO:45), IL13(E13Y)CAR-PAR140KMGMT (SEQ ID NO:46), and IL13)E13K.R109K)CAR-P140KMGMT (SEQ ID NO:47).

[0160] As will be appreciated by those of skill in the art, each of the nucleic acid sequences which encode for a signal peptide, an IL13 ligand, a hinge region, a transmembrane domain, and CD28 co-stimulatory domain, a CD3-zeta signaling domain, a self-cleaving peptide linker and an MGMT protein or variant thereof as disclosed herein can vary due to codon degeneracy without affecting the encoded protein. Moreover, the polypeptide sequences can also vary, such as through conservative amino acid substitutions, while not significantly affecting the function of the explicitly described proteins. Accordingly, also contemplated herein are variants of the each of the nucleotide sequences described herein such that the nucleic acid sequence of the present disclosure comprises sequences which are at least 75%, 80%, 82%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% identical to each of SEQ ID NOS:1-3, 7-24 and 34-35). Similarly, also contemplated and disclosed herein are polypeptides which are at least 75%, 80%, 82%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% identical to each of SEQ ID NOS:4-6, 25-33 and 36-41 and 43.

[0161] The percent identity of two nucleotide or amino acid sequences can be determined by aligning the sequences for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first sequence). The nucleotides or amino acids at corresponding positions are then compared, and the percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., %

identity = # of identical positions/total # of positions x 100). In certain embodiments, the length of the amino acid or nucleotide sequence aligned for comparison purposes is at least 30%, 40%, 50%, 60%,70%, 80%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the length of the reference sequence, for example, those sequences provided in FIGS. 7A (SEQ ID NO:1), 7B (SEQ ID NO:4), 8A (SEQ ID NO:2), 8B (SEQ ID NO:5), 9A (SEQ ID NO:3) and 9B (SEQ ID NO:6). The actual comparison of the two sequences can be accomplished by well-known methods, for example, using a mathematical algorithm. A preferred, non-limiting example of such a mathematical algorithm is described in Karlin et al., Proc. Natl. Acad. Sci. USA, 90:5873-5877 (1993). Such an algorithm is incorporated into the BLASTN and BLASTX programs (version 2.2) as described in Schaffer et al., Nucleic Acids Res., 29:2994-3005 (2001). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., BLASTN) can be used. In one embodiment, the database searched is a non-redundant (NR) database, and parameters for sequence comparison can be set at: no filters; Expect value of 10; Word Size of 3; the Matrix is BLOSUM62; and Gap Costs have an Existence of 11 and an Extension of 1. In another embodiment, the percent identity between two polypeptides or two polynucleotides is determined over the full-length of the polypeptide or polynucleotide of interest.

[0162] Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part of the GCG sequence alignment software package (Accelrys, San Diego, California). When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12 , and a gap penalty of 4 can be used. Additional algorithms for sequence analysis are known in the art and include ADVANCE and ADAM as described in Torellis and Robotti, Comput. Appl. Biosci., 10: 3-5 (1994); and FASTA described in Pearson and Lipman, Proc. Natl. Acad. Sci USA, 85: 2444-8 (1988).

[0163] In another embodiment, the percent identity between two amino acid sequences can be accomplished using the GAP program in the GCG software package using either a Blossom 63 matrix or a PAM250 matrix, and a gap weight of 12, 10, 8, 6, or 4 and a length weight of 2, 3, or 4. In yet another embodiment, the percent identity between two nucleic acid sequences can be accomplished using the GAP program in the GCG software package, using a gap weight of 50 and a length weight of 3.

[0164] Similarity between polypeptides is typically determined by conserved amino acid substitution. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Conservative substitutions are likely to be phenotypically silent. Typically seen as conservative substitutions are the replacements, one

for another, among the aliphatic amino acids Ala, Val, Leu, and Ile; interchange of the hydroxyl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitution between the amide residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among the aromatic residues Phe and Tyr. Guidance concerning which amino acid changes are likely to be phenotypically silent are found in Bowie et al., Science 247: 1306-1310 (1990).

[0165] A variant polypeptide can differ in amino acid sequence by one or more substitutions, deletions, insertions, inversions, fusions, and truncations or a combination of any of these. Further, variant polypeptides can be fully functional (*e.g.*, ability to infect cells and produce progeny virus) or can lack function in one or more activities (*e.g.*, ability to produce progeny virus). Fully functional variants typically contain only conservative variation or variation in non-critical residues or in non-critical regions. Functional variants can also contain substitution of similar amino acids that result in no change or an insignificant change in function. Alternatively, such substitutions may positively or negatively affect function to some degree. Non-functional variants typically contain one or more non-conservative amino acid substitutions, deletions, insertions, inversions, or truncations or a substitution, insertion, inversion, or deletion in a critical residue or critical region.

[0166] Amino acids that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham et al., Science, 244: 1081-1085 (1989)). The latter procedure introduces a single alanine mutation at each of the residues in the molecule (one mutation per molecule). The resulting mutant molecules are then tested for biological activity *in vitro*. Sites that are critical for polypeptide activity can also be determined by structural analysis, such as crystallization, nuclear magnetic resonance, or photoaffinity labeling (See Smith et al., J. Mol. Biol., 224: 899-904 (1992); and de Vos et al. Science, 255: 306-312 (1992)).

[0167] Further disclosed herein are compositions which comprise a substantially pure polypeptide comprising or consisting of a IL13CAR protein described herein as the sequence of amino acids 1-146 of SEQ ID NO:4, 5 or 6 and a polypeptide having preferably at least 75%, 80%, 82%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to the sequence of amino acids 1-146 of SEQ ID NO:4, 5 or 6, and a P140KMGMT variant described herein as the sequence of amino acids 400-606 of SEQ ID NO:1 and a polypeptide having preferably at least 75%, 80%, 82%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to the sequence of amino acids 400-606 of SEQ ID NO:1 as determined using the BLAST program and parameters described herein. In another embodiment, examples of polypeptides include a substantially pure polypeptide comprising or consisting of SEQ ID NOs: 4, 5 and/or 6; and a polypeptide having preferably at least 75%,

80%, 82%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% sequence similarity to SEQ ID NO:4, as determined using the BLAST program and parameters described herein.

[0168] In particular aspects, the disclosure is directed to an isolated polypeptide encoded by SEQ ID NO: 1 (IL13 CAR-P140KMGMT), SEQ ID NO: 2 (IL-13(E13Y) CAR-P140KMGMT), SEQ ID NO: 3 (IL-13(E13K R109K) CAR-P140KMGMT) or a combination thereof. In other aspects, the disclosure is directed to a polypeptide (an isolated polypeptide comprising an amino acid sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47 or a combination thereof.

[0169] A CAR polypeptide comprising a T cell receptor comprising one or more ligands (e.g., an antibody) to one or tumor antigens of a brain cancer is also contemplated. In a particular aspect, the CAR polypeptide further comprises one or more additional agents useful for treating a brain cancer. In other aspects, the invention is directed to isolated polypeptides, and fragments, derivatives, and variants thereof, as well as polypeptides encoded by nucleotide sequences described herein (e.g., other variants). As used herein, the term "polypeptide" refers to a polymer of amino acids, and not to a specific length; thus, peptides, oligopeptides, and proteins are included within the definition of a polypeptide.

[0170] The polypeptides can be synthesized using known protein synthesis methods. In one embodiment, the polypeptide is produced by recombinant DNA and recombinant protein expression and purification techniques. For example, a nucleic acid molecule encoding the polypeptide is cloned into an expression vector, the expression vector is introduced into a host cell, the polypeptide is expressed in the host cell and the desired protein is purified and formulated for packaging and administration.

[0171] As used herein, a polypeptide is said to be "isolated," "substantially pure," or "substantially pure and isolated" when it is substantially free of material, when it is isolated from recombinant or non-recombinant cells, or free of chemical precursors or other chemicals when it is chemically synthesized. In addition, a polypeptide can be joined to another polypeptide with which it is not normally associated in a cell (e.g., in a "fusion protein") and still be "isolated," "substantially pure," or "substantially pure and isolated." An isolated, substantially pure, or substantially pure and isolated polypeptide may be obtained, for example, using affinity purification techniques described herein, as well as other techniques described herein and known to those skilled in the art.

[0172] A polypeptide of the invention can be purified to homogeneity. It is understood, however, that preparations in which the polypeptide is not purified to homogeneity are useful. The critical feature is that the preparation allows for the desired function of the polypeptide, even in the presence of considerable amounts of other components. Thus, the invention encompasses various degrees of purity. In one embodiment, the language "substantially free

of material" includes preparations of the polypeptide having less than about 30% (by dry weight) other proteins (i.e., contaminating protein), less than about 20% other proteins, less than about 10% other proteins, less than about 5%, or less than about 1% other proteins.

[0173] When a polypeptide is recombinantly produced, it can also be substantially free of culture medium, i.e., culture medium represents less than about 20%, less than about 10%, or less than about 5% of the volume of the polypeptide preparation. The language "substantially free of chemical precursors or other chemicals" includes preparations of the polypeptide in which it is separated from chemical precursors or other chemicals that are involved in its synthesis. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of the polypeptide having less than about 30% (by dry weight) chemical precursors or other chemicals, less than about 20% chemical precursors or other chemicals, less than about 10% chemical precursors or other chemicals, or less than about 5% chemical precursors or other chemicals.

[0174] In one embodiment, a polypeptide of the invention comprises an amino acid sequence encoded by a nucleic acid molecule of SEQ ID NOs: 1, 3 and/or 5 and complements and portions thereof. The polypeptides of the invention also encompasses fragments and sequence variants having substantial homology to a polypeptide encoded by a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NOs: 1, 3, and/or 5 and complements and portions thereof.

Nucleic Acid Expression Constructs

[0175] Another aspect of the disclosure pertains to nucleic acid expression constructs or vectors, retroviral vectors and/or retroviral particles and their use. Recombinant DNA technology methods known to the ordinarily skilled artisan are used to design and generate the chimeric nucleic acid sequences which encode an IL13CAR, self-cleaving peptide, and an MGMT variant as described in detail herein. The chimeric nucleic acid construct is then cloned into a plasmid vector to allow, for example, sequencing to confirm the sequence of the construct. Once the desired sequence is confirmed, the chimeric nucleic acid construct is used to produce retroviral particles for transfection of a mammalian cell. Accordingly, chimeric constructs comprising nucleic acid sequences of SEQ ID NOS:1-3 and combinations of SEQ ID NOS:7-24, 34 and/or 34-35 as described herein are cloned into such plasmid vectors for later packaging into retroviral particles. In a particular aspect, the chimeric constructs and plasmid vectors comprise one or more nucleic acid sequences comprising SEQ ID NO:1, SEQ ID NO:2 and/or SEQ ID NO:3 as described above. A plasmid vector containing the IL13(WT)CAR-P140KMGMT sequence (SEQ ID NO:1), the IL13(E13Y)CAR-P140KMGMT (SEQ ID NO:2), or IL13(E13K.R109K)CAR-2A-P140KMGMT sequence (SEQ ID NO:3) was

generated by cloning fragments described herein into a pUC57 vector then into a pMFG vector as described in Example 1 prior to producing retroviral particles (Example 2).

[0176] FIG. 1B illustrates a plasmid vector comprising an IL13CAR construct wherein the MGMT gene has not yet been introduced. FIG. 2B illustrates the same plasmid backbone containing an IL13CAR construct and also containing an P140KMGMT coding sequence downstream of the IL13CAR coding sequence.

[0177] In an alternative embodiment, the nucleic acid encoding an IL13CAR as described herein is cloned into a first vector and the nucleic acid encoding an MGMT protein as described herein is cloned into a second vector. Accordingly, the present disclosure also describes a first vector (plasmid, expression, viral, retroviral, lentiviral, adenoviral, etc.) comprising a nucleic acid encoding an IL13CAR as described herein and a second vector (plasmid, expression, viral, retroviral, lentiviral, adenoviral, etc.) comprising a nucleic acid encoding an MGMT protein as described herein.

[0178] Examples of suitable nucleic acid constructs include a plasmid (*e.g.*, a circular double stranded DNA loop) and a viral vector (*e.g.*, a retroviral vector, a lentiviral vector, an adenoviral vector). Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors, expression vectors, are capable of directing the expression of nucleic acid to which they are operably linked. In general, expression constructs in recombinant DNA techniques are often in the form of plasmids. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses) that serve equivalent functions.

[0179] Preferred recombinant expression vectors of the invention comprise a nucleic acid molecule of the invention in a form suitable for expression of the nucleic acid molecule in a host cell. This means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operably linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, “operably linked” is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner that allows for expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). The term “regulatory sequence” is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel, Gene

Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences).

[0180] It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed and the level of expression of polypeptide desired. The expression vectors of the invention can be introduced into host cells to thereby produce polypeptides, including fusion polypeptides, encoded by nucleic acid molecules as described herein.

[0181] The recombinant expression vectors of the invention can be designed for expression of a polypeptide of the invention in prokaryotic or eukaryotic cells, e.g., bacterial cells, such as *E. coli*, insect cells (using baculovirus expression vectors), yeast cells or mammalian cells (primate (*e.g.*, human), murine (*e.g.*, mouse), feline, canine, rodent, ovine, bovine cells).

[0182] Production of retroviral particles containing the chimeric nucleic acid constructs were successfully generated to contain the chimeric nucleic acid sequence encoding IL13(E13K.R109K)CAR-P140KMGMT as described in Examples 1 and 2. The methods described in the examples and throughout the specification and in combination with methods known to the ordinarily skilled artisan can be used to produce retroviral particles containing any of the chimeric nucleic acid constructs as described herein. Transfection efficiency through the process can be monitored by measuring cell surface expression of the IL13 ligand by flow cytometry. Cells transduced with the IL13CAR-P140KMGMT construct can be enriched, for example, using a fluorescence activated cell sorter. FIGS. 3A and 3B show enrichment of PG13 cells transfected with the IL13(E13K.R109K)CAR-A2-P140KMGMT construct. FIG. 3A shows the relative number of cells expressing IL13 after the first transduction with the ecotropic retrovirus (e.g., 4.0%). FIG. 3B shows the relative number of cells expressing IL13 after the enrichment of cells transduced with the ecotropic retrovirus (e.g., 96.6%). Western blot analysis of cell lysates can be performed using routine methods to confirm and measure expression of the P140KMGMT protein by the host cell. As shown in FIG. 3C, enrichment of cells transduced with an IL13CAR-A2-P140KMGMT construct overexpresses the P140KMGMT protein as compared to untransduced cells or cells transduced with a construct only expressing an IL13CAR construct as depicted in FIGS. 1A-1B.

[0183] FIGS. 1A and 2A provide schematics of the IL13(E13K.R109K)CAR-P140KMGMT cloned into a plasmid vector while FIGS. 1B and 2B show a linear depiction of the chimera flanked by the 5' LTR and 3'LTR for integration into the host cell genome.

[0184] The nucleic acid constructs described herein are introduced into host mammalian cells to impart to the cell both a tumor cell killing function and resistance to a chemotherapeutic drug which is a DNA methylating agent such as TMZ. Introduction of the nucleic acid into the mammalian host cell is accomplished, for example, using a retroviral vector, for example, as described in Example 3. Retroviral vectors for transiently or stably transducing mammalian cells are well known in the art and described below as they are used in the presently described protein expression and therapeutic systems.

[0185] Certain embodiments employ viral vectors to transduce plasma cells such as T cells with the expression systems described herein. Examples of viral vectors include, without limitation, MFG vectors, adenovirus-based vectors, adeno-associated virus (AAV)-based vectors, retroviral vectors, retroviral-adenoviral vectors, and vectors derived from herpes simplex viruses (HSVs).

[0186] Typically, a minimal retroviral vector comprises certain 5'LTR and 3'LTR sequences, one or more genes of interest (to be expressed in the target cell), one or more promoters, and a cis-acting sequence for packaging of the RNA. Other regulatory sequences can be included, as described herein and known in the art. The viral vector is typically cloned into a plasmid that may be transfected into a packaging cell line, such as a eukaryotic cell (e.g., PG13 mouse fibroblast), and also typically comprises sequences useful for replication of the plasmid in bacteria. Certain viral vectors such as retroviral vectors employ one or more heterologous promoters, enhancers, or both. Certain embodiments employ an "internal" promoter/enhancer that is located between the 5' LTR and 3' LTR sequences of the viral vector, and is operably linked to the gene of interest. A "functional relationship" and "operably linked" mean, without limitation, that the gene is in the correct location and orientation with respect to the promoter and/or enhancer, such that expression of the gene will be affected when the promoter and/or enhancer is contacted with the appropriate regulatory molecules. Any enhancer/promoter combination may be used that either regulates (e.g., increases, decreases) expression of the viral RNA genome in the packaging cell line, regulates expression of the selected gene of interest in an infected target cell, or both.

[0187] A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoters are untranslated sequences that are located upstream (5') of the start codon of a selected gene of interest (typically within about 100 to 1000 bp) and control the transcription and translation of the coding polynucleotide sequence to which they are operably linked. Promoters may be inducible or constitutive. Inducible promoters initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, such as a change in temperature.

[0188] A variety of promoters are known in the art, as are methods for operably linking the promoter to the polynucleotide coding sequence. Both native promoter sequences and many heterologous promoters may be used to direct expression of the selected gene of interest. Certain embodiments employ heterologous promoters, because they generally permit greater transcription and higher yields of the desired protein as compared to the native promoter.

[0189] Certain viral vectors contain cis-acting packaging sequences to promote incorporation of the genomic viral RNA into the viral particle. Examples include psi-sequences. Such cis-acting sequences are known in the art.

[0190] Generation of viral vectors can be accomplished using any suitable genetic engineering techniques known in the art, including, without limitation, the standard techniques of restriction endonuclease digestion, ligation, transformation, plasmid purification, PCR amplification, and DNA sequencing, for example as described in Sambrook et al. (Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, N.Y. (1989)), Coffin et al. (Retroviruses. Cold Spring Harbor Laboratory Press, N.Y. (1997)) and "RNA Viruses: A Practical Approach" (Alan J. Cann, Ed., Oxford University Press, (2000)).

[0191] Any variety of methods known in the art may be used to produce suitable retroviral particles whose genome comprises an RNA copy of the viral vector. As one method, the viral vector may be introduced into a packaging cell line that packages the viral genomic RNA based on the viral vector into viral particles with a desired target cell specificity. The packaging cell line typically provides in trans the viral proteins that are required for packaging the viral genomic RNA into viral particles and infecting the target cell, including the structural gag proteins, the enzymatic pal proteins, and the envelope glycoproteins.

[0192] In certain embodiments, the packaging cell line may stably express certain of the necessary or desired viral proteins (e.g., gag, pol) (see, e.g., U.S. Pat. No. 6,218,181). In certain embodiments, the packaging cell line may be transiently transfected with plasmids that encode certain of the necessary or desired viral proteins (e.g., gag, pol, glycoprotein), including the measles virus glycoprotein sequences described herein. In one exemplary embodiment, the packaging cell line stably expresses the gag and pol sequences, and the cell line is then transfected with a plasmid encoding the viral vector and a plasmid encoding the glycoprotein. Following introduction of the desired plasmids, viral particles are collected and processed accordingly, such as by ultracentrifugation to achieve a concentrated stock of viral particles. Exemplary packaging cell lines include PG13 (ATCC CRL-10686), 293 (ATCC CCL X), HeLa (ATCC CCL 2), D17 (ATCC CCL 183), MDCK (ATCC CCL 34), BHK (ATCC CCL-10) and Cf2Th (ATCC CRL 1430) cell lines.

Host Cells

[0193] Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but also to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0194] A host cell can be any prokaryotic or eukaryotic cell. For example, a nucleic acid molecule of the invention can be expressed in bacterial cells (e.g., E. coli), insect cells, yeast, or mammalian cells. In one aspect, the host cell is a mammalian cell (primate (*e.g.*, human), murine (*e.g.*, mouse), feline, canine, rodent, ovine, bovine cells). In a particular aspect, the mammalian cell is an immune cell. In yet another aspect, the mammalian cell is a T cell. Other suitable host cells are apparent to those skilled in the art.

[0195] For purposes herein, the T cell can be any T cell, such as a cultured T cell, e.g., a primary T cell, or a T cell from a cultured T cell line, e.g., Jurkat, SupT1, etc., or a T cell obtained from a mammal. If obtained from a mammal, the T cell can be obtained from numerous sources, including but not limited to blood, bone marrow, lymph node, the thymus, or other tissues or fluids. T cells can also be enriched for or purified. The T cell may be a human T cell. The T cell may be a T cell isolated from a human. The T cell can be any type of T cell and can be of any developmental stage, including but not limited to, $CD4^+/CD8^+$ double positive T cells, $CD4^+$ helper T cells, e.g., Th_1 and Th_2 cells, $CD8^+$ T cells (e.g., cytotoxic T cells), peripheral blood mononuclear cells (PBMCs), peripheral blood leukocytes (PBLs), tumor infiltrating cells, memory T cells, naive T cells, and the like. The T cell may be a CD8+ T cell or a $CD4^+$ T cell.

[0196] In one embodiment, the host cell used in the compositions and methods of the invention is an NK-92 cell (NK-92 cell line ATCC Deposit No. PTA-6672).

[0197] Nucleic acid constructs can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "infection", "transformation", "transduction", and "transfection" are intended to refer to a variety of art-recognized techniques for introducing a foreign nucleic acid molecule (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in, for example, Sambrook et al., Molecular Cloning, A Laboratory Manual (2nd Ed., CSHP, New York (1989) and other laboratory manuals.

[0198] A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (express) one or more CARs of the present disclosure. Accordingly, the present disclosure further provides methods for producing a CAR using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of the present disclosure (into which a recombinant expression vector encoding a polypeptide of the present disclosure has been introduced) in a suitable medium such that the one or more CARs are produced (*e.g.*, expressed on the surface of the host cell).

[0199] For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., for resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those that confer resistance to drugs, such as G418, hygromycin, or methotrexate. Nucleic acid molecules encoding a selectable marker can be introduced into a host cell on the same vector as the nucleic acid molecule of the invention or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid molecule can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

[0200] As exemplified herein, contemplated is an immune cell, such as but not limited to a T cell, that expresses a IL13CAR and which is resistant to exposure to the chemotherapeutic agent temozolomide. Specifically, shown herein is that T cells transduced with a nucleic acid sequence encoding and expressing IL13CAR (SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6) and the P140KMGMT mutant (SEQ ID NO:33) as detailed above have a higher survival rate compared to T cells expressing a CAR expressing only IL13 in the presence of TMZ. Thus, in a particular aspect, the invention is directed to a CAR expressing IL13 and/or a variant of IL13 (*e.g.*, SEQ ID NO:4 (WT IL13 CAR), SEQ ID NO:5 (IL13E13YCAR) or SEQ ID NO:6 (IL13E13K.R109KCR) and/or R109K IL13CAR) and a MGMT mutant that chemoprotects a cell (*e.g.*, a P140KMGMT mutant (SEQ ID NO:33, SEQ ID NO:38, SE ID NO:39, SEQ ID NO:40, SEQ ID NO:41). In one embodiment, the cell is transduced with an IL13CAR-A2-MGMT construct wherein the MGMT protein is not a wildtype MGMT protein (SEQ ID NO:49).

Harvesting and Transfection of Host T Cells

[0201] T cells engineered with chimeric antigen receptors (CAR) to enable highly specific tumor recognition and killing have gained considerable attention following promising clinical results (Grupp et al., 2013, N Eng J Med, 368:1509-1518; Porter et al., 2011, N Eng J Med, 365:725-733; Sadelain et al., 2009, Curr Opin Immunol, 21:215-223). Reprogramming T cells with CAR genes provides an MHC-independent mechanism for docking with and lysing

tumor cells. Such modified T cells have been alternatively termed "designer T cells," "T-bodies," or "CAR-T cells" (Ma et al., 2002, Cancer Chemotherapy & Biological Response Modifiers: Elsevier Science, pp. 319-345; Park et al., 2011, Trends Biotech, 29:550-557; Ma et al., 2014, Prostate, 74:286-296).

[0202] In another aspect, the disclosure is directed to a method of producing a cell which expresses a CAR comprising a T cell receptor comprising one or more ligands (*e.g.*, an antibody) to one or tumor antigens of a brain cancer and an MGMT protein which increases viability of a cell transduced with the nucleic acid encoding the CAR and MGMT protein and exposed to a DNA methylating agent such as TMZ. In a particular aspect, the disclosure is directed to a method of producing a cell which expresses a CAR having an amino acid sequence of SEQ ID NO: 4 (IL13 CAR-P140KMGMT), SEQ ID NO:5 (IL-13(E13Y) CAR-P140KMGMT), SEQ ID NO:6 (IL-13(E13K R109K) CAR-P140KMGMT) or a combination thereof. The method comprises introducing a nucleic acid sequence comprising SEQ ID NO:1 (IL13(WT)CAR-P140KMGMT), SEQ ID NO:2(IL-13(E13Y) CAR-P140KMGMT) or SEQ ID NO:3 (IL-13(E13K R109K) CAR-P140KMGMT) into the cell; and maintaining the cell under conditions in which the CAR is expressed by the cell, thereby producing a cell which expresses a chimeric antigen receptor having an amino acid sequence of SEQ ID NO:4 (IL13(WT)CAR-P140KMGMT), SEQ ID NO:5 (IL-13(E13Y) CAR-P140KMGMT), SEQ ID NO:6 (IL-13(E13K R109K) CAR-P140KMGMT) or a combination thereof.

[0203] In a particular aspect, the nucleic acid sequence is introduced into the cell using a viral vector (*e.g.*, a retroviral vector, a lentiviral vector, an adenoviral vector or a combination thereof). In another aspect, the cell is a mammalian cell, such as a mammalian T cell (*e.g.*, a human T cell or a mouse T cell). In a particular aspect, the cell is an autologous cell or a human leukocyte antigen (HLA)-matched cell. In yet another aspect, the cell is obtained from one or more individuals with brain cancer (e.g., a high-grade malignant glioma such as a glioblastoma multiforme (GBM), an anaplastic astrocytoma or a pediatric glioma).

[0204] Therefore, an additional aspect relates to a recombinant T-cell that expresses at least one CAR and drug-resistance polypeptide according to the present disclosure. A particularly preferred transformed host cell is a transgenic T-precursor cell or a stem cell that is characterized in that it comprises a nucleic acid construct according to the present disclosure. Methods for transformation or transduction of host cells and/or stem cells are well known to the person of skill, and, for example, include electroporation or microinjection. A particularly preferred transformed host cell is a patient-unique T-cell, which is after the extraction transfected with a nucleic acid construct according to this disclosure. According to the disclosure, host cells in particular can be obtained by extracting one or several cells, preferably T-cells, in particular $CD8^+$-T-cells that are subsequently transfected or transduced

ex vivo with one or more nucleic acid constructs according to the present disclosure, in order to thereby obtain host cells according to the present disclosure.

[0205] Prior to expansion and genetic modification, a source of T cells is obtained from a subject. The term "subject" is intended to include living organisms in which an immune response can be elicited (e.g., mammals). Examples of subjects include humans and other primates dogs, cats, mice, rats, and transgenic rodent species. T cells can be obtained from a number of 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 certain aspects of the present invention, any number of T cell lines available in the art, may be used. In certain aspects of the present disclosure, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll™ separation. In one preferred aspect, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one aspect, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In one aspect of the invention, the cells are washed with phosphate buffered saline (PBS). In an alternative aspect, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Initial activation steps in the absence of calcium can lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers or other saline solution with or without buffer. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

[0206] In one aspect, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient or by counterflow centrifugal elutriation. A specific subpopulation of T cells, such as CD3+, CD28+, CD4+, CD8+, CD45RA+, and CD45RO+T cells, can be further isolated by positive or negative selection techniques. The skilled artisan would recognize that multiple rounds of selection can also be used in the context of this invention. In certain aspects, it may be desirable to perform the selection procedure and use the "unselected" cells in the activation and expansion process. "Unselected" cells can also be subjected to further rounds of selection.

[0207] Enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. One method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. In certain aspects, it may be desirable to enrich for or positively select for regulatory T cells which typically express CD4+, CD25+, CD62Lhi, GITR+, and FoxP3+. Alternatively, in certain aspects, T regulatory cells are depleted by anti-C25 conjugated beads or other similar method of selection.

Activation and Expansion of T Cells

[0208] Whether prior to or after genetic modification of the T cells to express a desirable CAR, the T cells can be activated and expanded generally using methods as described, for example, 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.

[0209] Once it is established that the transfected or transduced T cell is capable of expressing the IL13CAR as a surface membrane protein with the desired regulation and at a desired level, it can be determined whether the chimeric receptor is functional in the host cell to provide for the desired signal induction. Subsequently, the transduced T cells are reintroduced or administered to the subject to activate anti-tumor responses in the subject.

Pharmaceutical Compositions

[0210] In yet another aspect, the disclosure is directed to pharmaceutical compositions to facilitate administration of transduced T cells as described herein to a subject in need. The transduced T cells according to the disclosure can be made into a pharmaceutical composition or made implant appropriate for administration in vivo, with appropriate carriers or diluents, which further can be pharmaceutically acceptable. The means of making such a composition or an implant have been described in the art (see, for instance, Remington's Pharmaceutical Sciences, 16th Ed., Mack, ed. (1980)). Where appropriate, the transduced T cells can be formulated into a preparation in semisolid or liquid form, such as a capsule, solution, injection, inhalant, or aerosol, in the usual ways for their respective route of administration. Means known in the art can be utilized to prevent or minimize release and absorption of the composition until it reaches the target tissue or organ, or to ensure timed-release of the composition. Desirably, however, a pharmaceutically acceptable form is employed which does not ineffectuate the cells expressing the chimeric receptor. Thus, desirably the transduced T cells can be made into a pharmaceutical composition containing a balanced salt solution,

preferably Hanks' balanced salt solution, or normal saline. For instance, the compositions can be formulated with a physiologically acceptable carrier or excipient to prepare a pharmaceutical composition. The carrier and composition can be sterile. The formulation should suit the mode of administration.

[0211] Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions (e.g., NaCl), saline, buffered saline, alcohols, glycerol, ethanol, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, dextrose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrolidone, etc., as well as combinations thereof. The pharmaceutical preparations can, if desired, be mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like that do not deleteriously react with the active compounds.

[0212] The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrollidone, sodium saccharine, cellulose, magnesium carbonate, etc.

[0213] The composition can be formulated in accordance with the routine procedures as a pharmaceutical composition adapted for administration to human beings. For example, compositions for intravenous administration typically are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampule or sachette indicating the quantity of active compound. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water, saline or dextrose/water. Where the composition is administered by injection, an ampule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0214] Methods of introduction of these compositions include, but are not limited to, intracranial, intramedullary, intradermal, intramuscular, intraperitoneal, intraocular, intravenous, subcutaneous, topical, oral and intranasal. Other suitable methods of introduction can also include gene therapy (as described below), rechargeable or

biodegradable devices, particle acceleration devises ("gene guns") and slow release polymeric devices. The pharmaceutical compositions of this invention can also be administered as part of a combinatorial therapy with other compounds.

[0215] For topical application, nonsprayable forms, viscous to semi-solid or solid forms comprising a carrier compatible with topical application and having a dynamic viscosity preferably greater than water, can be employed. Suitable formulations include but are not limited to solutions, suspensions, emulsions, creams, ointments, powders, enemas, lotions, sols, liniments, salves, aerosols, etc., that are, if desired, sterilized or mixed with auxiliary agents, e.g., preservatives, stabilizers, wetting agents, buffers or salts for influencing osmotic pressure, etc. The compound may be incorporated into a cosmetic formulation. For topical application, also suitable are sprayable aerosol preparations wherein the active ingredient, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant, e.g., pressurized air.

[0216] Compounds described herein can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

Kits

[0217] The disclosure also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the present disclosure. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, that notice reflects approval by the agency of manufacture, use of sale for human administration. The pack or kit can be labeled with information regarding mode of administration, sequence of drug administration (e.g., separately, sequentially or concurrently), or the like. The pack or kit may also include means for reminding the patient to take the therapy. The pack or kit can be a single unit dosage of the combination therapy or it can be a plurality of unit dosages. In particular, the compounds can be separated, mixed together in any combination, present in a single vial or tablet. Compounds assembled in a blister pack or other dispensing means is preferred. For the purpose of this invention, unit dosage is intended to mean a dosage that is dependent on the individual pharmacodynamics of each compound and administered in FDA approved dosages in standard time courses.

Methods of Treatment

[0218] In another aspect, the disclosure is directed to a method of treating a malignancy in an individual in need thereof comprising administering one or more T cells that express an IL13CAR which comprises one or more ligands (*e.g.*, an antibody) to the IL13Rα2 protein, and an MGMT protein. In a particular aspect, the disclosure is directed to a method of treating brain cancer in an individual in need thereof comprising administering one or more T cells that harbor and express a nucleic acid sequence encoding a protein comprising SEQ ID NO:26, SEQ ID NO:36 or SEQ ID NO:37 (ligand), SEQ ID NO:28 (TM), SEQ ID NOS:29 and 30 (CD28 and CD3-zeta signaling domains), and optionally further comprising SEQ ID NO:27 (hinge). In another embodiment the nucleic acid sequence comprises SEQ ID NO:1 (IL13 CAR-P140KMGMT), SEQ ID NO:2(IL-13(E13Y) CAR-P140KMGMT), SEQ ID NO:3 (IL-13(E13K R109K) CAR-P140KMGMT) or a combination thereof. In one aspect, the T cells are autologous T cells or a human leukocyte antigen (HLA)-matched cell. In another aspect, the brain cancer is a high-grade malignant glioma such as high-grade malignant glioma is a glioblastoma multiforme (GBM), an anaplastic astrocytoma or a pediatric glioma. In one embodiment, the methods disclosed herein are used to treat cancer associated with detrimental IL13Rα2 expression.

[0219] Other cancers which have been demonstrated to have cells over-expressing IL13Rα2 include but are not limited to breast, pancreatic, head and neck, ovarian and colorectal. In another embodiment, the cancer is one that has metastasized. Accordingly, also contemplated are methods for treating one or more of these cancers by administering to the subject one or more T cells transduced with one or more of the IL13CAR-MGMT constructs as described above.

[0220] Since the T cells express a CAR and expresses a mutant MGMT that confers protection against the drug resistance of MGMT overexpression or of an MGMT variant (e.g., P140K), the method of treating brain cancer can further comprise administering one or more chemotherapeutic agents to the individual (the brain cancer patient) sequentially or simultaneously. In other words, the modified T cell is administered before, during or after administration of the chemotherapeutic agent. Examples of chemotherapeutic agents include temozolomide (TMZ), 1, 3-bis(2-chloroethyl)-1-nitrosurea (BCNU or carmustine), fotemustine and lomustine. In a particular aspect, the one or more T cells express a nucleic acid sequence comprising SEQ ID NO:1 (IL13 CAR-P140KMGMT), SEQ ID NO:2(IL-13(E13Y) CAR-P140KMGMT), SEQ ID NO:3 (IL-13(E13K R109K) CAR-P140KMGMT) or a combination thereof and the one or more chemotherapeutic agents are administered to the individual simultaneously. In a particular aspect, the individual is a mammal such as a human or other primate, or a rodent such as a mouse or rat.

[0221] The efficacy of T cells transduced with a construct encoding and expressing an IL13CAR-MGMT chimera is illustrated in part by Examples 4 and 5 below. Example 4 shows that isolated T cells transduced with a vector encoding the IL13CAR-2A-P140KMGMT protein have increased resistance (increased viability) when exposed to TMZ as compared to T cells transduced with a vector encoding the IL13CAR without co-expression of the P140KMGMT protein (e.g., see FIG. 5). Accordingly, envisioned is a method for increasing viability of an immune cell transduced with an IL13CAR-MGMT construct such as that described herein. In an exemplary embodiment, a T cell transduced with a retrovirus comprising the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3 is provided as is a method for treating a subject diagnosed with a brain cancer receiving either sequentially or simultaneously, treatment with TMZ.

[0222] Additional studies show that the IL13CAR-MGMT constructs disclosed herein are also effective in modifying T cells which can be administered to a subject, and which can increase survival of the subject. As shown by Example 5 and illustrated in FIG. 6, mice injected with U251MG glioma cells were treated with TMZ and with T cells transduced with a construct encoding an IL13CAR (with no MGMT) or an IL13CAR-A2-P140KMGMT construct. While expression of the IL13CAR in the absence of the P140KMGMT increased survival as compared to no administration of a CAR T cell, animals which were administered TMZ with a T cell transduced with a nucleic acid sequence encoding the IL13CAR-A2-P140KMGMT chimera has the highest rate of survival (FIG. 6). Accordingly, contemplated herein is a method for treating a subject diagnosed with a brain cancer comprising administering to the subject an immune cell expressing an IL13CAR-MGMT protein as described herein.

[0223] The T cells and/or chemotherapeutic agent can be administered to the individual using any suitable route of administration. Examples of suitable routes of administration include, but are not limited to, intracranial, intramedullary, intradermal, intramuscular, intraperitoneal, intraocular, intravenous, subcutaneous, topical, oral and intranasal delivery.

[0224] In one aspect, the method further comprises obtaining one or more T cells from the individual and introducing a chimeric nucleic acid sequence of the invention, *e.g.,* the nucleic acid sequence comprising SEQ ID NO:1 (IL13 CAR-P140KMGMT), SEQ ID NO:2 (IL-13(E13Y) CAR-P140KMGMT), SEQ ID NO:3 (IL-13(E13K R109K) CAR-P140KMGMT) or a combination thereof, into the T cells. Methods of obtaining T cells from an individual are known in the art and include, for example, plasmapheresis. In some aspects, the CAR T cells are grown (expanded) in the laboratory until they number *e.g.*, in the billions. The expanded population of CAR T cells can then be infused into the patient. After the infusion, the T cells multiply in the patient's body and, with guidance from their engineered receptor, recognize and kill cancer cells that harbor the antigen on their surfaces.

[0225] Host cells expressing IL13CAR and mutant MGMT described herein, are administered in a therapeutically effective amount (i.e., an amount that is sufficient to treat the disease, such as by ameliorating symptoms associated with the disease, preventing or delaying the onset of the disease, and/or also lessening the severity or frequency of symptoms of the disease). The amount that will be therapeutically effective in the treatment of a particular individual's disorder or condition will depend on the symptoms and severity of the disease, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of a practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

[0226] Desirably an effective amount or sufficient number of the isolated transfected or modified T cells is present in the composition and introduced into the subject such that long-term, specific, anti-tumor responses are established to reduce the size of a tumor or eliminate tumor growth or regrowth than would otherwise result in the absence of such treatment. Desirably, the amount of modified T cells administered to the subject causes a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 100% decrease in tumor size when compared to otherwise same conditions wherein the modified T cells are not present.

[0227] Accordingly, the amount of modified T cells administered should take into account the route of administration and should be such that a sufficient number of the transduced T cells will be introduced so as to achieve the desired therapeutic response. Furthermore, the amounts of each active agent included in the compositions described herein (e.g., the amount per each cell to be contacted or the amount per certain body weight) can vary in different applications. In general, the concentration of modified T cells desirably should be sufficient to provide in the subject being treated at least from about 1×10^6 to about 1×10^9 transduced T cells, even more desirably, from about 1×10^7 to about 5×10^8 transduced T cells, although any suitable amount can be utilized either above, e.g., greater than 5×10^8 cells, or below, e.g., less than 1×10^7 cells. The dosing schedule can be based on well-established cell-based therapies (see, e.g., Topalian and Rosenberg (1987) Acta Haematol. 78 Suppl 1:75-6; U.S. Pat. No. 4,690,915) or an alternate continuous infusion strategy can be employed.

EXAMPLES

IL13CAR-P140KMGMT for Temozolomide-resistant Glioblastoma Immunotherapy

Example 1: Expression Plasmid Construction

[0228] A cDNA encoding an IL13(WT)CAR construct, a cDNA encoding an IL13(E13Y) CAR construct, and cDNA encoding an IL13(E13K.R109K)CAR construct was inserted into the BamHI and NotI cloning sites of the MFG retroviral vector, as illustrated in FIGS. 1A and 1B for the IL13(WT)CAR construct, to generate the host plasmids IL13(WT)CAR-pMFG, IL13(E13Y)CAR-pMFG, and IL13(E13K.R109K)CAR-pMFG plasmids. (See Kong et al., (Clin Cancer Res, 2012, 18(21):5949-5960)). To generate a monocistronic transcript having both the IL13CAR and P140K.MGMT cDNA sequences, a 2A-P140KMGMT fragment with 5' *Not*I and 3' *Eag*I ends was synthesized by Genscript USA (Piscataway, NJ). The 2.3 Kb fragment was cloned into a pUC57 cloning vector for confirming the sequence. Once confirmed, it was transferred en bloc into the IL13CAR-pMFG retroviral vector at the 3' NotI site to generate each of the IL13(WT)CAR-2A-P140K.MGMT-pMFG, IL13(E13Y)CAR-2A-P140K.MGMT-pMFG, and IL13(E13K.R109K)CAR-2A-P140K.MGMT-pMFG plasmids. FIGS. 2A and 2B illustrate the IL13(E13K.R109K)CAR and IL13(E13K.R109K)CAR-2A-P140K.MGMT constructs and pMFG plasmid constructs.

Example 2: Production of Retroviral Particles

[0229] MFG retroviral particles containing a constructs encoding the IL13(E13K.R109K)CAR and IL13(E13K.R109K)CAR-2A-P140KMGMT constructs described in Example 1 were generated by using the "ping-pong" method. Each host plasmid from Example 1 was first transfected into phoenix-eco cells to generate the ecotropic retrovirus. The transfection efficiency was measured by flow cytometry of IL13 expression. Culture supernatant was saved and used to transduce amphotropic virus-encoding mouse fibroblast cell line PG13 (ATCC, Manassas, VA). Transduced PG13 cells were tested for IL13, and IL13 positive cells were enriched by fluorescence activated cell sorter (FIGS. 3A-3B). Overexpression of MGMT in IL13-enriched cells was also tested by western blot analysis of cell lysates (FIG. 3C).

[0230] As shown in FIGS. 3A and 3B, transduced cells expressed IL13 and were enriched such that about 97% of the cells expressed the IL13(E13K.R109K)CAR-2A-P140KMGMT construct. FIG. 3C further shows increased expression of the P140KMGMT in cells transfected with the IL13(E13K.R109K)CAR-2A-P140KMGMT as compared to untransfected cells or cells transfected with the IL13CAR-only construct.

[0231] Enriched cells were expanded under tissue culture conditions to harvest culture supernatant that contained high-titers of CAR-encoding amphotropic retrovirus.

Example 3: Genetic Modification of Human T Cells

[0232] The retroviral particles comprising the IL13(E13K.R109K)CAR and IL13(E13K.R109K)CAR-2A-P140KMGMT constructs as generated according to the method of Example 2 were used to transduce human T cells. Human PBMCs were isolated from blood-filter discards (Rhode Island Blood Center, Providence, RI). PBMCs were cultured in the presence of OKT3 (10 μg/ml) and IL2 (3000 U/ml) for 36-48 h to enrich T cell populations. Enriched T cells were spinfected with retrovirus containing culture supernatants, in the presence of protamine and IL2, in a retronectin-coated plate for 1h at room temperature. This step was repeated 3 times in the following 24 h. After 3 rounds of infection, the cells were allowed to grow in the retrovirus-containing medium for another 24 h, and then transferred to fresh RPMI-1640 medium containing 10% fetal bovine serum, antibiotics, and IL2 for future experiments. T cells, successfully transduced with the IL13(E13K.R109K)CAR (without P140KMGMT) and untransduced T cells were used as control group in all experiments. Approximately, 20-25% of T cells transfected with the retroviral particles comprising the IL13(E13K.R109K)CAR-2A-P140KMGMT construct were positive for the IL13(E13K.R109K)CAR-2A-P140KMGMT as measured by flow cytometry to detect IL13 on the cell surface (data not shown). The transduction efficiency for IL13(E13K.R109K)CAR-2A-P140KMGMT was about 69.2%, where untransduced T cells were used as the control..

Example 4: Temozolomide Resistance in Transduced T Cells

[0233] T cells transduced with IL13(E13K.R109K)CAR (FIG. 1A) or IL13(E13K.R109K)CAR-2A-P140KMGMT (FIG. 2A) as described above were incubated separately with increasing concentration of temozolomide (TMZ; 0-1000 μM) for 48 hrs. Culture media were changed every 24 h and supplemented with fresh TMZ. After treatment with TMZ, viability of the cells was analyzed by Trypan blue exclusion principle, as well as Annexin V/7AAD staining method. Frequency of Annexin V/7-AAD negative cells were measured by flow cytometry and the results represented cell viability. A survival curve was constructed to extrapolate the viability and concentration of TMZ activity. As shown in FIG. 4, T cells transduced with IL13(E13K.R109K)CAR-2A-P140KMGMT survived better as compared to IL13(E13K.R109K)CAR-transduced T cells after exposure to TMZ. This observation indicates that genetic modification of T cells with P140KMGMT-expressing CARs rendered chemoprotection to the modified T cells.

Example 5: Functional Characterization of IL13CAR-2A-P140KMGMT

[0234] Immunoregulatory function of the transduced cells was also analyzed by measuring secretion of the cytokines IL2 and IFNγ by the transduced cells when co-cultured

with glioma cells. T cells which had been transduced with IL13(E13K.R109K)CAR-2A-P140KMGMT retrovirus were cultured with or without 200 μM of TMZ for 48-72 hrs under normal tissue culture conditions (using RPMI1640 medium with 5% serum and IL2 (3000 U/ml) and 200μM TMZ). Next, the cells were cultured with U251MG glioma cells for 72 hrs (as described herein regarding U251MG co-culture). The culture supernatants were tested for cytokine secretion by ELISA.

[0235] Interleukin-2 (IL2) is a marker for T cell viability and proliferation, while Interferon-gamma (IFNγ) is a marker of functionality of cytotoxic T cells. As seen in FIGS. 5A and 5B, transduced cells secreted both IL2 and IFNγ in the absence or presence of TMZ. Moreover, the presence of TMZ did not significantly decrease the secretion of either cytokine by the transduced cells. TMZ-resistant T cells were able to maintain their normal cytotoxic function after exposure to TMZ, indicating that these genetic modification indeed rendered these cells resistant to TMZ-induced leukopenic cytotoxicity.

Example 6: In vivo efficacy of IL13CAR-2A-MGMT

[0236] The *in vivo* efficacy of the IL13E13K.R109K-2A-P140KMGMT construct was tested in mice using the viral particles generated as described in Example 2. Fifty athymic nude mice were injected subcutaneously (left flank) with U251MG glioma cells (40 mice; Groups I-IV) or PBS (10 mice). Four days after glioma implantation, 3 groups of mice (Groups I, II and III, 10 mice/group) were treated orally with TMZ (64 mg/kg/day by oral gavage) for 4 days. On the 5th day after glioma implantation, the 3 groups of mice that had been treated orally with TMZ were treated as follows: Group I: an intra-tumoral injection of the IL13E13K.R109KCAR-2A-P140KMGMT construct (TMZ-resistant); Group II: the IL13 IL13E13K.R109K construct with no MGMT (TMZ sensitive); Group III: treatment with PBS only (no injection of T cells). Group IV received no T cells or TMZ treatment. The mice were monitored for visual tumor growth, behavioral changes, and morbidity until day 90 after injection of the T cells at which time the mice were sacrificed as required by IACUC restrictions.

[0237] A survival curve drawn from the results of the mouse experiment showed that tumor-bearing mice that were treated with the IL13(E13K.R109K)CAR-2A-P140KMGMT T cells and TMZ (Group I) had a median survival of 73 days and 40% of animals survived in comparison to 61 days and 14% survival in Group II animals that had been treated with TMZ-sensitive the IL13(E13K.R109K)CAR T cells with no MGMT and TMZ. Tumor-bearing mice that received no treatment (Group IV) had a median survival of 29 days. Group III tumor-bearing animals that did not receive T cells but were orally treated with TMZ alone showed 20% survival rate but also demonstrated a lower median survival time of 36 days only, which

was considered to be a background anti-tumor effect of TMZ treatment (Figure 9 & 10). This observation indicate that Group I animals that receive 3G TMZ-resistant CARs were most efficient in eliminating tumors by synergistic effects of CAR immunotherapy and TMZ chemotherapy.

[0238] The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

[0239] While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

1. A chimeric nucleic acid sequence comprising

a first nucleic acid sequence encoding in an IL13CAR comprising

an IL13 ligand domain which binds the IL13α2 receptor (SEQ ID NO:44),

a transmembrane domain,

a cytoplasmic domain comprising a CD3-zeta signaling domain; and

a second nucleic acid sequence encoding an MGMT polypeptide,selected from the group consisting of SEQ ID NO:33, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:43.

2. The chimeric nucleic acid sequence according to claim 1, wherein the IL13CAR further comprises a signal peptide.

3. The chimeric nucleic acid sequence according to claim 1 or claim 2, wherein the IL13CAR further comprises a hinge region.

4. The chimeric nucleic acid sequence according to any one of claims 1-3, wherein the cytoplasmic domain further comprises a co-stimulatory domain selected from the group consisting of CD28, 4-1BB (CD137), and OX40 (CD134) .

5. The chimeric nucleic acid sequence according to claim 4, wherein the CD28 co-stimulatory domain is at least 90% identical to SEQ ID NO:29.

6. The chimeric nucleic acid sequence according to any one of claims 1-5, wherein the CD3-zeta signaling domain is at least 90% identical to SEQ ID NO:30.

7. The chimeric nucleic acid sequence according to any one of claims 1-5, further comprising a third nucleic acid sequence encoding a self-cleaving linker peptide.

8. The chimeric nucleic acid sequence of claim 7, wherein the third nucleic acid sequence is positioned between the first nucleic acid and second nucleic acid.

9. The chimeric nucleic acid sequence according to any one of claims 1-8, wherein the first nucleic acid further comprises a nucleic acid sequence encoding a dipeptide linker which is positioned between the IL13 ligand domain and the hinge domain, between the hinge domain and the transmembrane domain, between the transmembrane domain and the CD28 signaling domain, or between the CD28 signaling domain and the CD3-zeta signaling domain.

10. The chimeric nucleic acid sequence according to any one of claims 1-9, further comprising a nucleic acid sequence encoding a linker peptide between the CD3-zeta signaling domain and the self-cleaving peptide.

11. The chimeric nucleic acid sequence according to any one of claims 1-10, wherein the IL13 ligand domain comprises an amino acid sequence which is at least 90% identical to a sequence selected from the group consisting of SEQ ID NO:26, SEQ ID NO:36, and SEQ ID NO:37 and a variant thereof.

12. The chimeric nucleic acid sequence according to any one of claims 1-10, selected from the group consisting of SEQ ID NO:1 nucleotides 109 to 1839, SEQ ID NO:2 nucleotides 109 to 1839, and SEQ ID NO:3 nucleotides 109 to 1839.

13. A nucleic acid sequence comprising SEQ ID NO:1 (IL13 CAR-P140KMGMT), SEQ ID NO:2 (IL-13(E13Y) CAR-P140KMGMT), SEQ ID NO:3 (IL-13(E13K R109K) CAR-P140KMGMT), or a combination thereof.

14. A vector comprising the chimeric nucleic acid sequence of any one of claims 1-12.

15. The vector according to claim 14, wherein the vector is a viral vector.

16. The vector according to claim 15, wherein the viral vector is a retroviral vector.

17. A host cell comprising a chimeric nucleic acid sequence according to any one of claims 1-12.

18. The host cell according to claim 17, wherein the cell is a mammalian cell.

19. The host cell according to claim 17 or 18, wherein the cell is a T cell.

20. The host cell according to any one of claims 17-19, wherein the cell is an autologous cell or a human leukocyte antigen (HLA)- matched cell.

21. The host cell according to any one of claims 17-20, wherein the cell is obtained from one or more individuals with brain cancer.

22. A host cell comprising

a first nucleic acid sequence encoding in an IL13CAR comprising

an IL13 ligand domain which binds the IL13α2 receptor (SEQ ID NO:44),

a transmembrane domain,

a cytoplasmic domain comprising a CD3-zeta signaling domain; and

a second nucleic acid sequence encoding an MGMT polypeptide selected from the group consisting of SEQ ID NO:33, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41 and SEQ ID NO:43.

23. A pharmaceutical composition comprising the host cell according to any one of claims 17-21.

24. A pharmaceutical composition comprising

a first nucleic acid sequence encoding in an IL13CAR comprising

an IL13 ligand domain which binds the IL13α2 receptor (SEQ ID NO:44),

a transmembrane domain,

a cytoplasmic domain comprising a CD3-zeta signaling domain; and

a second nucleic acid sequence encoding an MGMT polypeptide selected from the group consisting of SEQ ID NO:33, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41 and SEQ ID NO:43.

25. A method for producing a mammalian cell which expresses an IL13CAR protein and an MGMT protein comprising:

a) introducing into the cell a nucleic acid sequence encoding the IL13CAR protein and the MGMT protein, wherein the IL13CAR protein comprises an IL13 ligand domain which binds the IL13α2 receptor (SEQ ID NO:44), a transmembrane domain, and a cytoplasmic domain comprising a CD3-zeta signaling domain; and

b) maintaining the cell under conditions in which the IL13CAR protein and the MGMT protein are expressed by the cell.

26. The method according to claim 25, wherein the nucleic acid sequence is introduced into the cell using a viral vector.

27. The method of claim 26, wherein the viral vector is selected from the group consisting of a retroviral vector, a lentiviral vector, an adenoviral vector or an adeno-associated viral vector.

28. A method for treating brain cancer in a subject in need thereof comprising administering to the subject one or more immune cells that express proteins encoded by

a first nucleic acid sequence encoding in an N-terminal to C-terminal direction an IL13CAR comprising

an IL13 ligand domain which binds the IL13α2 receptor (SEQ ID NO:44),

a transmembrane domain, and

a cytoplasmic domain comprising a CD3-zeta signaling domain; and

a second nucleic acid sequence encoding an MGMT polypeptide selected from the group consisting of SEQ ID NO:33, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41 and SEQ ID NO:43.

29. The method according to claim 28, wherein the brain cancer is a high-grade malignant glioma.

30. The method according to claim 28 or 29, wherein the subject is being treated with, has been treated with, or will be treated with a DNA-methylating chemotherapeutic agent.

31. The method according to claim 30, wherein the DNA-methylating chemotherapeutic agent is TMZ.

32. The method according to claim 30 or 31, wherein the DNA-methylating chemotherapeutic agent is administered before, during, or after the administering of the dose of the immune cell.

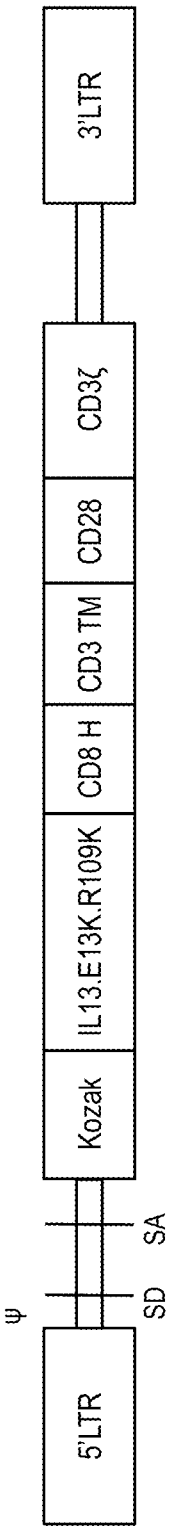
5'LTR
Ψ
SD
SA
Kozak
IL13.E13K.R109K
CD8 H
CD3 TM
CD28
CD3ζ
3'LTR

FIG. 1A

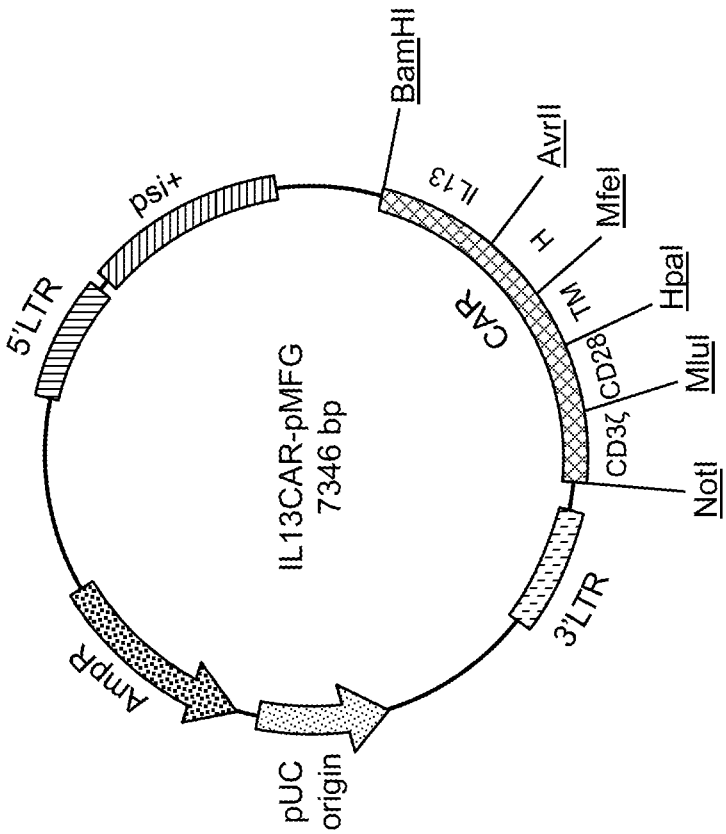
IL13CAR-pMFG
7346 bp
5'LTR
psi+
BamHI
IL13
AvrII
H
MfeI
TM
HpaI
CD28
MluI
CD3ζ
NotI
3'LTR
AmpR
pUC
origin

FIG. 1B

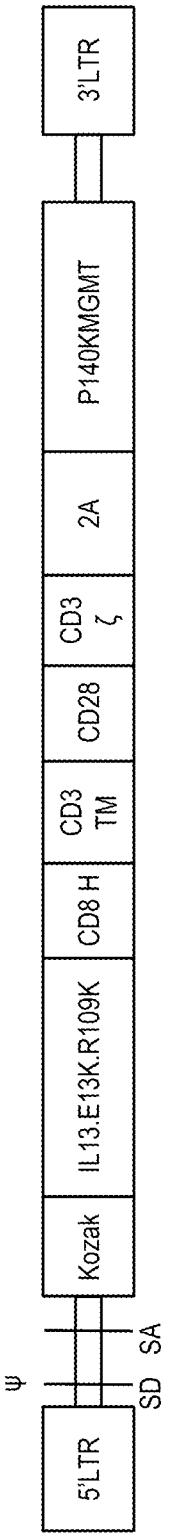
5'LTR
Ψ
SD
SA
Kozak
IL13.E13K.R109K
CD8 H
CD3 TM
CD28
CD3 ζ
2A
P140KMGMT
3'LTR

FIG. 2A

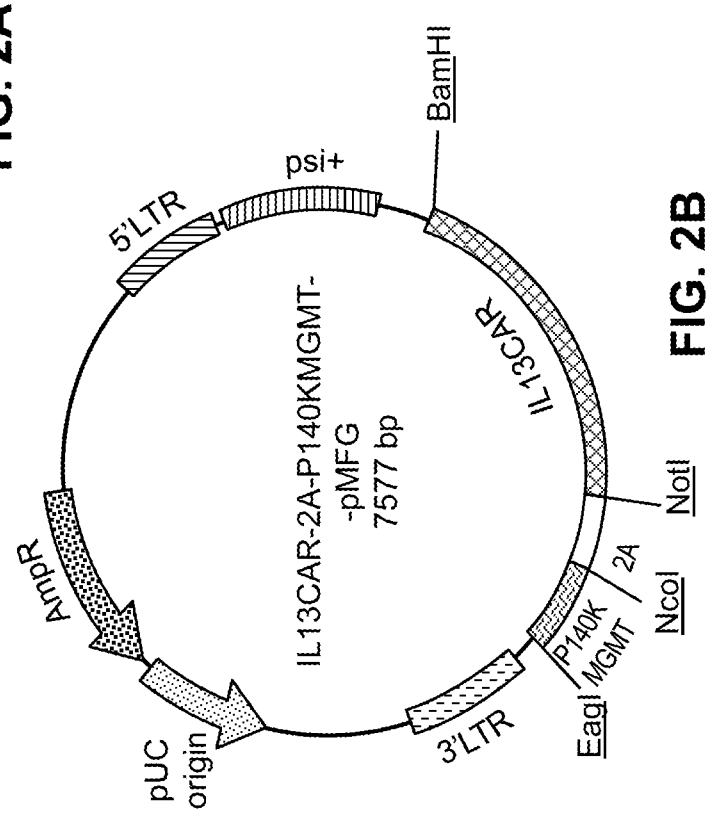
IL13CAR-2A-P140KMGMT-pMFG
7577 bp
5'LTR
psi+
BamHI
IL13CAR
NotI
2A
NcoI
P140K MGMT
EagI
3'LTR
pUC origin
AmpR

FIG. 2B

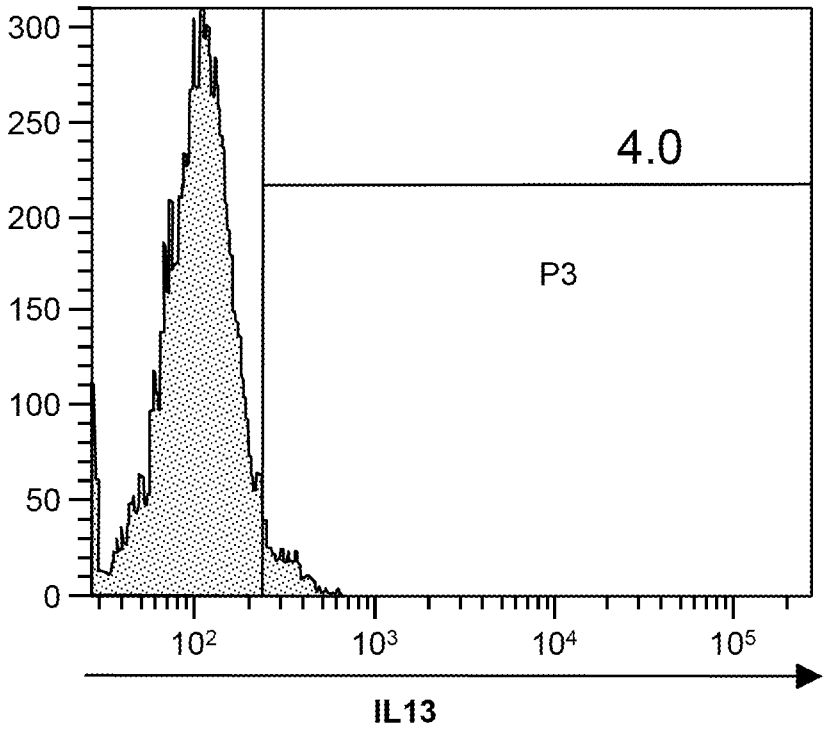

FIG. 3A

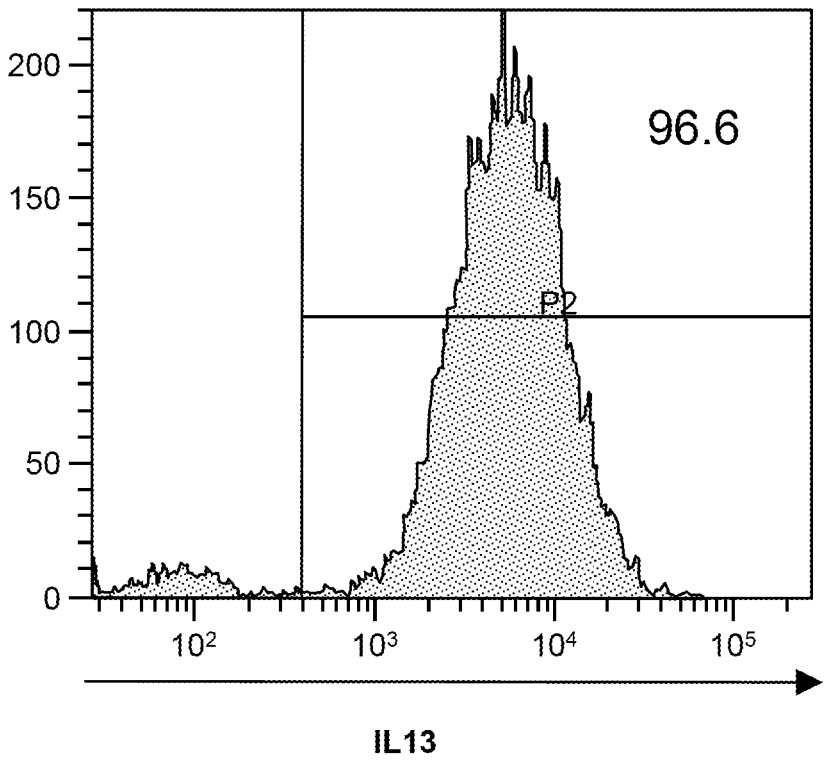
200
150
100
50
0
96.6
P2
10^2
10^3
10^4
10^5
IL13

FIG. 3B

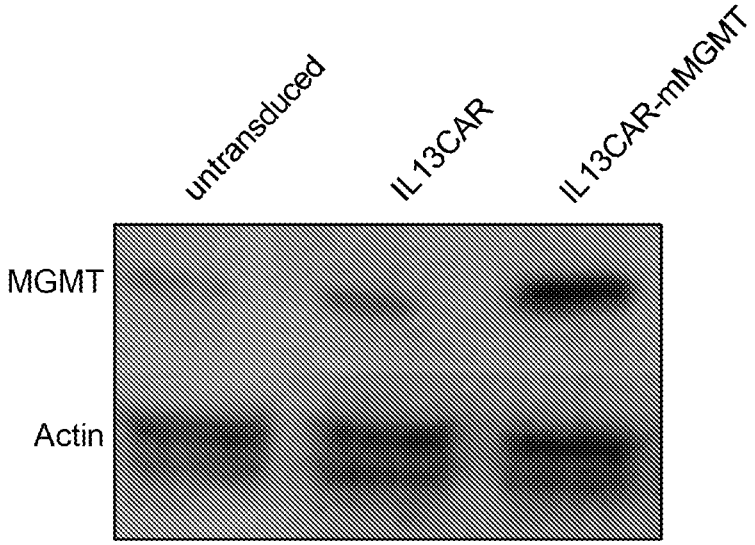
untransduced
IL13CAR
IL13CAR-mMGMT
MGMT
Actin

FIG. 3C

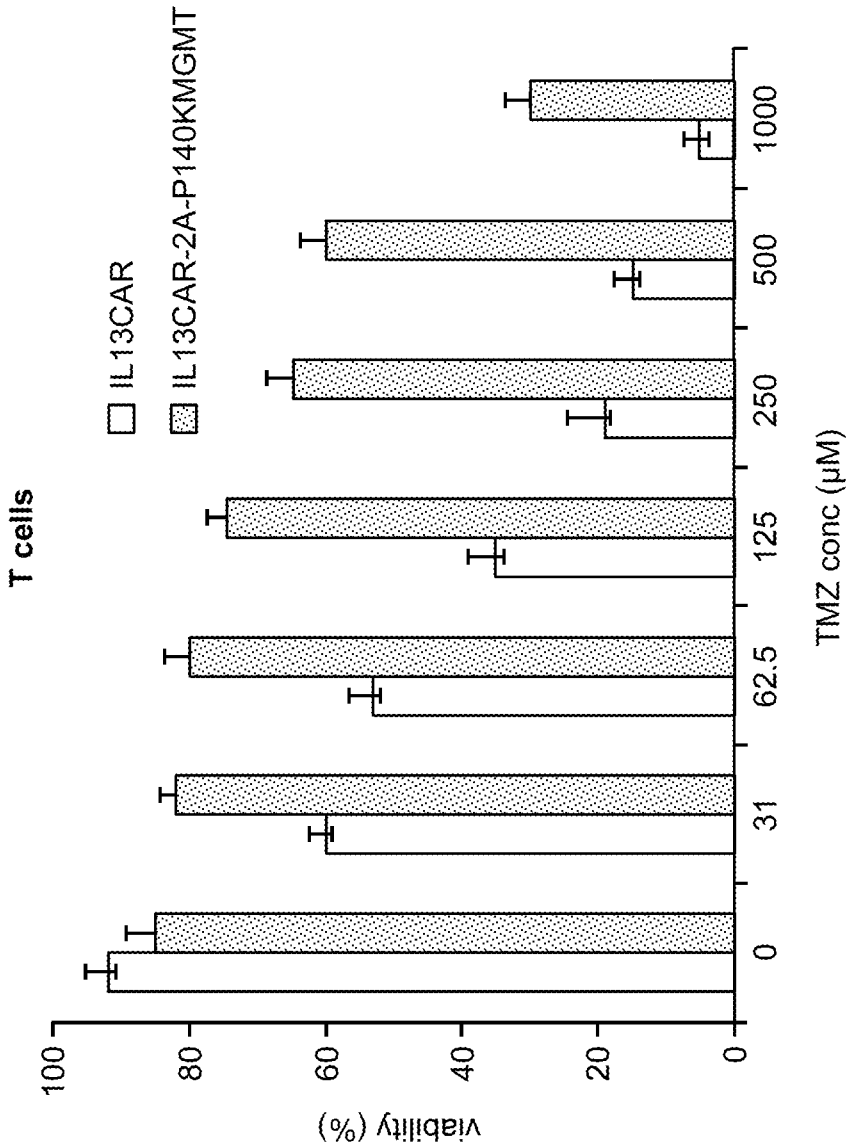
T cells
IL13CAR
IL13CAR-2A-P140KMGMT
viability (%)
100
80
60
40
20
0
0
31
62.5
125
250
500
1000
TMZ conc (μM)

FIG. 4

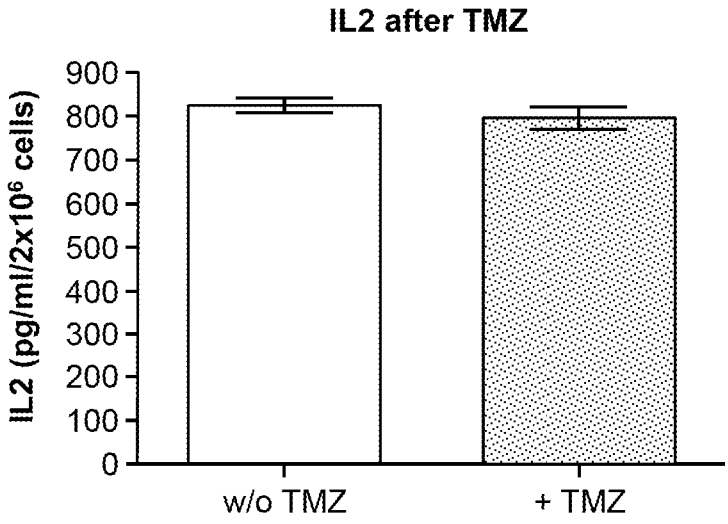
IL2 after TMZ
IL2 (pg/ml/2x10^6 cells)
900
800
700
600
500
400
300
200
100
0
w/o TMZ
+ TMZ

FIG. 5A

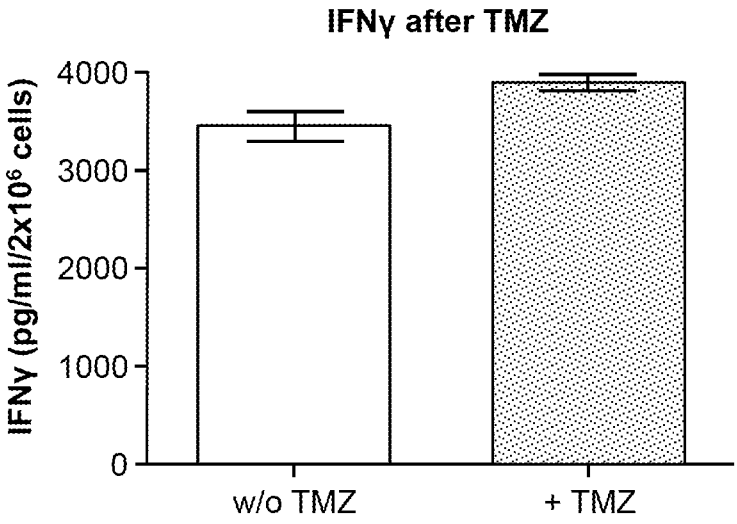
IFNγ after TMZ
IFNγ (pg/ml/2x10^6 cells)
4000
3000
2000
1000
0
w/o TMZ
+ TMZ

FIG. 5B

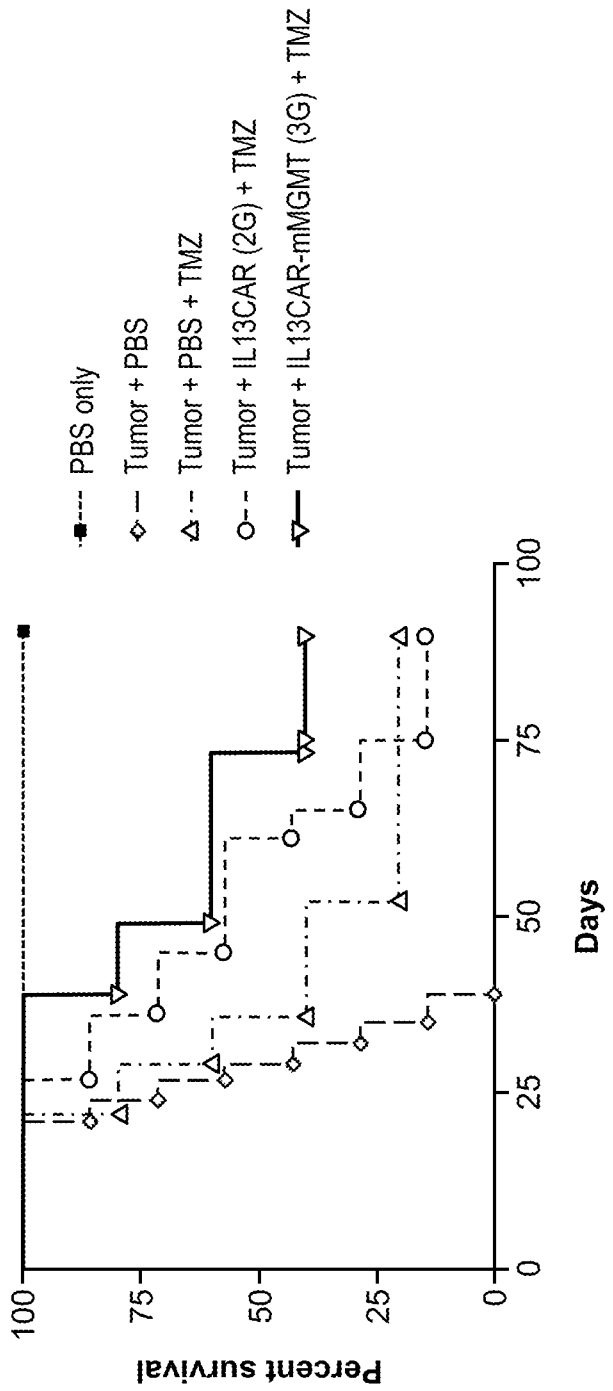
PBS only
Tumor + PBS
Tumor + PBS + TMZ
Tumor + IL13CAR (2G) + TMZ
Tumor + IL13CAR-mMGMT (3G) + TMZ
Percent survival
100
75
50
25
0
0
25
50
75
100
Days

FIG. 6

DNA Sequence of IL13CAR-P140KMGMT SEQ ID NO:1

GGA TCC GCC ACC **ATG** CAT CCG CTC CTC AAT CCT CTC CTG TTG GCA CTG GGC CTC ATG GCG CTT TTG TTG ACC ACG GTC ATT GCT CTC ACT	Signal
TGC CTT GGC GGC TTT GCC TCC CCA GGC CCT GTG CCT CCC TCT ACA GCC CTC AGG GAG CTC ATT GAG GAG CTG GTC AAC ATC ACC CAG AAC CAG AAG GCT CCG CTC TGC AAT GGC AGC ATG GTA TGG AGC ATC AAC CTG ACA GCT GGC ATG TAC TGT GCA GCC CTG GAA TCC CTG ATC AAC GTG TCA GGC TGC AGT GCC ATC GAG AAG ACC CAG AGG ATG CTG AGC GGA TTC TGC CCG CAC AAG GTC TCA GCT GGG CAG TTT TCC AGC TTG CAT GTC CGA GAC ACC AAA ATC GAG GTG GCC CAG TTT GTA AAG GAC CTG CTC TTA CAT TTA AAG AAA CTT TTT CGC GAG GGA CAG TTC AAC	IL13(WT)
CCT AGG AAG CCC ACC ACG ACG CCA GCG CCG CGA CCA CCA ACA CCG GCG CCC ACC ATC GCG TCG CAG CCC CTG TCC CTG CGC CCA GAG GCG TGC CGG CCA GCG GCG GGG GGC GCA GTG CAC ACG AGG GGG CTG GAC TTC GCC	Hinge
CAA TTG CTC TGC TAC CTG CTG GAT GGA ATC CTC TTC ATC TAT GGT GTC	TM
ATT CTC ACT GCC TTG TTC CTG AGA GTG GTT AAC TTC TGG GTG AGG AGT AAG AGG AGC AGG CTC CTG CAC AGT GAC TAC ATG AAC ATG ACT CCC CGC CGC CCC GGG CCC ACC CGC AAG CAT TAC CAG CCC TAT GCC CCA CCA CGC	CD28
GAC TTC GCA GCC TAT CGC TCC ACG CGT AAG TTC AGC AGG AGC GCA GAC GCC CCC GCG TAC CAG CAG GGC CAG AAC CAG CTC TAT AAC GAG CTC AAT CTA GGA CGA AGA GAG GAG TAC GAT GTT TTG GAC AAG AGA CGT GGC CGG GAC CCT GAG ATG GGG GGA AAG CCG AGA AGG AAG AAC CCT CAG GAA GGC CTG TAC AAT GAA CTG CAG AAA GAT AAG ATG GCG GAG GCC TAC AGT GAG ATT GGG ATG AAA GGC GAG CGC CGG AGG GGC AAG GGG CAC GAT GGC CTT TAC CAG GGT CTC AGT ACA GCC ACC AAG GAC ACC TAC GAC GCC CTT CAC	CD3ζ
ATG CAG GCC CTG CCC CCT CGC **TAA** CAG CCA GCG GCC GC A GAG GGC AGA	A2
GGA AGT CTT CTA ACA TGC GGT GAC GTG GAG GAG AAT CCC GGC CCT CCA	
TGG **ATG** GAC AAA GAT TGC GAG ATG AAG CGG ACC ACA CTG GAC TCC CCC CTG GGC AAA CTG GAG CTG TCT GGC TGT GAA CAG GGG CTG CAC GAG ATC AAA CTG CTG GGA AAG GGC ACT AGC GCC GCT GAT GCT GTG GAA GTG CCA GCT CCA GCT GCT GTG CTG GGA GGA CCT GAG CCA CTG ATG CAG TGC ACC GCC TGG CTG AAC GCT TAC TTC CAT CAG CCT GAA GCC ATC GAG GAA TTT CCC GTG CCT GCC CTG CAC CAT CCA GTG TTC CAG CAG GAG AGT TTT ACA AGG CAG GTG CTG TGG AAG CTG CTG AAA GTG GTG AAG TTC GGG GAA GTG ATT TCC TAC CAG CAG CTG GCT GCT CTG GCT GGA AAC CCA AAA GCT GCT CGG GCC GTG GGA GGA GCT ATG AGA GGC AAT CCA GTG AAA ATC CTG ATT CCC TGC CAC AGG GTG GTG TGT AGC TCC GGA GCT GTG GGG AAC TAT TCT GGG GGA CTG GCC GTG AAA GAA TGG CTG CTG GCT CAC GAG GGA CAT AGG CTG GGA AAG CCT GGC CTG GGA GGG TCT AGT GGA CTG GCT GGA GCT TGG CTG AAG GGA GCT GGA GCT ACC TCA GGA AGC CCA CCT GCC GGC CGG AAT	P140K MGMT
TGA CGG CCG	

FIG. 7A

Peptide Sequence of IL13CAR-P140KMGMT SEQ ID NO:4

MHPLLNPLLLALGLMALLLTTVIALTCLGGFA SPGPVPPSTALRELIEELVNITQNQKAPL CNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVR	IL13
DTKIEVAQFVKDLLLHLKKLFREGQFN PR KPTTTPAPRPPTPAPTIASQPLSLRPEAC	Hinge
RPAAGGAVHTRGLDFA QL LCYLLDGILFIYGVILTALFLRV VN FWVRSKRSRLLHSDYM	TM/CD28
NMTPRRPGPTRKHYQPYAPPRDFAAYRS TR KFSRSADAPAYQQGQNQLYNELNLGR REEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK	CD3ζ
GHDGLYQGLSTATKDTYDALHMQALPPR **STOP** QP AAA EGRGSLLTCGDVEENPGP M	A2
DKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGKGTSAADAVEVPAPAAVLGGPEPL MQCTAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQL AALAGNPKAARAVGGAMRGNPV K ILIPCHRVVCSSGAVGNYSGGLAVKEWLLAHEGH	P140K MGMT
RLGKPGLGGSSGLAGAWLKGAGATSGSPPAGRN **STOP**	

FIG. 7B

DNA Sequence of IL13 (E13Y) CAR-P140KMGMT SEQ ID NO:2

GGA TCC GCC ACC **ATG** CAT CCG CTC CTC AAT CCT CTC CTG TTG GCA CTG GGC CTC ATG GCG CTT TTG TTG ACC ACG GTC ATT GCT CTC ACT	Signal
TGC CTT GGC GGC TTT GCC TCC CCA GGC CCT GTG CCT CCC TCT ACA GCC CTC AGG TAC CTC ATT GAG GAG CTG GTC AAC ATC ACC CAG AAC CAG AAG GCT CCG CTC TGC AAT GGC AGC ATG GTA TGG AGC ATC AAC CTG ACA GCT GGC ATG TAC TGT GCA GCC CTG GAA TCC CTG ATC AAC GTG TCA GGC TGC AGT GCC ATC GAG AAG ACC CAG AGG ATG CTG AGC GGA TTC TGC CCG CAC AAG GTC TCA GCT GGG CAG TTT TCC AGC TTG CAT GTC CGA GAC ACC AAA ATC GAG GTG GCC CAG TTT GTA AAG GAC CTG CTC TTA CAT TTA AAG AAA CTT TTT CGC GAG GGA CAG TTC AAC	IL13(E13y)
CCT AGG AAG CCC ACC ACG ACG CCA GCG CCG CGA CCA CCA ACA CCG GCG CCC ACC ATC GCG TCG CAG CCC CTG TCC CTG CGC CCA GAG GCG TGC CGG CCA GCG GCG GGG GGC GCA GTG CAC ACG AGG GGG CTG GAC TTC GCC	Hinge
CAA TTG CTC TGC TAC CTG CTG GAT GGA ATC CTC TTC ATC TAT GGT GTC	TM
ATT CTC ACT GCC TTG TTC CTG AGA GTG GTT AAC TTC TGG GTG AGG AGT AAG AGG AGC AGG CTC CTG CAC AGT GAC TAC ATG AAC ATG ACT CCC CGC CGC CCC GGG CCC ACC CGC AAG CAT TAC CAG CCC TAT GCC CCA CCA CGC	CD28
GAC TTC GCA GCC TAT CGC TCC ACG CGT AAG TTC AGC AGG AGC GCA GAC GCC CCC GCG TAC CAG CAG GGC CAG AAC CAG CTC TAT AAC GAG CTC AAT CTA GGA CGA AGA GAG GAG TAC GAT GTT TTG GAC AAG AGA CGT GGC CGG GAC CCT GAG ATG GGG GGA AAG CCG AGA AGG AAG AAC CCT CAG GAA GGC CTG TAC AAT GAA CTG CAG AAA GAT AAG ATG GCG GAG GCC TAC AGT GAG ATT GGG ATG AAA GGC GAG CGC CGG AGG GGC AAG GGG CAC GAT GGC CTT TAC CAG GGT CTC AGT ACA GCC ACC AAG GAC ACC TAC GAC GCC CTT CAC	CD3ζ
ATG CAG GCC CTG CCC CCT CGC **TAA** CAG CCA GCG GCC GC A GAG GGC AGA	A2
GGA AGT CTT CTA ACA TGC GGT GAC GTG GAG GAG AAT CCC GGC CCT CCA	
TGG **ATG** GAC AAA GAT TGC GAG ATG AAG CGG ACC ACA CTG GAC TCC CCC CTG GGC AAA CTG GAG CTG TCT GGC TGT GAA CAG GGG CTG CAC GAG ATC AAA CTG CTG GGA AAG GGC ACT AGC GCC GCT GAT GCT GTG GAA GTG CCA GCT CCA GCT GCT GTG CTG GGA GGA CCT GAG CCA CTG ATG CAG TGC ACC GCC TGG CTG AAC GCT TAC TTC CAT CAG CCT GAA GCC ATC GAG GAA TTT CCC GTG CCT GCC CTG CAC CAT CCA GTG TTC CAG CAG GAG AGT TTT ACA AGG CAG GTG CTG TGG AAG CTG CTG AAA GTG GTG AAG TTC GGG GAA GTG ATT TCC TAC CAG CAG CTG GCT GCT CTG GCT GGA AAC CCA AAA GCT GCT CGG GCC GTG GGA GGA GCT ATG AGA GGC AAT CCA GTG AAA ATC CTG ATT CCC TGC CAC AGG GTG GTG TGT AGC TCC GGA GCT GTG GGG AAC TAT TCT GGG GGA CTG GCC GTG AAA GAA TGG CTG CTG GCT CAC GAG GGA CAT AGG CTG GGA AAG CCT GGC CTG GGA GGG TCT AGT GGA CTG GCT GGA GCT TGG CTG AAG GGA GCT GGA GCT ACC TCA GGA AGC CCA CCT GCC GGC CGG AAT	P140K MGMT
TGA CGG CCG	

FIG. 8A

Peptide Sequence of IL13 (E13Y) CAR-P140KMGMT SEQ ID NO:5

MHPLLNPLLLALGLMALLLTTVIALTCLGGFA SPGPVPPSTALRYLIEELVNITQNQKAPL	IL13
CNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVR	(E13y)
DTKIEVAQFVKDLLLHLKKLFREGQFN PR KPTTTPAPRPPTPAPTIASQPLSLRPEAC	Hinge
RPAAGGAVHTRGLDFA QL LCYLLDGILFIYGVILTALFLRV VN FWVRSKRSRLLHSDYM	TM/CD28
NMTPRRPGPTRKHYQPYAPPRDFAAYRS TR KFSRSADAPAYQQGQNQLYNELNLGR	CD3ζ
REEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK	
GHDGLYQGLSTATKDTYDALHMQALPPR **STOP** QP AAA EGRGSLLTCGDVEENPGP M	A2
DKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGKGTSAADAVEVPAPAAVLGGPEPL MQCTAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQL AALAGNPKAARAVGGAMRGNPV K ILIPCHRVVCSSGAVGNYSGGLAVKEWLLAHEGH RLGKPGLGGSSGLAGAWLKGAGATSGSPPAGRN **STOP**	P140K MGMT

FIG. 8B

DNA Sequence of IL13 (E13K.R109K) CAR-P140KMGMT SEQ ID NO:3

GGA TCC GCC ACC **ATG** CAT CCG CTC CTC AAT CCT CTC CTG TTG GCA CTG GGC CTC ATG GCG CTT TTG TTG ACC ACG GTC ATT GCT CTC ACT	Signal
TGC CTT GGC GGC TTT GCC TCC CCA GGC CCT GTG CCT CCC TCT ACA GCC CTC AGG AAG CTC ATT GAG GAG CTG GTC AAC ATC ACC CAG AAC CAG AAG GCT CCG CTC TGC AAT GGC AGC ATG GTA TGG AGC ATC AAC CTG ACA GCT GGC ATG TAC TGT GCA GCC CTG GAA TCC CTG ATC AAC GTG TCA GGC TGC AGT GCC ATC GAG AAG ACC CAG AGG ATG CTG AGC GGA TTC TGC CCG CAC AAG GTC TCA GCT GGG CAG TTT TCC AGC TTG CAT GTC CGA GAC ACC AAA ATC GAG GTG GCC CAG TTT GTA AAG GAC CTG CTC TTA CAT TTA AAG AAA CTT TTT AAG GAG GGA CAG TTC AAC	IL13 (E13K R109K)
CCT AGG AAG CCC ACC ACG ACG CCA GCG CCG CGA CCA CCA ACA CCG GCG CCC ACC ATC GCG TCG CAG CCC CTG TCC CTG CGC CCA GAG GCG TGC CGG CCA GCG GCG GGG GGC GCA GTG CAC ACG AGG GGG CTG GAC TTC GCC	Hinge
CAA TTG CTC TGC TAC CTG CTG GAT GGA ATC CTC TTC ATC TAT GGT GTC	TM
ATT CTC ACT GCC TTG TTC CTG AGA GTG GTT AAC TTC TGG GTG AGG AGT AAG AGG AGC AGG CTC CTG CAC AGT GAC TAC ATG AAC ATG ACT CCC CGC CGC CCC GGG CCC ACC CGC AAG CAT TAC CAG CCC TAT GCC CCA CCA CGC	CD28
GAC TTC GCA GCC TAT CGC TCC ACG CGT AAG TTC AGC AGG AGC GCA GAC GCC CCC GCG TAC CAG CAG GGC CAG AAC CAG CTC TAT AAC GAG CTC AAT CTA GGA CGA AGA GAG GAG TAC GAT GTT TTG GAC AAG AGA CGT GGC CGG GAC CCT GAG ATG GGG GGA AAG CCG AGA AGG AAG AAC CCT CAG GAA GGC CTG TAC AAT GAA CTG CAG AAA GAT AAG ATG GCG GAG GCC TAC AGT GAG ATT GGG ATG AAA GGC GAG CGC CGG AGG GGC AAG GGG CAC GAT GGC CTT TAC CAG GGT CTC AGT ACA GCC ACC AAG GAC ACC TAC GAC GCC CTT CAC	CD3ζ
ATG CAG GCC CTG CCC CCT CGC **TAA** CAG CCA GCG GCC GC A GAG GGC AGA GGA AGT CTT CTA ACA TGC GGT GAC GTG GAG GAG AAT CCC GGC CCT CCA	A2
TGG **ATG** GAC AAA GAT TGC GAG ATG AAG CGG ACC ACA CTG GAC TCC CCC CTG GGC AAA CTG GAG CTG TCT GGC TGT GAA CAG GGG CTG CAC GAG ATC AAA CTG CTG GGA AAG GGC ACT AGC GCC GCT GAT GCT GTG GAA GTG CCA GCT CCA GCT GCT GTG CTG GGA GGA CCT GAG CCA CTG ATG CAG TGC ACC GCC TGG CTG AAC GCT TAC TTC CAT CAG CCT GAA GCC ATC GAG GAA TTT CCC GTG CCT GCC CTG CAC CAT CCA GTG TTC CAG CAG GAG AGT TTT ACA AGG CAG GTG CTG TGG AAG CTG CTG AAA GTG GTG AAG TTC GGG GAA GTG ATT TCC TAC CAG CAG CTG GCT GCT CTG GCT GGA AAC CCA AAA GCT GCT CGG GCC GTG GGA GGA GCT ATG AGA GGC AAT CCA GTG AAA ATC CTG ATT CCC TGC CAC AGG GTG GTG TGT AGC TCC GGA GCT GTG GGG AAC TAT TCT GGG GGA CTG GCC GTG AAA GAA TGG CTG CTG GCT CAC GAG GGA CAT AGG CTG GGA AAG CCT GGC CTG GGA GGG TCT AGT GGA CTG GCT GGA GCT TGG CTG AAG GGA GCT GGA GCT ACC TCA GGA AGC CCA CCT GCC GGC CGG AAT	P140K MGMT
TGA CGG CCG	

FIG. 9A

Peptide Sequence of IL13 (E13K.R109K) CAR-P140KMGMT SEQ ID NO:6

MHPLLNPLLLALGLMALLLTTVIALTCLGGFA SPGPVPPSTALRKLIEELVNITQNQKAPL	IL13
CNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVR	(E13K R109K)
DTKIEVAQFVKDLLLHLKKLFKEGQFN PR KPTTTPAPRPPTPAPTIASQPLSLRPEAC	Hinge
RPAAGGAVHTRGLDFA QL LCYLLDGILFIYGVILTALFLRV VN FWVRSKRSRLLHSDYM	TM/CD28
NMTPRRPGPTRKHYQPYAPPRDFAAYRS TR KFSRSADAPAYQQGQNQLYNELNLGR	CD3ζ
REEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK	
GHDGLYQGLSTATKDTYDALHMQALPPR **STOP** QP AAA EGRGSLLTCGDVEENPGP M	A2
DKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGKGTSAADAVEVPAPAAVLGGPEPL MQCTAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQL AALAGNPKAARAVGGAMRGNPV K ILIPCHRVVCSSGAVGNYSGGLAVKEWLLAHEGH RLGKPGLGGSSGLAGAWLKGAGATSGSPPAGRN **STOP**	P140K MGMT

FIG. 9B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/63267

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K14/47; C12N 9/10; A61K 48/00 (2016.01)
CPC - A61K 38/1774, 38/45, 48/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8)- C07K 14/47, C12N 9/10, A61P 35/00, A61K 48/00 (2016.01)
CPC- A61K 38/1774, 38/45, 48/00, 45/06; C12Y 201/01063

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB; CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google/Google Scholar; NCBI/BLAST/PubMed; EBSCO; The Lens
Search terms used: IL13, nucleic acid, nucleotide, MGMT, CD3, IL13a2, CAR, chimeric, receptor, transmembrane

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2014/164554 A1 (BAYLOR COLLEGE OF MEDICINE); October 9, 2014; paragraphs [0015], [0045]-[0046], [0053], [0082], [0088], [0090]	25-27
Y	(KREBS, S et al.) T Cells Redirected to IL13Rα2 with IL13 Mutein-CARs have Antiglioma Activity but also Recognize IL13Rα1. Cytotherapy. 16 May 2014, Vol. 16, No. 8; pages 1121-1131; abstract; DOI:10.1016/j.jcyt.2014.02.012	25-27
Y	(ZHANG, JG et al.) Identification, Purification, and Characterization of a Soluble Interleukin (IL)-13-binding Protein: Evidence that it is Distinct from the Cloned Il-13 Receptor and Il-4 Receptor Alpha-chains. Journal of Biological Chemistry. 4 April 1997, Vol. 272, No. 14; pages 9747-9480; Genbank supplement, pages 1-3	25-27
A	(KALNINE, N et al.) Homo sapiens O-6-methylguanine-DNA Methyltransferase, Partial [Synthetic Construct]. National Center for Biotechnology Information. Genbank ontry. 13 May 2003 [retrieved on 27 February 2016]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/protein/30584785>	1-3, 22, 24, 28-31
A	US 2012/0148552 A1 (JENSEN, M) June 14, 2012; paragraphs [0009]-[0014]	1-3, 22, 24-31
P,Y	WO 2015/120363 A1 (EMORY UNIVERSITY, et al.) August 13, 2015; abstract; SEQ ID NO: 22	1-3, 22, 24-31

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2016 (06.04.2016)	13 MAY 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/63267

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

3. ☒ Claims Nos.: 4-12, 14-21, 23, 32
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Groups I+: Claims 1-3, 22, 24-25, 28-31, SEQ ID NO: 33 (MGMT amino acid sequence)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US15/63267

-***-Continued from Box No. III: Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+: Claims 1-3, 22, 24-31 are directed toward a chimeric nucleic acid sequence comprising a first nucleic acid sequence encoding in an IL 13CAR comprising an IL13 ligand domain which binds the IL13-alpha-2 receptor, a transmembrane domain, a cytoplasmic domain comprising a CD3-zeta signaling domain; and a second nucleic acid sequence encoding an MGMT polypeptide; as well as a host cell comprising the sequence, a pharmaceutical composition comprising the sequence and a method for treating brain cancer comprising administering cells that express the protein encoded by the nucleic acid sequence.

The sequence, host cell, pharmaceutical composition and method will be searched to the extent that they encompass SEQ ID NO: 33 (MGMT amino acid sequence). It is believed that Claims 1 (in-part), 2 (in-part), 3 (in-part), 22 (in-part), 24 (in-part), 25-27, 28 (in-part), 29 (in-part), 30 (in-part) and 31 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 33 (MGMT amino acid sequence). Applicant is invited to elect additional MGMT polypeptide sequence(s), with specified SEQ ID NO: for each, to be searched. Additional MGMT polypeptide sequence(s) will be searched upon the payment of additional fees. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An Exemplary Election would be: SEQ ID NO: 38 (MGMT amino acid sequence).

Groups II+: Claim 13 is directed toward a nucleic acid sequence.

The nucleic acid sequence can be searched to the extent that it comprises SEQ ID NO: 1 (IL13 CAR-P140KMGMT DNA sequence). It is believed that Claim 13 (in-part) encompasses this first named invention and thus this claim can be searched without fee to the extent that it encompasses SEQ ID NO: 1 (IL13 CAR-P140KMGMT). Applicant is invited to elect additional nucleic acid sequence(s), with specified SEQ ID NO: for each, to be searched. Additional nucleic acid sequence(s) can be searched upon the payment of additional fees. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An Exemplary Election would be: a nucleic acid encompassing SEQ ID NO: 2 (IL-13(E13Y) CAR-P140KMGMT DNA sequence).

The inventions listed as Groups I+-II+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Groups I + include SEQ ID NO: 33 (MGMT amino acid sequence), which is not present in any of Groups II+, the special technical features of Groups II+ include SEQ ID NO: 1 (IL13 CAR-P140KMGMT DNA sequence), which is not present in any of Groups I+.

No technical features are shared between the nucleic acid sequences of Groups II+ and, accordingly, these groups lack unity a priori.

Groups I+-II+ share the technical features including nucleic acid sequences. Groups I+ share the technical features including: a chimeric nucleic acid sequence comprising a first nucleic acid sequence encoding in an IL 13CAR; a host cell comprising a first nucleic acid sequence encoding in an IL13CAR; a pharmaceutical composition comprising a first nucleic acid sequence encoding in an IL 13CAR; and a method for treating brain cancer in a subject in need thereof comprising administering to the subject one or more immune cells that express proteins encoded by a first nucleic acid sequence encoding in an N-terminal to C-terminal direction an IL13CAR: the IL13CAR comprising an IL13 ligand domain which binds the IL13a2 receptor (SEQ ID NO: 44), a transmembrane domain, a cytoplasmic domain comprising a CD3-zeta signaling domain; and a second nucleic acid sequence encoding an MGMT polypeptide, selected from the group consisting of SEQ ID NO: 33, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, and SEQ ID NO: 43; and a method for producing a mammalian cell which expresses an IL13CAR protein and an MGMT protein comprising: a) introducing into the cell a nucleic acid sequence encoding the IL 13CAR protein and the MGMT protein, wherein the IL13CAR protein comprises an IL13 ligand domain which binds the IL13a2 receptor (SEQ ID NO: 44), a transmembrane domain, and a cytoplasmic domain comprising a CD3-zeta signaling domain; and b) maintaining the cell under conditions in which the IL13CAR protein and the MGMT protein are expressed by the cell. Groups II+ share the technical features including: a nucleic acid sequence.

-***-Continued on Next Supplemental Page-***-

-***-Continued from Previous Supplemental Page:

However, these shared technical features are previously disclosed by the publication entitled 'Review Article: Interleukin-13 Receptor Alpha 2-Targeted Glioblastoma Immunotherapy' by Sengupta, et al. (hereinafter 'Sengupta') and further in view of the publication entitled 'Isolation and Structural Characterization of a cDNA Clone Encoding The Human DNA Repair Protein For O6-Alkylguanine: UniProtKB Accession P16455' by Tano, et al. (hereinafter 'Tano') and US 8,822,647 B2 (JENSEN).

Sengupta discloses nucleic acid sequences (a retrovirus encoding a chimeric antigen receptor (nucleic acid sequences); page 3, column 2, paragraph 2); a chimeric nucleic acid sequence comprising a first nucleic acid sequence encoding an IL13CAR (a retrovirus (chimeric nucleic acid sequence) comprising a first nucleic acid sequence encoding in an IL13CAR; page 3, column 2, paragraph 2); a host cell (an autologous T cell (a host cell); page 2, column 2, paragraph 2) comprising a first nucleic acid sequence encoding an IL13CAR (comprising a retrovirus encoding an IL13CAR; page 3, column 2, paragraph 2); and a method for treating brain cancer in a subject in need thereof (a method of eliminating glioma targets in a subject; page 3, column 2, paragraph 2) comprising administering to the subject one or more immune cells (comprising administering to the subject CAR expressing T cells; page 3, column 2, paragraph 2) that express proteins encoded by a first nucleic acid sequence encoding an IL13CAR (that express a retrovirally encoded IL13CAR; page 3, column 2, paragraph 2): the IL13CAR comprising an IL13 ligand domain which binds the IL13(alpha)2 receptor (the IL13CAR comprising an IL13 ligand domain which binds the IL13(alpha)2 receptor; page 3, column 2, paragraph 2) and a cytoplasmic domain comprising a CD3-zeta signaling domain (page 3, column 2, paragraph 2); and a method for producing a mammalian cell which expresses an IL13CAR protein (producing IL13CAR expressing T-cells via retroviral transformation at high efficiency; page 3, column 2, paragraph 2) comprising: a) introducing into the cell a nucleic acid sequence encoding the IL13CAR protein (comprising delivering to the T-cells a retrovirus encoding the IL13CAR protein; page 3, column 2, paragraph 2), wherein the IL13CAR protein comprises an IL13 ligand domain which binds the IL13(alpha)2 receptor (wherein the IL13CAR protein comprises an IL13 ligand domain which binds the IL13 (alpha)2 receptor; page 3, column 2, paragraph 2), and a cytoplasmic domain comprising a CD3-zeta signaling domain (page 3, column 2, paragraph 2); and b) maintaining the cell under conditions in which the IL13CAR protein is expressed by the cell (wherein the cells express the IL13CAR; page 3, column 2, paragraph 2); wherein FDA-directed GBM standard care includes therapy with temozolomide (TMZ), which induces lymphopenia (wherein FDA-directed GBM standard care includes therapy with temozolomide (TMZ), which induces lymphopenia; page 4, column 1, paragraph 2), and reduced expression or deletion of O-6-methylguanine DNA-methyltransferase (MGMT) (and reduced expression or deletion of O-6-methylguanine DNA-methyltransferase (MGMT); page 4, column 2, paragraph 1); wherein mutation of proline at residue 140 of MGMT to lysine was protective chemoprotective against the effects of TMZ (page 4, column 2, paragraph 1); and using similar chemoprotection during GBM-targeted adoptive T cell-mediated immunotherapy (page 4, column 2, paragraph 1).

Sengupta does not disclose a pharmaceutical composition comprising a first nucleic acid sequence encoding an IL13CAR; in an N-terminal to C-terminal direction; a transmembrane domain; and a second nucleic acid sequence encoding an MGMT polypeptide, selected from the group consisting of SEQ ID NO: 33, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, and SEQ ID NO: 43; a method for producing a mammalian cell which expresses an MGMT protein; maintaining the cell under conditions in which the MGMT protein is expressed by the cell.

Tano discloses the amino acid sequence (pages 7, 8) of human O-6-methylguanine DNA-methyltransterase (of human O-6-methylguanine DNA-methyltransferase; page 1); wherein the sequence differs from SEQ ID NO: 33 of the instant PCT application by a single mutation of proline to lysine at residue 140 (having an amino acid sequence (wherein the sequence differs from SEQ ID NO: 33 of the instant PCT application by a single mutation of proline to lysine at residue 140), pages 7, 8; wherein the disclosed amino acid sequence is identical to SEQ ID NO: 33 of the instant PCT application, with the exception of a mutation of proline to lysine at residue 140).

Jensen discloses a pharmaceutical composition (a composition for anti-tumor effector functioning (a pharmaceutical composition); column 2, lines 19-22) comprising a first nucleic acid sequence encoding (column 3, lines 14-17) an IL13CAR (column 2, lines 19-47); and a transmembrane domain (column 2, lines 44-45).

It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of Sengupta, for including the use of a pharmaceutical composition comprising the nucleic acid encoding an IL13 CAR having a transmembrane domain, as previously disclosed by Jensen, including the retroviral CAR encoding construct of Sengupta, modified to include the transmembrane domain, with appropriate positioning of the domains in N-terminal to C-terminal, for producing a functional CAR molecule, as disclosed by Jensen, for enabling the administration of the construct to a subject in need thereof; wherein the construct may have further included a domain or polypeptide encoding an MGMT polypeptide comprising a proline to lysine mutation at position 140, as disclosed by Sengupta, for enabling chemoresistance to TMZ, in order to ensure the survival and proliferation of the CAR-expressing T cells. Additionally, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of Sengupta, for integrating the sequence of an appropriate MGMT polypeptide, such as the human sequence, as disclosed by Tano, for elucidating the sequence thereof, and illustrating the position of the mutated proline residue at position 140 to lysine, as disclosed by Sengupta, for enabling a laboratory practitioner to construct such a molecule, through routine experimentation and testing.

Since none of the special technical features of the Groups I+-II+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Sengupta, Tano and Jensen references, unity of invention is lacking.

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(54)发明名称

治疗癌症的方法和组合物

(57)摘要

本文描述了涉及免疫细胞的组合物和方法，所述免疫细胞表达结合IL13受体α-2(IL13Rα2)的嵌合抗原受体和O^6-甲基鸟嘌呤DNA甲基转移酶(MGMT)蛋白质这两者。包含IL13嵌合抗原受体(IL13CAR)或其变体以及MGMT蛋白质或其变体的病毒颗粒用于转染诸如T细胞之类的免疫细胞，向转染的细胞赋予IL13Rα2靶向活性和对化疗剂替莫唑胺(TMZ)的耐药性。本文描述的组合物和方法在诸如治疗高度恶性胶质瘤之类的癌症治疗方面有用。

CN 107709353 A

1.嵌合核酸序列,其包含:

编码IL13CAR的第一核酸序列,IL13CAR包含:

结合IL13α2受体的IL13配体结构域(SEQ ID NO:44),

跨膜结构域,

包含CD3-ζ信号传导结构域的细胞质结构域;以及

编码MGMT多肽的第二核酸序列,MGMT多肽选自:SEQ ID NO:33、SEQ ID NO:38、SEQ ID NO:39、SEQ ID NO:40、SEQ ID NO:41和SEQ ID NO:43。

2.如权利要求1所述的嵌合核酸序列,其中,IL13CAR还包含单个肽。

3.如权利要求1或2所述的嵌合核酸序列,其中,IL13CAR还包含铰链区。

4.如权利要求1至3中任一项所述的嵌合核酸序列,其中,所述细胞质结构域还包含选自CD28、4-1BB(CD137)和OX40(CD134)的共刺激结构域。

5.如权利要求4所述的嵌合核酸序列,其中,CD28共刺激结构域与SEQ ID NO:29具有至少90%的一致性。

6.如权利要求1至5中任一项所述的嵌合核酸序列,其中,CD3-ζ信号传导结构域与SEQ ID NO:30具有至少90%的一致性。

7.如权利要求1至5中任一项所述的嵌合核酸序列,其还包含编码自裂解连接体肽的第三核酸序列。

8.如权利要求7所述的嵌合核酸序列,其中,所述第三核酸序列位于所述第一核酸和所述第二核酸之间。

9.如权利要求1至8中任一项所述的嵌合核酸序列,其中,所述第一核酸还包含下述核酸序列,该核酸序列编码位于IL13结构域和铰链结构域之间、铰链结构域和跨膜结构域之间、跨膜结构域和CD28信号传导结构域之间或CD28信号传导结构域和CD3-ζ信号传导结构域之间的二肽连接体。

10.如权利要求1至9中任一项所述的嵌合核酸序列,其还包含编码位于CD3-ζ信号传导结构域和自裂解肽之间的连接体肽的核酸序列。

11.如权利要求1至10中任一项所述的嵌合核酸序列,其中,IL13配体结构域包含下述氨基酸序列,该氨基酸序列与选自SEQ ID NO:26、SEQ ID NO:36和SEQ ID NO:37及其变体的序列具有至少90%的一致性。

12.如权利要求1至10中任一项所述的嵌合核酸序列,其选自:SEQ ID NO:1核苷酸109至1839、SEQ ID NO:2核苷酸109至1839、以及SEQ ID NO:3核苷酸109至1839。

13.包含SEQ ID NO:1(IL13CAR-P140KMGMT)、SEQ ID NO:2(IL-13(E13Y)CAR-P140KMGMT)、SEQ ID NO:3(IL-13(E13K R109K)CAR-P140KMGMT)或其组合的核酸序列。

14.包含权利要求1至12中任一项所述的嵌合核酸序列的载体。

15.如权利要求14所述的载体,其中,所述载体是病毒载体。

16.如权利要求15所述的载体,其中,所述病毒载体是逆转录病毒载体。

17.包含权利要求1至12中任一项所述的嵌合核酸序列的宿主细胞。

18.如权利要求17所述的宿主细胞,其中,所述细胞是哺乳动物细胞。

19.如权利要求17或18所述的宿主细胞,其中,所述细胞是T细胞。

20.如权利要求17至19中任一项所述的宿主细胞,其中,所述细胞是自体细胞或人白细

胞抗原(HLA)-相合细胞。

21.如权利要求17至20中任一项所述的宿主细胞,其中,所述细胞获自患有脑癌的一个或多个个体。

22.宿主细胞,其包含:

编码IL13CAR的第一核酸序列,IL13CAR包含:

结合IL13α2受体的IL13配体结构域(SEQ ID NO:44),

跨膜结构域,

包含CD3-ζ信号传导结构域的细胞质结构域;以及

编码MGMT多肽的第二核酸序列,MGMT多肽选自:SEQ ID NO:33、SEQ ID NO:38、SEQ ID NO:39、SEQ ID NO:40、SEQ ID NO:41和SEQ ID NO:43。

23.包含权利要求17至21中任一项所述的宿主细胞的药物组合物。

24.药物组合物,其包含:

编码IL13CAR的第一核酸序列,IL13CAR包含:

结合IL13α2受体的IL13配体结构域(SEQ ID NO:44),

跨膜结构域,

包含CD3-ζ信号传导结构域的细胞质结构域;以及

编码MGMT多肽的第二核酸序列,MGMT多肽选自:SEQ ID NO:33、SEQ ID NO:38、SEQ ID NO:39、SEQ ID NO:40、SEQ ID NO:41和SEQ ID NO:43。

25.用于生成表达IL13CAR蛋白质和MGMT蛋白质的哺乳动物细胞的方法,所述方法包括:

a)将编码IL13CAR蛋白质和MGMT蛋白质的核酸序列引入至所述细胞,其中,IL13CAR蛋白质包含:结合IL13α2受体的IL13配体结构域(SEQ ID NO:44)、跨膜结构域、包含CD3-ζ信号传导结构域的细胞质结构域;以及

b)在由所述细胞表达IL13CAR蛋白质和MGMT蛋白质的条件下维持所述细胞。

26.如权利要求25所述的方法,其中,所述核酸序列通过病毒载体引入至所述细胞。

27.如权利要求26所述的方法,其中,所述病毒载体选自:逆转录病毒载体、慢病毒载体、腺病毒载体或腺相关病毒载体。

28.治疗有此需要的受治者体内的脑癌的方法,所述方法包括向所述受治者给药一种或多种表达由下列核酸序列编码的蛋白质的免疫细胞:

在N-端至C-端方向编码IL13CAR的第一核酸序列,IL13CAR包含:

结合IL13α2受体的IL13配体结构域(SEQ ID NO:44),

跨膜结构域,

包含CD3-ζ信号传导结构域的细胞质结构域;以及

编码MGMT多肽的第二核酸序列,MGMT多肽选自:SEQ ID NO:33、SEQ ID NO:38、SEQ ID NO:39、SEQ ID NO:40、SEQ ID NO:41和SEQ ID NO:43。

29.如权利要求28所述的方法,其中,所述脑癌是高度恶性胶质瘤。

30.如权利要求28或29所述的方法,其中,所述受治者正在接受DNA-甲基化化疗剂的治疗、已接受DNA-甲基化化疗剂的治疗或将要接受DNA-甲基化化疗剂的治疗。

31.如权利要求30所述的方法,其中,所述DNA-甲基化化疗剂是TMZ。

32.如权利要求30或31所述的方法,其中,所述DNA-甲基化化疗剂在给药一剂免疫细胞之前、给药一剂免疫细胞过程中或给药一剂免疫细胞之后给药。

治疗癌症的方法和组合物

[0001] 相关申请的交叉引用

[0002] 本申请要求2014年12月2日提交的美国临时申请US62/086,346的优先权权益，该美国临时申请的全部内容通过引用并入本文。

[0003] 序列的交叉引用

[0004] “序列表”以txt文件的形式随本申请一同提交，该“序列表”于2015年11月30日生成，并且命名为“0962018010WOseqlist.txt”(69892字节)，序列表的全部内容通过引用并入本文。

背景技术

[0005] 靶向免疫疗法近些年已成为治疗恶性肿瘤方面的有希望的研究领域并且获得了大量关注(Carpentier and Meng,2006,Curr Opin Oncol,18(6):631-636;Wainwright et al.,2012,Exp Opin Emerging Drugs;17(2):181-202)。最广泛研究的靶点之一是白介素-13受体α2(IL13Rα2)(Thaci et al.,2014,Neuro-Oncol,16(10):1304-1324)。IL13Rα2是白介素-13(IL13)的诱捕受体，缺乏在普遍存在的IL13Rα1上存在的信号传导链，因此阻止了任何IL13-介导的下游信号传导通路(Arima et al.,2005,J Biol Chem,280(26):24915-24922)。已报道IL13Rα2的表达的增加促进胶质瘤和其他肿瘤模型中的肿瘤恶化。IL13Rα2的表达是胶质瘤恶性分级和预后不良患者存活率的预后标志物(Brown et al.,2013,PLoS ONE,8(10):Article ID e77769)。大约二十年前已发现了IL13Rα2在MG上的选择性表达，从那时起IL13Rα2在MG上的选择性表达已作为治疗靶点(Debinski et al.,1999,Clin Canc Res,5(5):985-990)。

[0006] 胶质母细胞瘤是成人体内最常见的原发性脑肿瘤。在美国，每年被诊断为患有恶性原发性脑肿瘤的18,000位患者中超过一半的患者患有多形性胶质母细胞瘤。多形性胶质母细胞瘤是退行发育的巨细胞瘤，具有高增殖指数，微血管增殖和局灶性坏死。多形性胶质母细胞瘤的指征和症状基于多种因素(尺寸、生长速度、肿瘤在脑内部的位置)，并且多形性胶质母细胞瘤的指征和症状主要由头痛、痉挛、神经功能缺陷、精神状态的改变代表。多形性胶质母细胞瘤的预后仍然很差。大部分患者的存活时间少于2年。

[0007] 虽然采用目前的标准对胶质母细胞瘤(GBM)进行护理在存活率方面取得了很大的改善，所述目前的标准是手术、放疗和化疗的三重方案(Rolle et al.,2010,Neurosurgery Clin of North America,21(1):201-214;Ashby and Ryken,2006,Neurosurgical focus,20(4):E3)，但是，大多数患者的预后仍然很差(Stupp et al,2009,Lancet Oncol,10(5):459-466;Omuro and DeAngelis,2013,JAMA,310(17):1842-1850)。治疗GBM的主要限制是肿瘤在脑内部的位置，其妨碍细胞毒性药剂跨过血脑屏障的递送(Ashby and Ryken,2006,Neurosurgical Focus,20(4):E3)，以及强的免疫抑制环境(Rolle et al.,2012,Adv Exp Med Biol,746:53-76)和放化疗耐受的胶质瘤起始细胞(Bao et al,2006,Nature,444(7120):756-760;Frosina,Mol Canc Res,2009 7(7):989-999)。因此，本领域需要继续测试新的治疗策略以改善患者存活率、生活质量以及总体结果。

[0008] 因此，本文描述了用于更加有效地治疗恶性肿瘤细胞表达或过表达IL13Rα2的癌症(包括脑癌)的组合物和方法。

发明内容

[0009] 一方面，本文提供嵌合核酸序列，其中，嵌合核酸序列包括编码结合IL1Rα2受体(IL13Rα2)的IL13嵌合抗原受体(IL13CAR)的第一核酸和编码耐药性多肽的第二核酸，所述耐药性多肽是O^6-甲基鸟嘌呤DNA甲基转移酶(MGMT)蛋白质。

[0010] 在一种实施方式中，IL13CAR包括IL13Rα2的配体。在另一实施方式中，所述配体是IL13。在又一实施方式中，所述配体是结合IL13Rα2的IL13的片段。在再一实施方式中，配体是选择性结合IL13Rα2的抗体可变结构域或其片段。

[0011] 在一种实施方式中，MGMT蛋白质包括P140K取代。

[0012] 在一种实施方式中，嵌合核酸序列包括编码IL13CAR的第一核酸序列和编码MGMT蛋白质的第二核酸序列。

[0013] 在一种实施方式中，所述编码IL13CAR的第一核酸序列是编码MGMT多肽的5'-第二核酸序列。在可选的实施方式中，所述编码IL13CAR的第一核酸序列是编码MGMT多肽的3'-第二核酸序列。

[0014] 在一种实施方式中，所述编码IL13CAR的第一核酸序列在5'-3'方向上包括：编码IL13Rα2配体结构域的核酸序列，编码跨膜(TM)结构域的核酸序列，以及编码包括CD3ζ信号传导结构域的细胞质结构域的核酸序列。在另一实施方式中，所述第一核酸序列还包括编码铰链区的核酸序列，其中，所述铰链区位于IL13配体结构域和TM结构域之间。在又一实施方式中，所述第一核酸序列还包括编码CD28共刺激结构域的核酸序列，其中，CD28共刺激结构域位于TM结构域和CD3ζ链之间。在再一实施方式中，所述第一核酸序列还包括编码信号序列的核酸，其中，所述信号序列位于IL13Rα2配体结构域的N-端。

[0015] 在一种实施方式中，铰链结构域是CD8铰链结构域。在另一实施方式中，CD8铰链结构域包括SEQ ID NO:27。

[0016] 在一种实施方式中，细胞质结构域还包括一个或多个共刺激结构域。在一种实施方式中，共刺激结构域是CD28共刺激结构域。在另一实施方式中，CD28共刺激结构域位于TM结构域和CD3-ζ信号传导结构域之间。

[0017] 在一种实施方式中，细胞质结构域还包括一个或多个选自下列的共刺激结构域：OX-40共刺激结构域，HVEM共刺激结构域，41BB共刺激结构域，ICOS共刺激结构域，OX40共刺激结构域和CD27共刺激结构域。在一种实施方式中，另外的共刺激结构域位于CD28共刺激结构域和CD3ζ信号传导结构域之间。

[0018] 在一种实施方式中，信号序列是异质信号序列。在另一实施方式中，信号序列是IL13信号序列或其变体。在又一实施方式中，IL13信号序列包括SEQ ID NO:25。在再一实施方式中，编码信号序列的核酸序列包括SEQ ID NO:9。

[0019] 在一种实施方式中，IL13配体结合结构域包括成熟的IL13蛋白质(SEQ ID NO:26)。在另一实施方式中，IL13配体结合结构域由成熟的IL13的片段构成，其中，所述片段结合IL13Rα2蛋白质，该IL13Rα2蛋白质具有与成熟的IL13蛋白质(SEQ ID NO:26)的亲和性大致相同的亲和性。

[0020] 在一种实施方式中，编码IL13配体的核酸序列编码选自SEQ ID NO:26，SEQ ID NO:36和SEQ ID NO:37的多肽。在另一实施方式中，编码成熟的IL13多肽的核酸序列包括选自SEQ ID NO:10，SEQ ID NO:34和SEQ ID NO:35的核酸序列。

[0021] 在一种实施方式中，所述第一核酸在5’-3’方向上包括：编码IL13配体结构域的选自SEQ ID NO:10、SEQ ID NO:34或SEQ ID NO:35的核酸，编码TM结构域的包含SEQ ID NO:14或其变体的核酸，以及编码CD3-ζ信号传导结构域的包含SEQ ID NO:18或其变体的核酸。在另一实施方式中，所述第一核酸还包含编码IL13信号序列的SEQ ID NO:9或其变体，其中，SEQ ID NO:9的序列是编码IL13配体结构域的核酸序列的上游。在另一实施方式中，所述第一核酸还包含编码CD8铰链结构域的SEQ ID NO:12或其变体。在又一实施方式中，所述第一核酸还包含编码CD28共刺激结构域的SEQ ID NO:16或其变体的核酸。

[0022] 在一种实施方式中，所述第一核酸在5’-3’方向上包括：编码信号传导结构域的SEQ ID NO:9或其变体的核酸序列，编码IL13配体结构域的选自SEQ ID NO:10、SEQ ID NO:34或SEQ ID NO:35的核酸，编码CD8铰链结构域的SEQ ID NO:12或其变体的核酸序列，编码TM结构域的包含SEQ ID NO:14或其变体的核酸，编码CD28共刺激结构域的SEQ ID NO:16或其变体的核酸，以及编码CD3ζ信号传导结构域的SEQ ID NO:18或其变体的核酸序列。

[0023] 在一种实施方式中，所述编码CAR的第一核酸序列还包括编码位于成熟IL13配体和CD8铰链结构域之间的连接体的核酸序列。在另一实施方式中，编码位于成熟IL13配体和CD8铰链结构域之间的连接体的核酸序列由SEQ ID NO:11构成。

[0024] 在一种实施方式中，所述编码CAR的第一核酸序列还包括编码位于SEQ ID NO:12和SEQ ID NO:14之间的连接体的核酸序列。在另一实施方式中，编码位于SEQ ID NO:12和SEQ ID NO:14之间的连接体的核酸序列由SEQ ID NO:13构成。

[0025] 在一种实施方式中，所述编码CAR的第一核酸序列还包括编码位于SEQ ID NO:14和SEQ ID NO:16之间的连接体的核酸序列。在另一实施方式中，编码位于SEQ ID NO:14和SEQ ID NO:16之间的连接体的核酸序列由SEQ ID NO:15构成。

[0026] 在一种实施方式中，所述编码CAR的第一核酸序列还包括编码位于SEQ ID NO:16设SEQ ID NO:18之间的连接体的核酸序列。在另一实施方式中，编码位于SEQ ID NO:16和SEQ ID NO:18之间的连接体的核酸序列由SEQ ID NO:17构成。

[0027] 在一种实施方式中，所述编码MGMT蛋白质的第二核酸序列包括P140KMGMT(SEQ ID NO:22)。在另一实施方式中，所述编码MGMT蛋白质的第二核酸序列包括编码包含SEQ ID NO:33的蛋白质的核酸序列。

[0028] 在一种实施方式中，所述编码MGMT蛋白质的第二核酸序列包括选自G156A-MGMT(SEQ ID NO:38)、MGMT-2(SEQ ID NO:39)、MGMT-3(SEQ ID NO:40)和MGMT-5(SEQ ID NO:41)的氨基酸序列。在另一实施方式中，所述编码MGMT蛋白质的第二核酸序列包括编码包含G156A-MGMT(SEQ ID NO:38)、MGMT-2(SEQ ID NO:39)、MGMT-3(SEQ ID NO:40)和MGMT-5(SEQ ID NO:41)的蛋白质的核酸序列。

[0029] 在一种实施方式中，所述编码MGMT蛋白质的第二核酸序列不包括SEQ ID NO:48。在另一实施方式中，所述编码MGMT蛋白质的第二核酸序列不包括SEQ ID NO:49。

[0030] 在一种实施方式中，嵌合核酸序列还包括编码自裂解肽的核酸序列。在另一实施方式中，编码所述自裂解肽的核酸序列包括SEQ ID NO:21。在又一实施方式中，所述自裂解

肽包括SEQ ID NO:32的氨基酸序列。

[0031] 在一种实施方式中,嵌合核酸序列还包括Kozak序列。在另一实施方式中,Kozak序列包括SEQ ID NO:8。在又一实施方式中,Kozak序列位于编码IL13CAR和MGMT蛋白质的核酸序列的上游。在一种实施方式中,嵌合核酸序列还包括位于Kozak序列的上游的第一限制性内切酶位点。在另一实施方式中,Kozak序列的上游的限制性内切酶位点由SEQ ID NO:7构成。在一种实施方式中,所述嵌合核酸序列还包括位于编码IL13CAR和MGMT蛋白质的核酸序列的下游的第二内切酶位点。

[0032] 在一种实施方式中,嵌合核酸序列包括选自SEQ ID NO:1的核苷酸109-1836、SEQ ID NO:2的核苷酸109-1836和SEQ ID NO:3的核苷酸109-1836的核苷酸序列。在一种实施方式中,嵌合核酸序列包括选自SEQ ID NO:1的核苷酸13-1836、SEQ ID NO:2的核苷酸13-1836和SEQ ID NO:3的核苷酸13-1836的核苷酸序列。在一种实施方式中,嵌合核酸序列包括选自SEQ ID NO:1的核苷酸7-1842、SEQ ID NO:2的核苷酸7-1842和SEQ ID NO:3的核苷酸7-1842的核苷酸序列。在另一实施方式中,嵌合核酸序列包括选自SEQ ID NO:1、SEQ ID NO:2和SEQ ID NO:3的核苷酸序列。

[0033] 在一种实施方式中,所述嵌合核酸序列包括编码SEQ ID NO:4、SEQ ID NO:5、SEQ ID NO:6、SEQ ID NO:45、SEQ ID NO:46或SEQ ID NO:47的蛋白质的核苷酸序列。

[0034] 在一种实施方式中,IL13Rα2受体配体是以比野生型IL13(SEQ ID NO:26)的亲和性低5倍、10倍或100倍的亲和性与IL13Rα2结合的IL-13的变体或其片段。在可选的实施方式中,IL13Rα2受体配体是以比野生型IL13(SEQ ID NO:26)的亲和性高5倍、10倍或100倍的亲和性与IL13Rα2结合的IL13的变体或其片段。

[0035] 在一种实施方式中,IL13Rα2受体配体不等同于野生型IL13。在另一实施方式中,IL13Rα2受体配体不等同于SEQ ID NO:26。

[0036] 在一种实施方式中,当采用化疗药剂处理IL13-CAR-T转染的细胞时,与采用不表达耐药性多肽的化疗剂处理的细胞相比,P140KMGMT蛋白质有效增加表达耐药性多肽的细胞的体外和/或体内活力。

[0037] 在一种实施方式中,IL13Rα2受体配体不等同于野生型IL-13。在另一实施方式中,IL13Rα2受体配体不等同于SEQ ID NO:26。

[0038] 另一方面,本文提供了包括编码本文所述的IL13CAR和MGMT蛋白质的核酸序列的载体。

[0039] 在一种实施方式中,所述载体包括编码本文所述的IL13CAR、自裂解肽和MGMT蛋白质的单顺反子核酸序列。在另一实施方式中,所述自裂解肽包括2A肽。

[0040] 在可选的实施方式中,所述载体包括编码IL13CAR和MGMT蛋白质的多顺反子嵌合核酸序列。在另一实施方式中,所述编码IL13CAR和MGMT蛋白质的多顺反子嵌合核酸序列还包括位于编码IL13CAR的核酸序列和编码MGMT蛋白质的核酸序列之间的内部核糖体进入位点(IRES)。在又一实施方式中,所述编码IL13CAR和MGMT蛋白质的多顺反子嵌合核酸序列还包括位于编码IL13CAR的核酸序列和编码MGMT蛋白质的核酸序列之间的启动子。

[0041] 在一种实施方式中,所述载体是细菌质粒载体。在另一实施方式中,所述载体是表达载体。

[0042] 在一种实施方式中,所述载体是病毒载体。在另一实施方式中,所述病毒载体选

自:逆转录病毒载体、慢病毒载体、腺病毒载体和腺相关病毒载体。

[0043] 另一方面,本文提供由包括编码本文所述的嵌合抗原受体(CAR)和耐药性多肽的嵌合核酸序列的载体转染的细胞。

[0044] 在一种实施方式中,所述细胞选自:T细胞,NK细胞和NKT细胞。

[0045] 另一方面,本文提供在N-端至C-端方向上包括与本文所述的肿瘤抗原、跨膜结构域和细胞质信号传导结构域结合的配体的重组多肽。

[0046] 在一种实施方式中,所述在N-端至C-端方向上包括与肿瘤抗原、跨膜结构域和细胞质信号传导结构域结合的配体的重组多肽还包括位于CAR和耐药性多肽之间的自裂解肽。在另一实施方式中,所述耐药性多肽是CAR的N-端。在又一实施方式中,所述耐药性多肽是CAR的C端。

[0047] 另一方面,本文提供包括修饰的MGMT多肽的重组多肽,该修饰的MGMT多肽增加暴露于TMZ的细胞的活力,其中,所述细胞从基因上进行修饰以表达本文所述的CAR,并且,其中,所述细胞被给药于诊断为患有脑癌的患者。

[0048] 另一方面,本文提供包括本文所述的编码CAR的第一核酸和本文所述的编码MGMT蛋白质的第二核酸的组合物。

[0049] 在一种实施方式中,所述第一核酸编码CAR蛋白质,该CAR蛋白质包括本文所述的IL13配体结构域、本文所述的TM结构域和本文所述的包括CD3ζ信号传导结构域的细胞质结构域。在另一实施方式中,所述第一核酸还编码位于CAR蛋白质的IL13配体结合结构域的上游的信号序列。在又一实施方式中,所述第一核酸还编码本文所述的铰链区,其中,所述铰链区位于IL13配体结构域和CAR蛋白质的TM结构域之间。在再一实施方式中,所述第一核酸还编码位于TM结构域和CD3-ζ信号传导结构域之间的CD28共刺激结构域。在又一实施方式中,所述第一核酸还编码另外的共刺激结构域。在另一实施方式中,所述第一核酸还包括位于编码CAR蛋白质的核酸的上游的Kozak序列。

[0050] 在一种实施方式中,所述第二核酸编码具有选自SEQ ID NO:33、SEQ ID NO:38、SEQ ID NO:39、SEQ ID NO:40、SEQ ID NO:41和SEQ ID NO:43的氨基酸序列的MGMT蛋白质。

[0051] 在一种实施方式中,MGMT蛋白质不包括SEQ ID NO:49。

[0052] 在一种实施方式中,所述组合物包括嵌合核酸,该嵌合核酸包括所述第一核酸和所述第二核酸。

[0053] 在一种实施方式中,所述嵌合核酸还包括编码本文所述的自裂解连接体肽的核酸,其中,所述编码自裂解连接体肽的核酸位于所述第一核酸和所述第二核酸之间。

[0054] 在一种实施方式中,所述嵌合核酸还包括本文所述的内部核糖体进入位点(IRES),其中,IRES位于所述第一核酸和所述第二核酸之间。

[0055] 在一种实施方式中,嵌合核酸是双顺反子构建体,其包括位于编码CAR蛋白质的第一核酸的上游的第一启动子和位于编码MGMT蛋白质的第二核酸的上游的第二启动子。

[0056] 在一种实施方式中,所述组合物包括第一载体和第二载体,所述第一载体包括编码CAR蛋白质的第一核酸,所述第二载体包括编码MGMT蛋白质的第二核酸。在另一实施方式中,所述第一载体和所述第二载体均是质粒或表达载体。在又一实施方式中,所述第一载体和所述第二载体均为逆转录病毒颗粒。

[0057] 另一方面,本文提供包括编码本文所述的CAR的第一核酸和编码本文所述的MGMT

蛋白质的第二核酸的宿主细胞。在一种实施方式中，所述第一核酸编码CAR蛋白质，该CAR蛋白质包括本文所述的IL13配体结构域、本文所述的TM结构域和本文所述的包括CD3ζ信号传导结构域的细胞质结构域。在另一实施方式中，所述第一核酸还编码位于CAR蛋白质的IL13配体结合结构域的上游的信号序列。在又一实施方式中，所述第一核酸还编码本文所述的铰链区，其中，所述铰链区位于CAR蛋白质的IL13配体结构域和TM结构域之间。在再一实施方式中，所述第一核酸还编码位于TM结构域和CD3-ζ信号传导结构域之间的CD28共刺激结构域。在再一实施方式中，所述第一核酸还编码另外的共刺激结构域。在另一实施方式中，所述第一核酸还包括位于编码CAR蛋白质的核酸的上游的Kozak序列。

[0058] 在一种实施方式中，所述第二核酸编码MGMT蛋白质，该MGMT蛋白质具有选自SEQ ID NO:33、SEQ ID NO:38、SEQ ID NO:39、SEQ ID NO:40、SEQ ID NO:41和SEQ ID NO:43的氨基酸序列。在另一实施方式中，MGMT蛋白质不是SEQ ID NO:49。

[0059] 在一种实施方式中，所述宿主细胞包括嵌合核酸，该嵌合核酸包括所述第一核酸和所述第二核酸。

[0060] 在一种实施方式中，所述嵌合核酸还包括编码本文所述的自裂解连接体肽的核酸，其中，所述编码自裂解连接体肽的核酸位于所述第一核酸和所述第二核酸之间。

[0061] 在一种实施方式中，所述嵌合核酸还包括本文所述的内部核糖体进入位点(IRES)，其中，IRES位于所述第一核酸和所述第二核酸之间。

[0062] 在一种实施方式中，所述嵌合核酸是双顺反子(dicistronic)构建体，其包括位于编码CAR蛋白质的第一核酸的上游的第一启动子和位于编码MGMT蛋白质的第二核酸的上游的第二启动子。

[0063] 在一种实施方式中，所述宿主细胞包括第一载体和第二载体，所述第一载体包括编码CAR蛋白质的第一核酸，所述第二载体包括编码MGMT蛋白质的第二核酸。在另一实施方式中，所述第一载体和所述第二载体均为本文所述的质粒或表达载体。在又一实施方式中，所述第一载体和所述第二载体均为本文所述的逆转录病毒颗粒。

[0064] 另一方面，本文提供包括重组多肽以及药学上可接受的赋形剂的组合物，其中，所述重组多肽在N-端至C-端方向上包括本文所述的信号序列、本文所述的IL13配体、本文所述的跨膜结构域、本文所述的细胞质信号传导结构域、本文所述的MGMT蛋白质。在另一实施方式中，所述重组多肽还包括本文所述的铰链结构域，其中，所述铰链结构域位于IL13配体结构域和跨膜结构域之间。在又一实施方式中，所述重组多肽还包括本文所述的自裂解肽，其中，所述自裂解多肽位于细胞质信号传导结构域和MGMT蛋白质之间。

[0065] 在一种实施方式中，所述组合物是药物组合物。

[0066] 另一方面，本文提供用于治疗诊断患有癌症的受治者的方法。

[0067] 在一种实施方式中，所述方法包括从所述受治者中获取细胞，采用编码本文所述的IL13CAR和本文所述的MGMT蛋白质的一种或多种核酸转导所述细胞，在由所述细胞表达所述核酸的条件下维持所述细胞，以及将治疗有效量的表达IL13CAR和MGMT蛋白质的细胞给药于患者。

[0068] 在一种实施方式中，引入至所述细胞包括使用包括编码IL13CAR的核酸的第一载体和包括编码MGMT蛋白质的核酸的第二载体。在另一实施方式中，引入至所述细胞包括使用包括编码IL13CAR和MGMT蛋白质的核酸的载体。

[0069] 在一种实施方式中，所述细胞被包括本文所述的IL13CAR-P140KMGMT嵌合构建体的载体转导。

[0070] 在一种实施方式中，所述受治者是哺乳动物。在另一实施方式中，所述哺乳动物是灵长类、人类或鼠类。

[0071] 在一种实施方式中，使用选自逆转录病毒载体、慢病毒载体、腺病毒载体或其组合的病毒载体将一种或多种核酸引入至所述细胞。

[0072] 在一种实施方式中，所述细胞是T细胞。

[0073] 在一种实施方式中，所述T细胞采用血浆分离术获得。

[0074] 在一种实施方式中，所述受治者已被诊断患有选自脑癌、乳腺癌、胰腺癌、头颈癌、卵巢癌和结肠直肠癌的癌症。在另一实施方式中，所述癌症已经转移。

[0075] 在一种实施方式中，所述受治者已被诊断患有高度恶性胶质瘤。在另一实施方式中，所述受治者已被诊断患有多形性胶质母细胞瘤(GMB)、间变性星形细胞瘤或儿童胶质瘤。

[0076] 在一种实施方式中，脑癌是胶质母细胞瘤。在另一实施方式中，脑癌是高度星形细胞瘤。

[0077] 在一种实施方式中，乳腺癌是基底样乳腺癌。

[0078] 在一种实施方式中，所述方法还包括采用一种或多种化疗剂治疗所述受治者。在另一实施方式中，所述方法包括采用替莫唑胺(TMZ)治疗所述受治者。

[0079] 在一种实施方式中，所述一种或多种化疗剂在给药一剂修饰的细胞之前、给药一剂修饰的细胞过程中和/或给药一剂修饰的细胞之后给药于所送受治者。

[0080] 在一种实施方式中，所述给药是颅内给药、髓内给药、皮内给药、皮下给药、局部给药或静脉内给药。

[0081] 另一方面，本文提供用于生成表达本文所述的IL13CAR-P140KMGMT构建体的细胞的方法，该方法包括将编码IL13CAR-P140KMGMT嵌合蛋白质的核酸序列引入至所述细胞，在由所述细胞表达IL13CAR-P140KMGMT嵌合蛋白质的条件下维持所述细胞。

[0082] 在一种实施方式中，所述细胞是哺乳动物细胞。在另一实施方式中，所述哺乳动物细胞是人细胞、灵长类动物细胞或鼠类细胞。

[0083] 在一种实施方式中，所述细胞是T细胞。在另一实施方式中，所述细胞选自体细胞或人白细胞抗原(HLA)-相合细胞。

[0084] 在一种实施方式中，所述细胞获自被诊断为患有脑癌或恶性肿瘤的一个或多个受治者。

[0085] 另一方面，本文提供包括IL13CAR-P140KMGMT构建体的细胞群。在另一实施方式中，所述细胞群中至少约50％、60％、70％、80％、90％或95％的细胞表达本文提供的IL13CAR-P140KMGMT构建体。

[0086] 一方面，本发明涉及编码嵌合抗原受体(CAR)(具有嵌合抗原受体(CAR)，包括嵌合抗原受体(CAR)，基本由嵌合抗原受体(CAR)构成或由嵌合抗原受体(CAR)构成)的(一种或多种)分离的核酸序列，所述嵌合抗原受体(CAR)包括T细胞受体(基本由T细胞受体构成，由T细胞受体构成)，所述T细胞受体表达脑癌的一种或多种肿瘤抗原的一种或多种配体(例如，抗体)。在一些方面，CAR还表达用于治疗脑癌的一种或多种额外的药剂。

[0087] 另一方面，本发明涉及表达构建体，所述表达构建体包括编码CAR的一种或多种核酸序列(基本由编码CAR的一种或多种核酸序列构成，由编码CAR的一种或多种核酸序列构成)，CAR包括表达脑癌的一种或多种肿瘤抗原(例如，癌症抗原结合结构域)的一种或多种配体(例如，抗体)的T细胞受体。在一些方面，CAR还表达用于治疗脑癌的一种或多种额外的药剂。

[0088] 另一方面，本发明涉及包含表达构建体(基本由表达构建体构成，由表达构建体构成)的宿主细胞，所述表达构建体包括编码包含T细胞受体的CAR的一种或多种核酸序列，所述T细胞受体表达脑癌的一种或多种肿瘤抗原的一种或多种配体(例如，抗体)。在一些方面，CAR还表达用于治疗脑癌的一种或多种额外的药剂。

[0089] 另一方面，本发明涉及生成表达CAR的细胞的方法，所述CAR包括T细胞受体(基本由T细胞受体构成，由T细胞受体构成)，所述T细胞受体包括脑癌的一种或多种肿瘤抗原的一种或多种配体(例如，抗体)。在特定的方面，CAR还包括用于治疗脑癌的一种或多种额外的药剂。

[0090] 另一方面，本发明涉及包含T细胞受体(具有T细胞受体，基本由T细胞受体构成，由T细胞受体构成)的CAR多肽，所述T细胞受体包括脑癌的一种或多种肿瘤抗原的一种或多种配体(例如，抗体)。在特定的方面，CAR还包括用于治疗脑癌的一种或多种额外的药剂。

[0091] 另一方面，本发明涉及治疗有这种治疗需求的个体体内的脑癌的方法，所述方法包括给药一种或多种T细胞(基本由给药一种或多种T细胞构成，由给药一种或多种T细胞构成)，所述一种或多种T细胞表达包含T细胞受体的CAR，所述T细胞受体表达脑癌的一种或多种肿瘤抗原的一种或多种配体(例如，抗体)。在一些方面，CAR还表达用于治疗脑癌的一种或多种额外的药剂。

[0092] 本发明还涉及包含本文提供的组合物的药物组合物。

附图说明

[0093] 通过下文对本发明的实施方式的更加具体的描述，如在附图中所举例说明的，上述内容将会变得明白。在附图中的不同的视图中，相同的附图标记指代相同的部分。附图不必须按照比例，重点是举例说明本发明的实施方式。

[0094] 图1A提供了IL13E13K.R109K CAR核酸构建体的示意图。

[0095] 图1B提供了包含IL13E13K.R109K CAR的pMFG宿主质粒的质粒图谱。

[0096] 图2A提供了IL13E13K.R109K CAR-2A-P140KMGMT的示意图。

[0097] 图2B提供了包含IL-13-CAR-2A-P140KMGMT的pMFG宿主质粒的质粒图谱。

[0098] 图3A至图3C举例说明富集之前(图3A)和富集之后(图3B)由包含IL13CAR构建体和IL13CAR-2A-P140KMGMT构建体的病毒转导的PG13细胞的FACS分析。图3C举例说明细胞溶解产物的western印迹分析。

[0099] 图4提供了显示由包含IL13CAR-2A-P140KMGMT构建体的逆转录病毒转导的且暴露于TMZ的T细胞的活力的图。

[0100] 图5A和图5B举例说明由本文所述的IL13CAR-2A-MGMT构建体转染的细胞内的IL2(图5A)和IFNγ(图5B)的分泌。

[0101] 图6举例说明带有肿瘤的并且被给药本文所述的嵌合构建体和/或化疗剂的小鼠

的活力。

[0102] 图7A至图7B提供了IL13CAR-P140KMGMT构建体的嵌合核酸序列(图7A,SEQ ID NO:1)和氨基酸序列(图7B,SEQ ID NO:4)。

[0103] 图8A至图8B提供了IL13(E13Y)CAR-P140KMGMT构建体的嵌合核酸序列(图8A,SEQ ID NO:2)和氨基酸序列(图8B,SEQ ID NO:5)。

[0104] 图9A至图9B提供了IL13(E13K.R109K)CAR-P140KMGMT构建体的嵌合核酸序列(图9A,SEQ ID NO:3)和氨基酸序列(图9B,SEQ ID NO:6)。

具体实施方式

[0105] 本文描述了(一种或多种)嵌合抗原受体(CAR)的生成,该嵌合抗原受体包含通过基因工程修饰的T细胞受体,从而表达癌症的一种或多种肿瘤抗原的一种或多种配体(例如,抗体或细胞表面蛋白质的其他配体)。具体而言,本文描述的CAR蛋白质包括与在癌细胞表面上表达的蛋白质结合的配体结合结构域。优选地,肿瘤抗原不在未患病的细胞或正常细胞的表面上表达,或以比在癌细胞或其他患病的细胞上表达的水平低得多的水平在未患病的细胞或正常细胞上表达。修饰为表达得到的CAR的T细胞通过CAR的新的特异性重新定向以攻击表达由CAR识别的表面抗原(例如,受体)的肿瘤。本文还表现出,CAR可进一步包括用于治疗脑癌的一种或多种额外的药剂(例如,克服脑癌细胞对治疗的耐药性的药剂)。具体而言,本文所述的组合物和方法被设计为治疗其中恶性肿瘤细胞表达IL13α2受体(IL13Rα2)的脑癌。因此,使用表达并展示IL13Rα2的配体的CAR来举例说明本文公开的内容,例如,细胞因子白介素13(IL13)或选择性结合IL13Rα2的抗体的可变结构域。IL13CAR采用O(6)-甲基鸟嘌呤-DNA-甲基转移酶(MGMT)基因表达。在一种实施方式中,MGMT基因被修饰为编码蛋白质,该蛋白质向宿主细胞(例如,T细胞)赋予替莫唑胺(TMZ,一种用于治疗脑癌的化疗剂)耐药性或提高宿主细胞(例如,T细胞)对替莫唑胺(TMZ)的耐药性。

[0106] 在一种优选的实施方式中,IL13CAR包括在位置13(相对于SEQ ID NO:26进行编号)突变以将谷氨酸改变为酪氨酸的IL13。在可选的优选实施方式中,IL13在位置13进行突变以将谷氨酸改变为赖氨酸并且在位置109进行突变以将精氨酸改变为赖氨酸(氨基酸位置13和位置109是相对于例如SEQ ID NO:26)。在一种实施方式中,IL13被突变,使得位置109的氨基酸从精氨酸变为赖氨酸。

[0107] 在一种优选的实施方式中,修饰的MGMT基因编码MGMT变体,该MGMT变体在本文中称为P140KMGMT(SEQ ID NO:33),其保护表达IL13CAR-P140KMGMT的T细胞不受采用诸如TMZ之类的甲基化剂进行治疗导致的细胞毒性的影响。

[0108] 在一种实施方式中,IL13CAR和P140KMGMT蛋白质通过单顺反子构建体表达,该构建体被转录为生成编码单一蛋白质的单一转录子,所述单一蛋白质在N-端至C-端方向上包括IL13CAR,自裂解肽(2A)和P140KMGMT。在翻译该融合蛋白之后,自裂解肽的裂解产生主要集中在细胞核和IL13CAR的P140KMGMT蛋白质,其中,IL13配体结构域在宿主细胞的表面上展示。然而,应当理解的是,IL13CAR和P140KMGMT蛋白质可通过独立的核酸表达。例如,编码IL12CAR和P140KMGMT蛋白质的核酸可位于分离的载体中,所述载体随后被引入至相同的细胞,或者编码IL12CAR和P140KMGMT蛋白质的核酸可被克隆至作为独立的单顺反子构建体(例如,具有它们自己的启动子的构建体)的单一载体中。

[0109] 在存在TMZ的条件下，与IL13CAR转导的不表达P140KMGMT的T细胞相比，由IL13CAR-2A-P140KMGMT构建体转导的T细胞更好地存活(例如，实施例6，图6)。因此，本文还公开了编码CAR和耐药性多肽(MGMT蛋白质)的核酸序列，一种或多种核酸或包含所述核酸的逆转录病毒载体，由一种或多种载体转染或转导的细胞，以及通过在修饰的T细胞中共表达修饰的MGMT基因提高暴露于诸如TMZ之类的化疗剂的基因修饰的T细胞的活力的方法。

[0110] 本文还涉及用于治疗诊断为患有癌症的受治者的方法，其中，所述癌症包括表达IL13Rα2的细胞。例如，诸如高度恶性胶质瘤之类的脑癌细胞和基底样乳腺癌细胞相对于相同组织的未患病的细胞或非癌细胞过表达IL13Rα2蛋白质。本发明要求保护的组合物在向受治者给药甲基化化疗剂和基因修饰的免疫细胞(例如，T细胞)这两者时特别有用，所述基因修饰的免疫细胞表达IL13CAR和修饰的MGMT基因。相对于仅采用化疗剂或基因修饰的T细胞进行治疗的受治者，本发明的方法可缩短治疗时间并且可更快更有效地实现胶质瘤消除。因此，一方面，本发明涉及编码用于T细胞的嵌合抗原受体(CAR)的(一种或多种)分离的核酸序列，所述T细胞表达癌症的一种或多种肿瘤抗原的一种或多种配体(例如，抗体)。在一些方面，T细胞还表达用于治疗脑癌的一种或多种额外的药剂。

[0111] 释义

[0112] 本文使用的“嵌合抗原受体(CAR)”是指包含一种或多种细胞外癌症抗原结合结构域，一种或多种跨膜结构域和一种或多种用于活化T细胞的细胞质信号传导结构域的分子，并且该分子对表达癌症配体(例如，癌症抗原)的细胞具有特异性。当将CAR引入至T细胞中时，CAR将重新定向T细胞的特异性。在特定的方面，CAR被表达为单个分子。

[0113] 本文使用的“癌症抗原结合结构域”是指结合由癌细胞表达的一种或多种抗原(一种或多种癌症抗原)的结构域。在特定的方面，所述癌症抗原结合结构域是特异性(选择性)结合癌症抗原并且不结合不被非癌细胞(例如，正常细胞，健康细胞，野生型细胞)表达的非特异性靶点的结合结构域。

[0114] 可使用多种癌症抗原结合结构域，并且可使用已知的方法生成或从商业来源获得多种癌症抗原结合结构域。癌症抗原结合结构域可以是例如核酸，肽(蛋白质)，抗体，有机分子，合成的分子，等等。这些癌症抗原结合结构域例如可衍生自文库和/或获自天然来源。

[0115] 本文使用的术语“IL13CAR”包括包含本文所述的或本领域已知的IL13配体的CAR，包括但不限于：IL13的变体(包括，但不限于：IL13的功能片段(例如，可结合IL13Rα2的IL13的片段))以及诸如选择性结合IL13Rα2的免疫球蛋白结构域之类的其他IL13配体。类似地，本文使用的术语“MGMT”包括野生型MGMT和本文所描述的或本领域已知的任何MGMT变体。

[0116] 本文针对剂量或量所使用的术语“治疗有效”是指向有此需要的受治者给药之后足以产生期望的活性的化合物的量或药物组合物的量，所述药物组合物(例如，包含诸如T淋巴细胞和/或NK细胞之类的免疫细胞的组合物)包含本文公开的嵌合受体，并且还包括耐药性多肽。在本文公开的内容的范围内，术语“治疗有效”是指足以延缓表现、抑制恶化、减轻或缓解至少一种由本文公开的方法治疗的失调的症状的化合物或药物组合物的量。应当注意的是，当给药活性成分的组合时，组合的有效量可包括或可不包括每个成分单独给药时的有效量。

[0117] 与本文公开的组合物联合使用的词组“药学上可接受的”是指生理学上可耐受的并且在向哺乳动物(例如，人类)给药时通常不会产生不良反应的分子实体和这些组合物的

其他成分。优选地，本文使用的术语“药学上可接受的”是指由联邦药监局或州政府批准的或在美国药典中列出的或其他通常识别的处方用于哺乳动物并且更加特别优选地用于人类。

[0118] 本文使用的术语“受治者”是指任何哺乳动物。在优选的实施方式中，所述受治者是人类。

[0119] 在其他方面，癌症抗原结合结构域是全部抗体或抗体的生物活性部分。本文使用的术语“抗体”是指免疫球蛋白分子和免疫球蛋白分子的免疫活性部分，即，包含选择性结合抗原的抗原结合位点的分子。本文使用的“选择性结合”是指抗体能够结合样本中的抗原或其片段并且不能充分结合样本中的其他分子(例如，抗原)。免疫球蛋白分子的免疫活性部分的实例包括Fab片段(例如，F(ab)，F(ab')$_2$)，可变片段(例如，单链可变区(scFv)，di-scFv，单结构域抗体片段(sdAb)，双特异性片段(例如，双特异性T细胞衔接器(BiTE)))。这些片段可获自商业来源和/或通过例如采用诸如胃蛋白酶之类的酶处理抗体产生。

[0120] 抗体可以是多克隆抗体或单克隆抗体，其结合(例如，选择性结合)癌细胞表达的一种或多种抗原。本文使用的“多克隆抗体”是结合特异性抗原的一批抗体中的抗体，所述一批抗体中的每个抗体识别不同的表位。本文使用的“单克隆抗体”或“单克隆抗体组合物”是指仅包含能够与一种或多种抗原的特定表位发生免疫反应的一类抗原结合位点的抗体分子群。因此，单克隆抗体组合物通常表现出对与其发生免疫反应的特定抗原具有单一结合亲和性。

[0121] 多克隆抗体可如上所述的通过采用一种或多种期望的癌症抗原(例如，IL13Rα2蛋白质的细胞外结构域)对合适的受治者进行免疫来制备。免疫的受治者体内的抗体滴度可通过标准技术(例如，酶联免疫吸附分析(ELISA))使用固定的多肽随时间进行监测。如果需要的话，针对癌症抗原的抗体分子可从哺乳动物中分离出来(例如，从组织、血液中分离出来)并且进一步通过已知的技术(例如，蛋白质A层析法)进行纯化，从而获得IgG片段。本领域普通技术人员能够制备选择性结合IL13Rα2蛋白质的细胞外结构域的多克隆抗体。

[0122] 在免疫之后的合适时间，例如，当抗体滴度最高时，抗体生成细胞可获自受治者并且通过标准技术用于制备单克隆抗体，所述标准技术例如，最初由Kohler和Milstein描述的杂交瘤技术(Nature 256:495-497(1975)，人B细胞杂交瘤技术(Kozbor et al., Immunol.Today 4:72(1983))，EBV-杂交瘤技术(Cole et al.,Monoclonal Antibodies and Cancer Therapy,Alan R.Liss,Inc.,pp.77-96(1985))或三源杂交瘤(trioma)技术。用于生成杂交瘤的技术是本领域熟知的(通常参见，Current Protocols in Immunology, Coligan et al.,(eds.)John Wiley&Sons,Inc.,New York,NY(1994))。简言之，将持续增殖细胞系(通常是骨髓瘤)融合至来自由上述免疫原免疫的哺乳动物的淋巴细胞(通常是脾细胞)并且筛选得到的杂交瘤细胞的培养物上清液以识别产生结合本发明的多肽的单克隆抗体的杂交瘤。

[0123] 为了产生癌症抗原的单克隆抗体，可应用用于融合淋巴细胞和持续增殖细胞系的许多熟知的操作规程中的任何一种(参见，例如，Current Protocols in Immunology, supra;Galfre et al.,Nature,266:55052(1977);R.H.Kenneth,in Monoclonal Antibodies:A New Dimension In Biological Analyses,Plenum Publishing Corp.,New York,New York(1980);and Lerner,Yale J.Biol.Med.54:387-402(1981))。而且，本领域

普通技术人员将会理解的是,这些方法的许多改良也是有用的。

[0124] 在用于制备分泌单克隆抗体的杂交瘤的一种可选方法中,癌症抗原的单克隆抗体可通过由多肽筛选重组组合免疫球蛋白文库(例如,抗体噬菌体展示文库)而被识别和分离,从而分离结合癌症抗原的免疫球蛋白文库成员。用于生成和筛选噬菌体展示文库的试剂盒是商售的(例如,Pharmacia重组噬菌体抗体系统,批号:27-9400-01;和Stratagene SurfZAP™噬菌体展示试剂盒,批号:240612)。此外,特别适用于生成和筛选抗体展示文库的方法和试剂的实例可在例如US5,223,409;WO 92/18619;WO 91/17271;WO 92/20791;WO 92/15679;WO 93/01288;WO 92/01047;WO 92/09690;WO 90/02809;Fuchs等人,Bio/Technology 9:1370-1372(1991);Hay等人,Hum.Antibod.Hybridomas 3:81-85(1992);Huse等人,Science 246:1275-1281(1989);以及Griffiths等人,EMBO J.12:725-734(1993)中找到。

[0125] 此外,包括人和非人部分的可使用标准重组DNA技术制备的诸如嵌合的和人源化的单克隆抗体之类的重组抗体在本发明的范围内。这些嵌合的和人源化的单克隆抗体可由本领域已知的重组DNA技术生成。

[0126] 上述用于生成多克隆抗体或单克隆抗体的常规方法中的任何一种可容易地应用于生成选择性结合IL13Rα2蛋白质的细胞外结构域单克隆抗体的方法中。得到的抗体或其片段的可变结构域随后可用于生成IL13配体结构域,该IL13配体结构域以与IL13配体结构域(SEQ ID NO:26)结合IL13Rα2蛋白质的亲和性相同的亲和性结合IL13Rα2蛋白质。

[0127] 本发明的构建体

[0128] 本发明至少部分基于表达对IL13Rα2具有特异性的CAR的免疫细胞,其中,所述细胞对诸如TMZ之类的化疗剂具有耐药性。

[0129] 在一些实施方式中,本发明提供编码对脑癌细胞具有选择性的CAR蛋白质和耐药性多肽这两者的核酸(本文中也称为嵌合核酸序列)。在优选的实施方式中,CAR蛋白质是IL13CAR并且耐药性多肽是MGMT蛋白质,该MGMT蛋白质能够赋予表达MGMT蛋白质的细胞TMZ耐药性。嵌合核酸序列可如本文所述的那样构建,从而编码IL13CAR蛋白质和MGMT耐药性蛋白质这两者。下文提供对IL13CAR蛋白质的结构域或序列区域和MGMT蛋白质的结构域或序列区域以及每个结构域或区域的非限定性实例的描述。IL13CAR蛋白质是线性嵌合(融合)蛋白质,其在N-端至C-端方向上包括:选择性结合患病细胞(例如脑癌细胞)上的IL13Rα2的IL13配体结构域、跨膜结构域和细胞内信号传导结构域。在一些实施方式中,本文所述的IL13CAR蛋白质可包括位于IL13配体结构域的N端的信号结构域和/或位于配体结构域和跨膜结构域之间的铰链区。

[0130] 在一些实施方式中,包含1,2,3,4,5,6,7,8,9,10个氨基酸残基的短肽连接体被包括在IL13CAR中,以分离CAR蛋白质的区域或结构域(例如,信号序列,IL13配体结构域,铰链区,跨膜结构域,CD28共刺激结构域,CD3-ζ信号传导结构域或另外的共刺激结构域)。应当理解的是,短肽连接体可存在于任何两个区域(结构域)之间,不论位于任何其他两个区域(结构域)之间的短肽连接体的存在或不存在。例如,小的肽连接体可存在于CAR中,其中,连接体使信号序列(如果存在的话)和IL13配体结构域分离,使IL13配体结构域和跨膜结构域分离,使IL13配体结构域和铰链区(如果存在的话)分离,使铰链区(如果存在的话)和跨膜结构域分离,使跨膜结构域和CD28共信号传导结构域(如果存在的话)分离,使CD28共信号

传导结构域(如果存在的话)和CD3ζ信号传导结构域分离,和/或使CD3-ζ信号传导结构域和另外的共刺激结构域(如果存在的话)分离。肽连接体可以是由如下核酸序列编码的氨基酸,所述核酸序列包含限制性内切酶位点或如下其他特征,所述特征允许编码IL13CAR-自裂解肽-MGMT嵌合蛋白质的区域或结构域中的每个区域(单顺反子的情况)或结构域的核酸进行连接。这些结构域和连接体中的每一个在下文详细描述。

[0131] 一方面,癌症抗原结合结构域是肽或蛋白质(例如,结合在癌细胞表面上表达的抗原的配体;结合在癌细胞表面上表达的受体的配体)。在优选的实施方式中,受体是IL132α受体并且CAR被构建为包括选择性结合IL132α受体的配体。在特定的方面,癌症抗原结合结构域是白介素13(IL13)或具有一个或多个插入、缺失或点突变的IL-13的变体(例如,E13K IL13;R109K IL13和/或E13Y IL13)。可选地,癌症抗原结合结构域可以是选择性结合IL13Rα2的抗体或其片段(例如scFv片段)的可变结构域。

[0132] 下表1和表2分别提供了本文所述的可在本发明的组合物(例如嵌合核酸序列)和方法中使用的核酸和多肽的总结。在一种实施方式中,本发明包括包含表1所涉及的核酸序列的任何组合的嵌合核酸序列。在一种实施方式中,本发明包括编码表2所涉及的氨基酸序列的任何组合的嵌合核酸序列。

[0133] 表1

[0134] 核酸序列的序列标识符

[0135]

SEQ ID NO	描述	相对于SEQ ID NO:1的核苷酸位置

[0136]

1	IL13(WT)CAR-P140K 全长 (SEQ ID NO:1)	
2	IL13(E13Y)CAR-P140K全长 (SEQ ID NO:2)	
3	IL13(E13K.R109K)CAR-P140K全长(SEQ ID NO:3)	
7	限制性内切酶位点	1-6
8	Kozak 序列	7-12
9	IL13 信号序列	13-108
10	成熟的 IL13	109-450
11	二肽连接体-1	451-456
12	CD8 铰链区	457-591
13	二肽连接体-2	592-597
14	CD3ζ 跨膜结构域	598-666
15	二肽连接体-3	667-672
16	CD28 共刺激结构域	673-804
17	二肽连接体-4	805-810
18	CD3ζ 信号传导结构域	811-1140
19	终止密码子	1141-1143
20	5 氨基酸连接体/限制性位点/阅读框调节	1144-1158
21	自裂解肽	1159-1212
22	P140K MGMT 耐药性多肽	1213-1839
23	终止密码子	1840-1842
24	限制性内切酶位点	1843-1846
34	带有E13Y 突变的成熟IL13	109-450
35	带有E13KR109K的成熟IL13	109-450
48	MGMT 耐药性多肽P140	

[0137] 表2

[0138] 多肽序列的序列标识符

[0139]

SEQ ID NO	**描述**	**相对于SEQ ID NO:4 的氨基酸位置**

[0140]

4	IL13(WT)CAR-P140K 全长 (SEQ ID NO:4)	
5	IL13(E13Y)CAR-P140K 全长 (SEQ ID NO:5)	
6	IL13(E13K.R109K)CAR-P140K 全长 (SEQ ID NO:6)	
25	IL13CAR-P140K – IL13 信号序列	1-32
26	IL13CAR-P140K – wt 成熟 IL13	33-146
	二肽连接体-1	147-148
27	IL13CAR-P140K – CD8 铰链区	149-193
	二肽连接体-2	194-195
28	IL13CAR-P140K – CD3ζ 跨膜结构域	196-218
	二肽连接体-3	219-220
29	IL13CAR-P140K – CD28 共刺激结构域	221-264
	二肽连接体-4	265-266
30	IL13CAR-P140K – CD3ζ 信号传导结构域	267-376
31	5 氨基酸连接体/阅读框调节/限制性位点	377-381
32	IL13CAR-P140K – 2A 自裂解肽	382-399
33	IL13CAR-P140K – P140K MGMT 耐药性蛋白质	400-606
36	带有E13Y 突变的成熟IL13	33-146
37	带有E13KR109K的成熟IL13	33-146
38	G156A-MGMT	
39	MGMT-2	
40	MGMT-3	
41	MGMT-5	
43	MGMT (GenBank NP_002403-(P140K))	
44	IL13Rα2	
45	IL13(WT)CAR-P140K 全长 (SEQ ID NO:45)	
46	IL13(E13Y)CAR-P140K 全长 (SEQ ID NO:46)	
47	IL13(E13K.R109K)CAR-P140K 全长 (SEQ ID NO:47)	
49	MGMT P140	

[0141] 配体

[0142] IL13CAR配体(可选地,配体结构域)是选择性结合患病细胞表达的IL13受体(例如,IL13Rα2)的肽、多肽或蛋白质。患病细胞可以是肿瘤细胞或其他癌细胞或恶性肿瘤细胞,并且,可选地,患病细胞表达的蛋白质在本文中是指癌症抗原。在一些实施方式中,癌症抗原是不被健康组织细胞表达的或非常少(由mRNA表达分析确定的小于50%,40%,30%,

20％或10％)的健康组织细胞表达的蛋白质。在一种实施方式中,患病细胞是脑癌细胞,例如,胶质母细胞瘤细胞。在特定方面,癌症抗原结合结构域为结合(例如,特异性或选择性结合)IL-13R(例如,IL13Rα2,例如,GenBank Acc.No.NP_000631;SEQ ID NO:44)的全部抗体或抗体的生物活性部分。在其他方面,配体(癌症抗原结合结构域)是定向针对(结合,特异性或选择性结合)IL-13R(例如,IL13Rα2)的scFv。

[0143] 本领域已知多种由癌(例如,肿瘤)细胞表达的抗原。一方面,癌症抗原是脑肿瘤抗原,例如,在肿瘤表面上表达的脑肿瘤抗原。在特定的方面,脑肿瘤抗原由高度恶性胶质瘤表达(例如,胶质瘤/恶性脑肿瘤抗原)。高度恶性胶质瘤的具体实例包括多形性胶质母细胞瘤(GBM),间变性星形细胞瘤和儿童胶质瘤。由高度恶性胶质瘤的肿瘤细胞表达的抗原的具体实例包括EGFRvIII,EphA2,Her-2和IL-13R(例如,IL13Rα2)。

[0144] 一方面,本发明的方法和组合物靶定的癌症抗原是IL13受体α-2(IL13Rα2),在GBM肿瘤上过表达但在正常脑组织中少量表达或不表达的多形性胶质母细胞瘤(GBM)相关蛋白(Thaci et al.,Neuro-Onco,16(10):1304-1312(2014);Sengupta et al.,Biomed Res Int,2014:952128(2014))。一方面,IL13CAR表达或包含配体结构域,该配体结构域是结合IL13α2受体的诸如E13K IL13(SEQ ID NO:36)和/或R109K IL13(SEQ ID NO:37)之类的IL13和/或IL13突变体(Kong et al.,Clin Cancer Res,18(21):5949-5960(2012))。也已发现IL13Rα2蛋白质上调乳腺癌(Papageorgis et al.,2015,Breast Canc Res,17:98-112),包括头颈癌中的乳腺癌转移(Joshi et al.,2000,Cancer Res,60:1168-1172;Kawakami et al,2003,9:6381-6388),并且IL13Rα2蛋白质还表现出促进胰腺癌、卵巢癌和结肠直肠癌的浸润和转移(Fujisawa et al.,2009,Int J Cancer,69:8678-8695;Barderas et al.,2012,Cancer Res,72:2780-2790)。因此,本文描述的组合物和方法在用于治疗诊断患有恶性肿瘤的受治者的方法中有用,其中,恶性肿瘤的类型包括但不限于:脑癌,头颈癌,乳腺癌,胰腺癌,卵巢癌和结肠直肠癌。

[0145] 本领域普通技术人员可生成特异性结合IL13Rα2的配体。例如,进行常规实验,例如通过采用IL13Rα2的细胞外结构域免疫小鼠或通过噬菌体展示,生成选择性结合IL13Rα2的抗体。带有期望的选择性结合活性的抗体的可变结构域随后可用于产生单链可变结构域(scFv)。在一种实施方式中,以与野生型IL13、IL13(E13Y)、IL13(R109K)或IL13(E13K.R109K)相同的亲和性选择性结合IL13Rα2的scFv可用作本文公开的IL13CAR构建体中的配体。

[0146] 在一种实施方式中,IL13CAR具有IL13配体结构域,该IL13配体结构域包括与SEQ ID NO:26具有至少90％,95％,96％,97％,98％,99％或99.5％一致性的多肽或其功能片段,其中:a)SEQ ID NO:26的位置13的氨基酸是谷氨酸;b)SEQ ID NO:26的位置13的氨基酸是酪氨酸;或c)SEQ ID NO:26的位置13的氨基酸是赖氨酸并且SEQ ID NO:26的位置109的氨基酸是精氨酸。在一种实施方式中,IL13CAR IL13配体结构域包括SEQ ID NO:26,SEQ ID NO:36或SEQ ID NO:37或其功能片段。

[0147] 根据本文公开的内容的核酸编码上文所述的IL13多肽。在一种实施方式中,编码IL13多肽的核酸选自SEQ ID NO:10,SEQ ID NO:34和SEQ ID NO:35。

[0148] IL13CAR还任选地包括允许对IL13CAR蛋白质进行加工并在宿主细胞表面进行展示的信号(前导)肽。在一种实施方式中,信号肽是配体蛋白质的天然生成的信号肽。例如,

配体结构域包括全长IL13蛋白质，该全长IL13蛋白质包括其信号序列(包括SEQ ID NO:25的氨基酸序列的信号序列或由SEQ ID NO:25的氨基酸序列构成的信号序列)。在一种实施方式中，信号序列包括与SEQ ID NO:25具有95％，96％，97％，98％，99％或99.5％的一致性的序列。根据本文公开的内容的核酸编码信号肽。在一种实施方式中，编码信号肽的核酸由SEQ ID NO:9构成。

[0149] 本领域普通技术人员会理解的是，可使用异质信号序列，其来自分泌的蛋白质或跨膜蛋白质的信号肽，所述分泌的蛋白质或跨膜蛋白质不是IL13。信号肽(信号序列)是膜结合多肽或分泌的多肽的必需部分，所述膜结合多肽或分泌的多肽是多肽膜转位所需要的并且在膜转位之后或膜转位过程中被加工。信号序列具有大约13和36个氨基酸的长度并且在氨基末端包含至少一个正残基。信号序列的中心是10至15个残基的强疏水部分并且由例如Nunnari,j.,et al.,Curr.Opin.Cell Biol.4(1992)573-580和Gilmore,R.,et al,Ann.N.Y.Acad.Sci.674(1992)27-37进行描述。信号肽的一些实例包括但不限于:VHCAMP,CD40,CD40L或TNF-R的信号肽。信号肽被切割整合至靶细胞的膜。本发明还涉及可使用异质信号序列，例如，IgG样蛋白质的信号序列。

[0150] 铰链区

[0151] 本文公开的IL13CAR可包括间隔体区域，也称为铰链区或铰链结构域，其位于IL13配体结构域(抗原结合结构域)和跨膜结构域之间。本文公开的内容所涵盖的IL13CAR可包括或可不包括铰链区。本文描述的CAR的铰链区是如下肽序列，其通常是柔性的，足以允许抗原结合结构域定位于不同方向以促进抗原识别。如本领域技术人员所理解的，可确定其他合适的间隔体。例如，长度为2个氨基酸至10个氨基酸的短寡肽连接体或短多肽连接体，其可形成位于CAR的跨膜结构域和细胞质结构域之间的连接。铰链区的实例是来自免疫球蛋白的铰链区(例如，IgG1的铰链区)，包括但不限于:来自免疫球蛋白样蛋白质或结构域的铰链区，免疫球蛋白的CH2CH3区域和CD3的部分。在一种实施方式中，铰链区包括来自CD8α链的Ig样结构域。

[0152] 在IL13CAR的一种实施方式中，铰链区包括SEQ ID NO:27的氨基酸序列或由SEQ ID NO:27的氨基酸序列构成。可选地，铰链区包括与SEQ ID NO:27具有95％，96％，97％，98％，99％或99.5％一致性的序列或由与SEQ ID NO:27具有95％，96％，97％，98％，99％或99.5％一致性的序列构成。根据本文公开的核酸编码铰链区。在一种实施方式中，编码铰链区的核酸包括SEQ ID NO:12。在一种实施方式中，编码IL13CAR的核酸包括与SEQ ID NO:12具有至少90％，95％，96％，97％，98％，99％或99.5％一致性的核酸。

[0153] 跨膜结构域

[0154] 如本文所述，CAR包括一种或多种跨膜结构域。通常，跨膜结构域是跨过膜的疏水区域(例如，疏水α螺旋)。可使用多种跨膜结构域中的任何一种。合适的跨膜结构域的实例包括但不限于:CD3(例如，CD3-ζ跨膜结构域)或CD28跨膜结构域。跨膜结构域可衍生自天然来源或重组来源。在来源为天然来源的条件下，结构域衍生自任何膜结合蛋白或跨膜蛋白。一方面，每当CAR已结合靶点时，跨膜结构域能够将信号传导至细胞内结构域。本发明中具体使用的跨膜结构域可包括至少如下的跨膜结构域，例如T细胞受体的α链、β链或ζ链的跨膜结构域，CD28，CD3E，CD45，CD4，CD5，CD8，CD9，CD16，CD22，CD33，CD37，CD64，CD80，CD86，CD134，CD137，CD154的跨膜结构域。

[0155] 在一种实施方式中，IL13CAR的跨膜结构域包括人CD3ζ链的至少一部分或由人CD3ζ链的至少一部分构成，例如，SEQ ID NO:28。在一种实施方式中，跨膜结构域包括下列序列或由下列序列构成，所述序列是与SEQ IDNO:28具有95％，96％，97％，98％，99％或99.5％一致性的序列。根据本文公开的内容的核酸编码跨膜结构域。在一种实施方式中，编码跨膜结构域的核酸包括SEQ ID NO:14或与SEQ ID NO:14具有至少90％，95％，96％，97％，98％，99％或99.5％一致性的核酸。

[0156] 细胞质信号传导结构域

[0157] 如本文所述，IL13CAR具有结合抗原之后将活化信号和/或刺激信号传送至T细胞的细胞质结构域(例如，导致T细胞的活化，启动，扩增，持续和/或增加以及T细胞针对肿瘤抗原的响应(例如，细胞毒性所需的信号))。细胞质结构域包括一种或多种T细胞受体的信号传导结构域或一个或多个与T细胞受体相关的信号传导结构域(例如，共刺激结构域)和/或一种或多种T细胞受体的活化结构域或一种或多种与T细胞受体相关的活化结构域。合适的细胞质信号传导结构域的实例包括但不限于：包含免疫受体酪氨酸活化基序(ITAM)的CD3-ζ，FcεRIγ，CD28(例如，嵌合CD28)，4-IBB(CD137)，DAP10，OX40(CD134)，CD4，CD27，CD244，可诱导T-cell共刺激物(ICOS)，白细胞C-端SRC激酶(LCK)，和CD137(例如，Sadelain et al.，Cancer Discov，3(4)：388-398(2013)；Lee et al.，Clin Cancer Res，18(10)：2780-2790(2012))的那些细胞质信号传导结构域。一种或多种这些细胞质结构域的存在可通过使ZAP70、TNF受体相关因子1(TRAF1)、PI3K以及生长因子受体结合的蛋白质2(GRB2)与CAR的细胞质结构域中的元件相关联来启动通路，从而触发信号传导中间体和基因转录。

[0158] “共刺激结构域”或“共刺激信号传导结构域”是指包含共刺激分子的细胞内结构域的CAR的一部分。共刺激分子是细胞表面分子，其不同于在结合抗原之后提供T淋巴细胞的有效活化和T淋巴细胞的功能所需的另一信号的抗原受体或Fc受体。共刺激结构域经历构象改变，该改变通过例如CD3-ζ信号传导结构域产生对细胞的活化信号。共刺激配体可包括但不限于：CD7，B7-1(CD80)，B7-2(CD86)，PD-L1，PD-L2，4-1BBL，OX40L，可诱导共刺激配体(ICOS-L)，细胞间粘合分子(ICAM)，CD30L，CD40，CD70，CD83，HLA-G，MICA，M 1CB，HVEM，淋巴毒素β受体，3/TR6，ILT3，ILT4，结合Toll配体受体的激动剂或抗体以及特异性结合B7-H3的配体。IL13CAR内的一种或多种共刺激结构域的内容物可提高表达IL13CAR的T细胞的效力和扩增。在一种实施方式中，共刺激结构域尤其还包括特异性结合存在于T细胞上的共刺激分子的抗体，例如但不限于：CD27，CD28，4-IBB，OX40，CD30，CD40，PD-1，ICOS，淋巴细胞功能相关抗原-1(LFA-1)，CD2，CD7，LTGHT，NKG2C，B7-H3，特异性结合CD83的配体。

[0159] 对本领域技术人员而言明显的是，IL13CAR可具有任何数量的活化结构域和/或刺激结构域。信号传导结构域中的每一个通过肽键连接以形成IL13CAR构建体的细胞质结构域。一方面，IL13CAR具有活化结构域和刺激结构域。另一方面，IL13CAR具有一个、两个、三个、四个、五个等等活化结构域和一个、两个、三个、四个、五个等等刺激结构域。

[0160] 在一种实施方式中，本文公开的IL13CAR包括人CD28蛋白质和/或人CD3-ζ链的信号传导结构域。在一种实施方式中，IL13CAR包括CD28和CD3-ζ链信号传导结构域，其中，CD28共刺激结构域在CD3-ζ信号传导结构域的N-端。本发明还涉及CAR细胞质结构域，其中，CD3-ζ结构域在CD28共刺激结构域的N-端。如本文所述的，CD28共刺激结构域包括SEQ ID NO:29的序列。CD3-ζ信号传导结构域包括SEQ ID NO:30的序列或由SEQ ID NO:30的序列构

成。根据本文公开内容的核酸编码CD28共刺激结构域。在一种实施方式中，编码CD28共刺激结构域的核酸由SEQ ID NO:16构成。根据本文公开内容的核酸编码CD3-ζ信号传导结构域。在一种实施方式中，编码CD3-ζ信号传导结构域的核酸由SEQ ID NO:18构成。这些信号传导结构域中的每一个可包含一个或多个缺失、插入或点突变(天然或人工)，其中，每个结构域的信号传导功能与不包含一个或多个缺失、插入或点突变的蛋白质的信号传导功能大致相同。

[0161] 在一种实施方式中，IL13CAR的细胞质结构域由包含SEQ ID NO:1的核苷酸673-1140的核酸序列编码或由包含SEQ ID NO:1的核苷酸673-1143的核酸序列编码。在另一实施方式中，IL13CAR包含细胞质结构域，该细胞质结构域包含SEQ ID NO:4的氨基酸残基221-376。

[0162] 图1A举例说明了IL13CAR构建体的实施方式，其中，IL13配体是包含E13K取代和R109K取代的变体。

[0163] 耐药性多肽

[0164] 本发明公开的内容至少部分涉及既编码上文详细描述的对脑癌细胞具有选择性的CAR蛋白质又编码耐药性多肽的核酸，其中，CAR和耐药性多肽这两者的表达对于治疗癌症而言是有用的。例如，CAR和耐药性多肽可在下述细胞中表达，该细胞被给药至正在采用化疗剂和/或提高化疗剂的细胞毒性的药剂进行治疗的受治者。化疗剂的实例包括：通常用于治疗恶性胶质瘤(例如，多形性胶质母细胞瘤(GBM))的1,3-双(2-氯乙基)-1-亚硝基脲(BCNU或卡莫司汀)，福莫司汀(fotemustine)，洛莫司汀和替莫唑胺(TMZ)。一些化疗剂的细胞毒性作用涉及能够重新排布形成致死性链内交联的O^6-甲基鸟嘌呤病变的形成。然而，这些甲基化试剂的有效性受到DNA修复蛋白质的肿瘤过表达的限制，该DNA修复蛋白质为O^6-甲基鸟嘌呤DNA甲基转移酶(MGMT)，其是从治疗后的细胞的DNA中除去细胞毒性O^6-烷基鸟嘌呤加合物的蛋白质。因此，表达高水平MGMT的肿瘤细胞部分或完全耐受TMZ化疗的杀伤。防止甲基化试剂的效力降低的一种方式是使患者接收TMZ化疗剂联合MGMT抑制剂(具体是O^6-苄基鸟嘌呤)的治疗。然而，由于O^6-苄基鸟嘌呤对造血细胞的毒性作用，因此，使O^6-苄基鸟嘌呤的剂量受到限制。

[0165] 替莫唑胺(TMZ)是抗胶质瘤化疗药物，其对包括T细胞在内的造血细胞具有细胞毒性作用。FDA对TMZ的标准剂量进行了指导，因此，抗胶质瘤治疗中的不可避免的步骤是通过使T细胞的DNA进行甲基化而杀伤T细胞，这与TMZ破坏肿瘤细胞的方式非常相同(Sengupta et al.,Clin Dev Immunol,831090(2012))。然而，野生型MGMT在细胞中的过表达或一种或多种MGMT突变体(例如，G156A，P140K)在细胞中的表达将保护该细胞不受诸如TMZ之类的甲基化试剂的影响(Woolford et al.,J Gene Med,8(1):29-31(2006))。因此，由上述核酸编码的耐药性多肽是赋予TMZ耐受性的MGMT或MGMT变体。这些TMZ耐药性变体包括但不限于：P140K-MGMT(SEQ ID NO:33)，P140K-MGMT(SEQ ID NO:43)，G156A-MGMT(SEQ ID NO:38)，MGMT-2(SEQ ID NO:39)，MGMT-3(SEQ ID NO:40)和MGMT-5(SEQ ID NO:41)(Fontes et al.,Mol Cancer Ther,5(1):121-128)。全文所描述的MGMT变体(例如，P140K和G156A)中的氨基酸取代的指定位置是基于SEQ ID NO:33的序列的氨基酸位置。MGMT-2变体具有S152H，A154G，Y158H，G160S和L162V取代。MGMT-3变体具有C150Y，A154G，Y158F，L162P和K165R取代。MGMT-5变体具有N157T，Y158H和A170S取代。

[0166] 本文公开且使用的特别优选的构建体是P140K-MGMT变体。根据本发明公开的内容的核酸编码MGMT变体。在一种实施方式中，编码P140K-MGMT的核酸由SEQ ID NO:22构成。本发明还涉及编码G156A-MGMT(SEQ ID NO:38)，MGMT-2(SEQ ID NO:39)，MGMT-3(SEQ ID NO:40)或MGMT-5(SEQ ID NO:41)的核酸，上述中的每一个可存在于IL13CAR-MGMT构建体中。本文还涉及包含SEQ ID NO:43的MGMT蛋白质序列(GenBank登录号:NP_002403，其具有P140K点突变)和对应于G156A-MGMT(SEQ ID NO:38)，MGMT-2(SEQ ID NO:39)，MGMT-3(SEQ ID NO:40)或MGMT-5(SEQ ID NO:41)的突变的任何点突变。

[0167] 由本文公开的嵌合核酸序列编码的MGMT或其变体位于编码IL13CAR蛋白质的核酸序列的一部分的下游或上游。在一种实施方式中，嵌合核酸序列是单顺反子的，其中，构建体包括单个启动子序列以驱动单个转录子的转录，这进而被翻译成稍后被裂解的单个蛋白质。这种单顺反子构建体的使用需要位于IL13CAR和MGMT蛋白质之间的自裂解元件。例如，当在宿主细胞中表达嵌合核酸序列时，嵌合核酸序列最初产生如下单个蛋白质，所述单个蛋白质在N-端至C-端方向上包括CAR(例如，上述IL13CAR)、自裂解肽以及本文所述的MGMT蛋白质或MGMT变体。该蛋白质随后被裂解，从而生成单个的CAR蛋白质和MGMT蛋白质。CAR蛋白质被加工并在细胞表面上展示，而MGMT蛋白质可被保留在细胞核内部。可选的构建体在N-端至C-端方向上包括MGMT蛋白质、自裂解蛋白质和CAR。

[0168] 在一种实施方式中，CAR和MGMT多肽部分被自裂解肽分离。自裂解序列的一个实例是2A元件，其包括来自口蹄疫病毒的2A序列。在示例性的实施方式中，自裂解序列包括SEQ ID NO:32的序列或由SEQ ID NO:32的序列构成。

[0169] 在可选的实施方式中，编码上述CAR和上述MGMT或其变体这两者的核酸序列是多顺反子的，其中，所述核酸序列包括编码CAR的核酸序列和编码MGMT或其变体的核酸序列，该核酸序列由诸如内部核糖体进入位点(IRES)之类的非蛋白质编码序列分离。可使用的IRES序列的实例包括但不限于：脑脊髓炎病毒(EMCV)的IRES元件，口蹄疫病毒(FMDV)的IRES元件，泰勒小鼠脑脊髓炎病毒(TMEV)的IRES元件，人鼻病毒(HRV)的IRES元件，可萨基病毒(CSV)的IRES元件，脊髓灰质炎病毒(POLIO)的IRES元件，甲肝病毒(HAV)的IRES元件，丙肝病毒(HCV)的IRES元件和瘟疫病毒(例如，猪瘟病毒(HOCV)和牛病毒性腹泻病毒(BVDV))的IRES元件(参见，例如，Le等人，Virus Genes 12:135-147,1996；和Le等人，Nuc.AcidsRes.25:362-369,1997，其全部内容通过引用并入本文)。

[0170] 多顺反子嵌合核酸的可选实施方式是第二启动子位于编码IL13CAR的核酸序列和编码MGMT或其变体的核酸序列之间的嵌合核酸。在该实施方式中，不存在编码自裂解肽的核酸序列。

[0171] 本发明还涉及编码IL13CAR和MGMT变体的核酸均位于单独的核酸载体内。即，本发明还涉及包括第一载体和第二载体的系统或试剂盒，所述第一载体包括编码本文所述的IL13CAR的核酸，所述第二载体编码本文所述的MGMT蛋白质。

[0172] 本领域技术人员将会理解的是，编码至少IL13CAR和MGMT蛋白质或其变体的核酸序列可进一步包括额外的成分以促进和/或提高宿主细胞中CAR和/或MGMT的表达和功能。例如，CAR可还包括(一个或多个)启动翻译的序列(例如，Kozak序列和/或启动子序列)以及用于同源性重组的序列(逆转录病毒5'LTR；逆转录病毒3'LTR)。Kozak序列可在包括编码本文所述的IL13CAR-2A-MGMT构建体的核酸序列的嵌合核酸构建体中使用。

[0173] 根据本发明公开的内容使用的嵌合核酸序列通过在5'至3'方向上连接或结合下列元件而生成:Kozak序列,编码信号序列的核酸,编码IL13配体的核酸,编码铰链区的核酸,编码跨膜结构域的核酸,编码CD28共刺激(信号传导)结构域的核酸,编码CD3-ζ信号传导结构域的核酸,编码自裂解肽(例如,2A肽)的核酸以及编码MGMT蛋白质的核酸,上述核酸中的每一个在上文中描述。嵌合核酸序列可通过合成包括并编码上述元件的单个序列而生成。可选地,上述元件中的每一个可使用本领域普通技术人员已知的方法独立地生成。例如,每个元件通过使用PCR进行扩增,其中,PCR被设计成根据需要在每个元件的5'端和3'端上生成限制性内切酶位点,并且,使用合适的内切酶对各个元件进行消化并将各个元件连接在一起以获得所需的构建体。因此,生成IL13CAR-P140KMGMT构建体的这种方法使得短连接体肽存在于各个元件之间。

[0174] 例如,编码2,3,4,5或6氨基酸的核酸可位于配体结构域和铰链区之间,铰链区和跨膜结构域之间,跨膜结构域和CD28共刺激结构域之间,CD28共刺激结构域和CD3-ζ信号传导结构域之间,CD3-ζ信号传导结构域和/或自裂解肽之间。

[0175] 在示例性的实施方式中,IL13CAR包括位于配体和铰链结构域之间的连接体,该连接体由两个氨基酸,脯氨酸-精氨酸构成。铰链区和跨膜结构域之间的连接体是谷氨酸-赖氨酸。跨膜结构域和CD28共刺激结构域之间的连接体是缬氨酸-苏氨酸。CD28共刺激结构域和CD3-ζ信号传导结构域之间的连接体是苏氨酸-精氨酸。CD3-ζ信号传导结构域和2A肽之间的连接体是谷氨酸-脯氨酸-丙氨酸-丙氨酸-丙氨酸。本发明涉及上述连接体中的每一个可独立于其他连接体存在于IL13CAR蛋白质中或者独立于其他连接体不存在于IL13CAR蛋白质中。

[0176] 因此,用于转染诸如T细胞之类的宿主细胞并且用于抑制或防止表达IL13Rα2且暴露于TMZ的细胞的生长的IL13CAR-P140KMGMT构建体的优选的实施方式包括但不限于:IL13(WT)CAR-P140KMGMT(SEQ ID NO:1的核苷酸序列;SEQ ID NO:4的氨基酸序列);IL13(E13Y)CAR-P140KMGMT(SEQ ID NO:2的核苷酸序列;SEQ ID NO:5的氨基酸序列);以及IL13(E13K.R109K)CAR-P140KMGMT(SEQ ID NO:3的核苷酸序列;SEQ ID NO:6氨基酸序列)。图2A提供了IL13(E13K.R109K)CAR-P140KMGMT构建体的示意图。这些IL13CAR-P140KMGMT构建体的其他实施方式包括但不限于:编码蛋白质IL13(WT)CAR-P140KMGMT(SEQ ID NO:45),IL13(E13Y)CAR-PAR140KMGMT(SEQ ID NO:46),和IL13(E13K.R109K)CAR-P140KMGMT(SEQ ID NO:47)的那些构建体。

[0177] 本领域技术人员将会理解的是,编码本文公开的信号肽、IL13配体、铰链区、跨膜结构域和CD28共刺激结构域、CD3-ζ信号传导结构域、自裂解肽连接体和MGMT蛋白质或其变体的核酸序列中的每一个可在不影响所编码的蛋白质的条件下由于密码子简并而发生改变。而且,多肽序列也可例如通过保守氨基酸取代而发生改变,同时不显著影响明确描述的蛋白质的功能。因此,本发明还涉及本文所述的核苷酸序列中的每一个的变体,这样,本文公开的核酸序列包括与SEQ ID NO:1-SEQ ID NO:3,SEQ ID NO:7-SEQ ID NO:24和SEQ ID NO:34-SEQ ID NO:35中的每一个具有至少75%,80%,82%,85%,90%,92%,94%,95%,96%,97%,98%,99%或99.5%一致性的序列。类似地,本文还涉及并公开了与SEQ ID NO:4-SEQ ID NO:6,SEQ ID NO:25-SEQ ID NO:33和SEQ ID NO:36-SEQ ID NO:41以及SEQ ID NO:43中的每一个具有至少75%,80%,82%,85%,90%,92%,94%,95%,96%,97%,

98％,99％或99.5％的一致性的多肽。

[0178] 出于优化比较目的,两个核苷酸或氨基酸序列的百分比一致性可通过序列比对进行确定(例如,可在第一序列的序列中引入空位)。对应位置的核苷酸或氨基酸随后进行比较,并且两个序列之间的百分比一致性是序列共享相同位置的数目的函数(即,一致性％＝相同位置的#/位置的总#x 100)。在一些实施方式中,出于比较目的而比对的氨基酸或核苷酸序列的长度是参比序列的长度的至少30％,40％,50％,60％,70％,80％,85％,90％,92％,94％,95％,96％,97％,98％,99％或100％,例如,图7A(SEQ ID NO:1)、图7B(SEQ ID NO:4)、图8A(SEQ ID NO:2)、图8B(SEQ ID NO:5)、图9A(SEQ ID NO:3)和图9B(SEQ ID NO:6)中提供的那些序列。两个序列的实际比较可通过熟知的方法,例如,使用数学算法完成。这些数学算法的优选的非限定性的实例在Karlin等人的文章(Proc.Natl.Acad.Sci.USA,90:5873-5877(1993))中描述。这种算法并入了BLASTN和BLASTX程序(2.2版本)中,BLASTN和BLASTX程序在Schaffer等人的文章(Nucleic Acids Res.,29:2994-3005(2001))中描述。当使用BLAST和Gapped BLAST程序时,可使用各个程序(例如,BLASTN)的默认参数。在一种实施方式中,所检索的数据库是非冗余(NR)数据库,并且序列比较参数可设定为:无过滤;Expect Value＝10;Word Size＝3;矩阵(Matrix)为BLOSUM62;并且Gap Cost:Existence＝11,Extension＝1。在另一实施方式中,在目标多肽或多核苷酸的全长上确定两个多肽或两个多核苷酸之间的百分比一致性。

[0179] 用于序列比较的数学算法的另一优选的非限定的实例是Myers和Miller的算法(CABIOS(1989))。这种算法并入ALIGN程序(2.0版本)中,其是GCG序列比对软件包(Accelrys,San Diego,California)的一部分。当使用用于比较氨基酸序列的ALIGN程序时,可使用PAM120权重余数表,缺口长度罚分12和缺口罚分4。序列分析的其他算法是本领域已知的并且包括Torellis和Robotti,Comput.的文章(Appl.Biosci.,10:3-5(1994))中描述的ADVANCE和ADAM以及Pearson和Lipman的文章(Proc.Natl.Acad.SciUSA,85:2444-8(1988))中描述的FASTA。

[0180] 在另一实施方式中,两个氨基酸序列之间的百分比一致性可使用GCG软件包中的GAP程序,使用Blossom63矩阵或PAM250矩阵,缺口权重为12,10,8,6或4以及长度权重为2,3或4来完成。在又一实施方式中,两个核酸序列之间的百分比一致性可使用GCG软件包中的GAP程序,使用缺口权重为50和长度权重为3来完成。

[0181] 多肽之间的类似性通常由保守氨基酸取代来确定。这些取代是由类似特性的另一氨基酸取代多肽中的给定氨基酸的那些取代。保守取代可能是表型沉默的。通常看到的保守取代是脂肪族氨基酸Ala、Val、Leu和Ile中的一个代替另一个,羟基残基Ser和Thr的互换,酸性残基Asp和Glu的交换,酰胺基残基Asn和Gln之间的取代,碱性残基Lys和Arg的交换以及芳香族残基Phe和Tyr之间的代替。关于氨基酸改变可能是表型沉默的指导在Bowie et al.,Science 247:1306-1310(1990)中找到。

[0182] 变体多肽可通过一个或多个取代、缺失、插入、反向、融合和截断或者这些的任何组合而在氨基酸序列方面产生差异。进一步而言,变体多肽可以是完全功能性的(例如,能够感染细胞并产生子代病毒)或可在一种或多种活性方面缺乏功能(例如,能够产生子代病毒)。完全功能变体通常仅包含保守改变或在非关键残基或非关键区域包含改变。功能变体还可包含类似氨基酸的取代,这种取代不会产生功能上的改变或产生不显著的功能改变。

可选地，这种取代可在一定程度上对功能产生正面或负面的影响。非功能性变体通常包含一个或多个非保守氨基酸取代，缺失，插入，反向或截断或者包括在关键残基或关键区域中的取代，插入，反向或缺失。

[0183] 功能必需的氨基酸可通过本领域已知的方法识别，例如，定点突变或丙氨酸扫描突变(Cunningham et al.,Science,244:1081-1085(1989))。后者的步骤在分子中的每个残基上引入单个丙氨酸突变(每个分子一个突变)。得到的突变分子随后进行体外生物活性检测。对于多肽活性而言关键的位点还可通过结构分析进行确定，例如，结晶、核磁共振或光亲和标记(参见，Smith et al.,J.Mol.Biol.,224:899-904(1992)；和de Vos et al.Science,255:306-312(1992))。

[0184] 本文进一步公开了包含基本上纯的多肽的组合物，所述基本上纯的多肽包括本文所述的IL13CAR蛋白质和本文所述的P140KMGMT变体或由本文所述的IL13CAR蛋白质和本文所述的P140KMGMT变体构成，该IL13CAR蛋白质为SEQ ID NO:4,SEQ ID NO:5或SEQ ID NO:6的氨基酸1-146的序列和优选地与SEQ ID NO:4,SEQ ID NO:5或SEQ ID NO:6的氨基酸1-146的序列具有至少75％,80％,82％,85％,90％,92％,94％,95％,96％,97％,98％或99％序列一致性的多肽，该P140KMGMT变体为SEQ ID NO:1的氨基酸400-606的序列以及优选地与SEQ ID NO:1的氨基酸400-606的序列具有至少75％,80％,82％,85％,90％,92％,94％,95％,96％,97％,98％或99％序列一致性的多肽，上述序列一致性使用本文所述的BLAST程序以及本文所述的参数确定。在另一实施方式中，多肽的实例包括包含SEQ ID NO:4,SEQ ID NO:5和/或SEQ ID NO:6的基本上纯的多肽或包括由SEQ ID NO:4,SEQ ID NO:5和/或SEQ ID NO:6构成的基本上纯的多肽，以及优选地与SEQ ID NO:4具有至少75％,80％,82％,85％,90％,92％,94％,95％,96％,97％,98％或99％序列类似性的多肽，所述序列类似性使用本文所述的BLAST程序和参数确定。

[0185] 在特定的方面，本文公开的内容涉及由SEQ ID NO:1(IL13 CAR-P140KMGMT),SEQ ID NO:2(IL-13(E13Y)CAR-P140KMGMT),SEQ ID NO:3(IL-13(E13K R109K)CAR-P140KMGMT)或其组合编码的分离的多肽。在其它方面，本文公开的内容涉及包含SEQ ID NO:4,SEQ ID NO:5,SEQ ID NO:6,SEQ ID NO:45,SEQ ID NO:46,SEQ ID NO:47或其组合的氨基酸序列的多肽(分离的多肽)。

[0186] 本发明还涉及包含T细胞受体的CAR多肽，所述T细胞受体包含脑癌的一种或多种肿瘤抗原的一种或多种配体(例如，抗体)。在特定的方面，CAR多肽还包含用于治疗脑癌的一种或多种额外的药剂。在其他方面，本发明涉及分离的多肽及其片段、衍生物和变体，以及由本文所述的核苷酸序列编码的多肽(例如，其他变体)。本文使用的术语“多肽”是指氨基酸的聚合物，并且不涉及特定长度，因此，肽、寡肽和蛋白质包括在多肽的定义范围内。

[0187] 多肽可使用已知的蛋白质合成方法合成。在一种实施方式中，多肽由重组DNA和重组蛋白质表达以及纯化技术生成。例如，编码多肽的核酸分子被克隆至表达载体，所述表达载体被引入宿主细胞内，在所述宿主细胞内表达所述多肽并且纯化和配制所需的蛋白质，用于包装和给药。

[0188] 本文使用的涉及多肽的“分离的”，“基本上纯的”或“基本上纯的且分离的”是指在从重组或非重组细胞中分离所述多肽时所述多肽基本不含其它物质，或化学合成所述多肽时所述多肽不含化学前体或其它化学药品。此外，多肽可加至细胞中通常不与其相关的另

一多肽中(例如,在融合蛋白中)并且仍然是“分离的”,“基本上纯的”或“基本上纯的且分离的”。例如,可使用本文所述的亲和性纯化技术以及本文所述的和本领域技术人员已知的其他技术获得分离的、基本上纯的或基本上纯的且分离的多肽。

[0189] 本发明的多肽可被纯化为同质性的。然而,应当理解的是,其中多肽没有被纯化为同质性的制剂也是有用的。重要特性在于制剂允许多肽的期望的功能,甚至在存在显著量的其他成分时,制剂也允许多肽的期望的功能。因此,本发明包括各种不同的纯度。在一种实施方式中,“基本不含其他物质”包括含有少于约30%(按干重量计)的其他蛋白质(即,污染蛋白质),少于约20%的其他蛋白质,少于约10%的其他蛋白质,少于约5%的其他蛋白质,或少于约1%的其他蛋白质的多肽的制剂。

[0190] 当重组生成多肽时,所述多肽也可以是基本不含培养基的,即,培养基占少于约20%的多肽制剂体积,少于约10%的多肽制剂体积,或少于约5%的多肽制剂体积。“基本不含化学前体或其他化学药品”包括从多肽的合成中所涉及的化学前体或其他化学药品中分离出来的多肽的制剂。在一种实施方式中,“基本不含化学前体或其他化学药品”包括具有少于约30%(按干重量计)化学前体或其他化学药品,少于约20%化学前体或其他化学药品,少于约10%化学前体或其他化学药品,或者少于约5%化学前体或其他化学药品的多肽的制剂。

[0191] 在一种实施方式中,本发明的多肽包括由SEQ ID NO:1,SEQ ID NO:3和/或SEQ ID NO:5的核酸分子编码的氨基酸序列以及补体及其一部分。本发明的多肽还包括如下片段和序列变体,所述片段和序列变体与由包含SEQ ID NO:1,SEQ ID NO:3和/或SEQ ID NO:5的核苷酸序列的核酸分子编码的多肽以及补体及其一部分具有实质的同源性。

[0192] 核酸表达构建体

[0193] 本发明公开的内容的另一方面属于核酸表达构建体或载体、逆转录病毒载体和/或逆转录病毒颗粒及其应用。本领域普通技术人员已知的重组DNA技术方法被用于设计和生成编码本文所述的IL13CAR、自裂解肽和MGMT变体的嵌合核酸序列。随后,嵌合核酸构建体被克隆至质粒载体以进行,例如,排序,从而确认构建体的序列。一旦确认了期望的序列,那么使用嵌合核酸构建体生成用于转染哺乳动物细胞的逆转录病毒颗粒。因此,包含本文所述的SEQ ID NO:1-SEQ ID NO:3的核酸序列以及SEQ ID NO:7-SEQ ID NO:24、SEQ ID NO:34和/或SEQ ID NO:34-SEQ ID NO:35的组合的嵌合构建体被克隆至这些质粒载体中,用于稍后包装进入逆转录病毒颗粒。在特定的方面,嵌合构建体和质粒载体包括包含上述SEQ ID NO:1、SEQ ID NO:2和/或SEQ ID NO:3的一个或多个核酸序列。如实施例1所描述的,包含IL13(WT)CAR-P140KMGMT序列(SEQ ID NO:1)、IL13(E13Y)CAR-P140KMGMT(SEQ ID NO:2)或IL13(E13K.R109K)CAR-2A-P140KMGMT序列(SEQ ID NO:3)的质粒载体通过将本文所述的片段克隆至pUC57载体中随后克隆至pMFG载体中而产生,然后生成逆转录病毒颗粒(实施例2)。

[0194] 图1B举例说明包含IL13CAR构建体的质粒载体,其中尚未引入MGMT基因。图2B举例说明包含IL13CAR构建体并且还包含位于IL13CAR编码序列的下游的P140KMGMT编码序列的相同的质粒骨架。

[0195] 在可选的实施方式中,编码本文所述的IL13CAR的核酸被克隆至第一载体并且编码本文所述的MGMT蛋白质的核酸被克隆至第二载体。因此,本发明还描述了第一载体(质

粒,表达,病毒,逆转录病毒,慢病毒,腺病毒,等等)和第二载体(质粒,表达,病毒,逆转录病毒,慢病毒,腺病毒,等等),所述第一载体包含编码本文所述的IL13CAR的核酸,所述第二载体包含编码本文所述的MGMT蛋白质的核酸。

[0196] 合适的核酸构建体的实例包括质粒(例如,环状双链DNA环)和病毒载体(例如,逆转录病毒载体,慢病毒载体,腺病毒载体)。一些载体能够自动复制至宿主细胞中,将所述载体引入所述宿主细胞中(例如,具有复制的细菌源的细菌载体和附加型哺乳动物载体)。其他载体(例如,非附加型哺乳动物载体)在引入宿主细胞中之后被整合至宿主细胞基因组中,从而沿着宿主基因组进行复制。而且,一些载体(表达载体)能够将核酸的表达定向至它们可操作地连接的核酸。总体而言,重组DNA技术中的表达构建体通常是质粒形式。然而,本发明意在包括表达载体的这些其他形式,例如,提供等同功能的病毒载体(例如,复制缺陷型逆转录病毒,腺病毒和腺相关病毒)。

[0197] 本发明的优选的重组表达载体包括具有适于表达宿主细胞中的核酸分子的形式的本发明的核酸分子。这意味着重组表达载体包括基于待用于表达的宿主细胞选择的一个或多个调控序列,所述调控序列可操作地连接至待表达的核酸序列。在重组表达载体中,“可操作地连接的”是指目标核苷酸序列以允许核苷酸序列表达的方式连接至调控序列(例如,当载体引入宿主细胞中时在体外转录/翻译系统中或在宿主细胞中)。术语“调节性序列”意在包括启动子,强化子和其他表达控制元件(例如,多聚腺苷酸信号)。这些调控序列在例如Goeddel,Gene Expression Technology:Methods in Enzymology 185,Academic Press,San Diego,CA(1990)中描述。调控序列包括将核苷酸序列的连续表达定向在多种类型的宿主细胞中的那些调控序列和将核苷酸序列的表达仅定向在一些宿主细胞中的那些调控序列(例如,组织特异性调控序列)。

[0198] 本领域技术人员应当理解的是,表达载体的设计可取决于如下因素,例如,待转化的宿主细胞的选择和期望的多肽的表达水平。本发明的表达载体可被引入宿主细胞,从而产生多肽,包括由本文所述的核酸分子编码的融合多肽。

[0199] 本发明的重组表达载体可设计为用于本发明的多肽在原核细胞或真核细胞(例如,细菌细胞,例如,大肠杆菌细胞,昆虫细胞(使用杆状表达载体),酵母细胞或哺乳动物细胞(灵长类动物细胞(例如,人类细胞),鼠类细胞(例如,小鼠细胞),猫科动物细胞,犬类动物细胞,啮齿类动物细胞,羊细胞,牛细胞))中的表达。

[0200] 成功地生成包含嵌合核酸构建体的逆转录病毒颗粒以包含编码实施例1和实施例2所述的IL13(E13K.R109K)CAR-P140KMGMT的嵌合核酸序列。实施例和说明书全文描述的方法结合本领域普通技术人员已知的方法可用于生成包含本文所述的任何嵌合核酸构建体的逆转录病毒颗粒。加工过程中的转染效率可通过由流失细胞仪测量IL13配体的细胞表面表达来监测。采用IL13CAR-P140KMGMT构建体转导的细胞可使用例如荧光活化的细胞分类器来富集。图3A和图3B显示了由IL13(E13K.R109K)CAR-A2-P140KMGMT构建体转染的PG13细胞的富集。图3A显示了在采用亲嗜性逆转录病毒第一次转导之后表达IL13的细胞的相对数量(例如,4.0%)。图3B显示了在富集采用亲嗜性逆转录病毒转导的细胞之后表达IL13的细胞的相对数量(例如,96.6%)。使用常规方法进行细胞溶解产物的Western印迹分析,从而确定并测量宿主细胞表达的P140KMGMT蛋白质。如图3C所示,与图1A至图1B所示的未转导的细胞或由仅表达IL13CAR构建体的构建体转导的细胞相比,由IL13CAR-A2-P140KMGMT构建

体转导的细胞的富集会过表达P140KMGMT蛋白质。

[0201] 图1A和图2A提供了克隆至质粒载体的IL13(E13K.R109K)CAR-P140KMGMT的示意图，而图1B和图2B显示了用于整合至宿主细胞基因组中的5'LTR和3'LTR的侧翼的嵌合体的线性描述。

[0202] 本文描述的核酸构建体被引入宿主哺乳动物细胞中，以赋予细胞肿瘤细胞杀伤功能和化疗药物耐药性，所述化疗药物是诸如TMZ之类的DNA甲基化药剂。使用例如实施例3所述的逆转录病毒载体完成将核酸引入至哺乳动物宿主细胞。瞬时或稳定转导哺乳动物细胞的逆转录病毒载体是本领域已知的并且在下文中描述了将其用于目前描述的蛋白质表达和治疗系统中。

[0203] 一些实施方式利用病毒载体通过本文所述的表达系统转导诸如T细胞之类的浆细胞。病毒载体的实例包括但不限于:MFG载体，基于腺病毒的载体，基于腺相关病毒(AAV)的载体，逆转录病毒载体，逆转录病毒-腺病毒载体和源自单纯性疱疹病毒(HSV)的载体。

[0204] 通常，最小逆转录病毒载体包括一些5'LTR和3'LTR序列，一种或多种目标基因(待在目标细胞中表达)，一个或多个启动子和用于包装RNA的顺式作用序列。可包括本文所述的和本领域已知的其他调控序列。病毒载体通常被克隆至可被转染至包装细胞系中的质粒中并且通常还包括用于质粒在细菌中的复制的序列，所述包装细胞系例如真核细胞(例如，PG13小鼠纤维母细胞)。诸如逆转录病毒载体之类的一些病毒载体使用一个或多个异源性启动子，增强子或这两者。一些实施方式使用位于病毒载体的5'LTR和3'LTR序列之间的并且可操作地连接至目标基因的“内部”启动子/增强子。“功能关系”和“可操作地连接”是指，但不限于，基因相对于启动子和/或增强子位于准确的位置和方位上，这样，当启动子和/或增强子与合适的调控分子接触时，基因的表达将会受到影响。任何增强子/启动子组合可用于调节(例如，增加，降低)包装细胞系中的病毒RNA基因组的表达，调节感染的目标细胞中所选择的目标基因的表达，或这两者都调节。

[0205] 启动子是由DNA序列形成的表达控制元件，该DNA序列允许发生RNA聚合酶和转录的结合。启动子是位于所选择的目标基因(通常在约100bp至1000bp范围内)的起始密码子的上游(5')并且控制与其可操作地连接的编码多核苷酸序列的转录和翻译的未翻译的序列。启动子可以是可诱导的或组成性的。可诱导的启动子响应培养条件的一些改变(例如，温度的改变)在DNA的控制下通过DNA启动转录水平的增加。

[0206] 本领域已知多种启动子和用于将启动子可操作地连接至多核苷酸编码的序列的方法。天然启动子序列和许多异源性启动子可用于所选择的目标基因的定向表达。一些实施方式使用异源性启动子，因为相对于天然启动子，异源性启动子通常允许更好的转录并产生更高产量的期望的蛋白质。

[0207] 一些病毒载体包括顺式作用包装序列，从而促进将基因组病毒RNA并入病毒颗粒。实例包括psi-序列。这些顺式作用序列是本领域已知的。

[0208] 可使用本领域已知的任何合适的基因工程技术完成病毒载体的产生，包括但不限于:限制性内切酶消化，连接，转换，质粒纯化，PCT扩增以及DNA排序的标准技术，例如，下列文献中所描述的:Sambrook等人的文献(Molecular Cloning:A Laboratory Manual.Cold Spring Harbor Laboratory Press,N.Y.(1989))，Coffin等人的文献(Retroviruses.Cold Spring Harbor Laboratory Press,N.Y.(1997))和"RNA Viruses:A Practical

Approach"(Alan J.Cann,Ed.,Oxford University Press,(2000))。

[0209] 本领域已知任何多种可用于产生合适的逆转录病毒颗粒的方法,所述逆转录病毒的基因组包括病毒载体的RNA拷贝。作为一种方法,基于并入具有期望的靶细胞特异性的病毒颗粒中的病毒载体,病毒载体可引入包装病毒基因组RNA的包装细胞系中。所述包装细胞系通常提供反式病毒蛋白,该病毒蛋白是将病毒基因组RNA包装至病毒颗粒中并感染靶细胞所需的,包括结构空位蛋白质、酶pal蛋白和包膜糖蛋白。

[0210] 在一些实施方式中,包装细胞系可稳定地表达一些必需的或期望的病毒蛋白(例如,gag,pol)(参见,例如,美国专利US6,218,181)。在一些实施方式中,包装细胞系可被编码一些必需或期望病毒蛋白(例如,gag,pol)的质粒瞬时转染,包括本文所述的麻疹病毒糖蛋白序列。在一种示例性的实施方式中,包装细胞系稳定地表达gag和pol序列,并且细胞系随后被编码病毒载体的质粒和编码糖蛋白的质粒转染。下文介绍期望的质粒,例如通过超离心收集并因此加工病毒颗粒,从而得到病毒颗粒的浓缩母液。示例性的包装细胞系包括PG13(ATCC CRL-10686),293(ATCC CCL X),HeLa(ATCC CCL 2),D17(ATCC CCL 183),MDCK(ATCC CCL 34),BHK(ATCC CCL-10)和Cf2Th(ATCC CRL 1430)细胞系。

[0211] 宿主细胞

[0212] 本发明的另一方面关于已引入本发明的重组表达载体的宿主细胞。术语"宿主细胞"和"重组宿主细胞"在本文中互换使用。应当理解的是,这些术语不仅仅涉及特定的受治者,而且还涉及这种细胞的子代或潜在的子代。因为可由于突变或环境影响而在后代中产生某些修饰,这些子代事实上可能不等同于母细胞,但是这些子代仍然包括在本文使用的术语的范围内。

[0213] 宿主细胞可以是任何原核细胞或真核细胞。例如,本发明的核酸分子可在细菌细胞(例如,大肠杆菌)、昆虫细胞、酵母或哺乳动物细胞中表达。一方面,宿主细胞是哺乳动物细胞(灵长类动物细胞(例如,人类细胞),鼠科动物细胞(例如,小鼠细胞),猫科动物细胞,犬类动物细胞,啮齿类动物细胞,羊细胞,牛细胞)。在特定的方面,哺乳动物细胞是免疫细胞。又一方面,哺乳动物细胞是T细胞。对于本领域技术人员而言其他合适的宿主细胞是明显的。

[0214] 就本文的目的而言,T细胞可以是任何T细胞,诸如培养的T细胞,例如,原始T细胞,或来自培养的T细胞系的T细胞(例如,Jurkat,SupT1,等等),或获自哺乳动物的T细胞。如果T细胞获自哺乳动物,那么T细胞可以自多种来源获得,包括但不限于:血液、骨髓、淋巴结、胸腺或其他组织或体液。T细胞还可被富集或纯化。T细胞可以是人T细胞。T细胞可以是从人体分离的T细胞。T细胞可以是任何类型的T细胞并且可以是任何发育阶段的T细胞,包括但不限于:$CD4^+/CD8^+$双正电荷T细胞、$CD4^+$辅助T细胞(例如,Th_1细胞和Th_2细胞)、$CD8^+$T细胞(例如,细胞毒性T细胞)、外周血单核细胞(PBMC)、外周血白细胞(PBL)、肿瘤浸润细胞、记忆T细胞、初始T细胞等等。T细胞可以是$CD8^+$T细胞或$CD4^+$T细胞。

[0215] 在一种实施方式中,本发明的组合物和方法中使用的宿主细胞是NK-92细胞(NK-92细胞系,ATCC保藏号:PTA-6672)。

[0216] 核酸构建体可通过常规转化或转染技术引入至原核细胞或真核细胞中。本文使用的术语"感染","转化","转导"和"转染"是指用于将外源性核酸分子(例如,DNA)引入至宿主细胞的本领域已知的多种技术,包括:磷酸钙或氯化钙共沉淀、DEAE-葡聚糖介导的转染、

脂质转染或电穿孔。用于转化或转染宿主细胞的合适的方法可在例如Sambrook et al., Molecular Cloning,A Laboratory Manual(2nd Ed.,CSHP,New York(1989)和其他实验指南中找到。

[0217] 本发明的宿主细胞(例如培养基中的原核宿主细胞或真核宿主细胞)可用于生成(表达)本发明公开的一种或多种CAR。因此,本发明公开的内容还提供了使用本发明的宿主细胞生成CAR的方法。在一种实施方式中,所述方法包括在合适的培养基中培养本发明公开的宿主细胞(在所述宿主细胞中已引入了编码本发明公开的多肽的重组表达载体),从而生成一种或多种CAR。

[0218] 对于哺乳动物细胞的稳定转染而言,本领域已知的是,基于所使用的表达载体和转染技术,仅仅一小部分细胞可将外源性DNA整合至其基因组中。为了识别和选择这些整合素,编码可选择的标记物(例如,对于抗生素具有耐受性)的基因通常沿着目标基因被引入宿主细胞中。优选的可选择的标记物包括赋予耐药性的那些标记物,例如,G418、潮霉素或甲氨蝶呤。编码可选择的标记物的核酸分子可在与本发明的核酸分子相同的载体上被引入至宿主细胞或可在单独的载体上被引入至宿主细胞。由引入的核酸分子稳定转染的细胞可通过药物选择来识别(例如,已合并了可选择的标记物基因的细胞将会存活,而其他细胞死亡)。

[0219] 如本文举例说明的,本发明涉及免疫细胞,例如,但不限于,表达IL13CAR并且对暴露于化疗药剂替莫唑胺具有耐受性的T细胞。具体而言,本发明已显示出,在TMZ存在的情况下,由编码并表达上文详细描述的IL13CAR(SEQ ID NO:4,SEQ ID NO:5 or SEQ ID NO:6)和P140KMGMT突变体(SEQ ID NO:33)的核酸序列转导的T细胞相对于表达仅表达IL13的CAR的T细胞具有更高的存活率。因此,在特定的方面,本发明涉及表达IL13和/或IL13的变体(例如,SEQ ID NO:4(WT IL13 CAR),SEQ ID NO:5(IL13E13YCAR)或SEQ ID NO:6(IL13E13K.R109KCR)和/或R109K IL13CAR)以及对细胞产生化学保护的MGMT突变体(例如,P140KMGMT突变体(SEQ ID NO:33,SEQ ID NO:38,SE ID NO:39,SEQ ID NO:40,SEQ ID NO:41))的CAR。在一种实施方式中,细胞由IL13CAR-A2-MGMT构建体转导,其中,MGMT蛋白质不是野生型MGMT蛋白质(SEQ ID NO:49)。

[0220] 宿主细胞的收获和转染

[0221] 采用嵌合抗原受体(CAR)工程化的、能够产生高度特异性肿瘤识别和杀伤的T细胞在获得了有希望的临床结果之后获得巨大关注(Grupp et al.,2013,N Eng J Med,368:1509-1518;Porter et al.,2011,N Eng J Med,365:725-733;Sadelain et al.,2009,Curr Opin Immunol,21:215-223)。带有CAR基因的重编程T细胞为对接和溶解肿瘤细胞提供了独立于MHC的机理。可选地,这些修饰的T细胞被称为“设计T细胞(designer T cells)”、“T-bodies”或“CAR-T细胞”(Ma et al.,2002,Cancer Chemotherapy&Biological Response Modifiers:Elsevier Science,pp.319-345;Park et al.,2011,Trends Biotech,29:550-557;Ma et al.,2014,Prostate,74:286-296)。

[0222] 另一方面,本发明公开的内容涉及用于生成表达CAR和MGMT蛋白质的细胞的方法,CAR包含T细胞受体,所述T细胞受体包含脑癌的一种或多种肿瘤抗原的一种或多种配体(例如,抗体),所述MGMT蛋白质增加由编码CAR和MGMT蛋白质的核酸转导的并且暴露于诸如TMZ之类的DNA甲基化药剂的细胞的活力。在特定方面,本发明公开的内容涉及生成表达CAR的

细胞的方法，CAR具有SEQ ID NO:4(IL13 CAR-P140KMGMT)、SEQ ID NO:5(IL-13(E13Y)CAR-P140KMGMT)、SEQ ID NO:6(IL-13(E13K R109K)CAR-P140KMGMT)或其组合的氨基酸序列。所述方法包括将包含SEQ ID NO:1(IL13(WT)CAR-P140KMGMT)、SEQ ID NO:2(IL-13(E13Y)CAR-P140KMGMT)或SEQ ID NO:3(IL-13(E13K R109K)CAR-P140KMGMT)的核酸序列引入细胞，并且在由细胞表达CAR的条件下维持所述细胞，从而生成表达下述嵌合抗原受体的细胞，该嵌合抗原受体具有SEQ ID NO:4(IL13(WT)CAR-P140KMGMT)、SEQ ID NO:5(IL-13(E13Y)CAR-P140KMGMT)、SEQ ID NO:6(IL-13(E13K R109K)CAR-P140KMGMT)或其组合的氨基酸序列。

[0223] 在特定的方面，使用病毒载体(例如，逆转录病毒载体，慢病毒载体，腺病毒载体或其组合)将核酸序列引入细胞中。另一方面，细胞是哺乳动物细胞，例如，哺乳动物T细胞(例如，人T细胞或小鼠T细胞)。在特定的方面，细胞是自体细胞或人白细胞抗原(HLA)-相合细胞。再一方面，细胞获自患有脑癌(例如，高度恶性胶质瘤，例如，多形性胶质母细胞瘤(GBM)，间变性星形细胞瘤或儿童胶质瘤)的一个或多个个体。

[0224] 因此，其他方面涉及表达根据本发明公开的内容的至少一种CAR和耐药性多肽的重组T细胞。特别优选的转化宿主细胞是转基因T前体细胞或干细胞，其特征在于，所述转基因T前体细胞或干细胞包括根据本发明的核酸构建体。用于转化或转导宿主细胞和/或干细胞的方法是本领域技术人员熟知的，并且例如，包括电穿孔或微注射。特别优选的转化的宿主细胞是患者个性化T细胞，其在提取之后，由根据本发明公开的内容的核酸构建体转染。根据本发明公开的内容，宿主细胞尤其可通过提取一个或多个细胞而获得，优选地，通过提取T细胞，尤其是$CD8^+$-T细胞而获得宿主细胞，所述$CD8^+$-T细胞随后被根据本发明公开内容的一种或多种核酸构建体离体转染或转导，从而获得根据本发明公开内容的宿主细胞。

[0225] 在进行扩增和基因修饰之前，T细胞的来源获自受治者。术语“受治者”意在包括免疫反应可被诱发的生物体(例如，哺乳动物)。受治者的实例包括人和其他灵长类动物，狗，猫，小鼠，大鼠和转基因啮齿类动物。T细胞可自多种来源获得，包括外周血单核细胞，骨髓，淋巴结组织，脐带血，胸腺组织，感染部位的组织，腹水，胸腔积液，脾脏组织和肿瘤。在本发明的一些方面，可使用本领域可获得任何数量的T细胞。在本发明公开的内容的一些方面，可使用本领域技术人员已知的任何技术(例如，Ficoll™分离)自从受治者采集到的血液单元获得T细胞。在一个优选的方面，来自个体的循环血液的细胞可通过分离术获得。分离术产品通常包含包括T细胞在内的淋巴细胞，单核细胞，粒细胞，B细胞，其他成核的白细胞，红细胞以及血小板。一方面，通过分离术收集的细胞可被洗涤以除去血浆碎片并将细胞放置于合适的缓冲液或培养基中用于下一加工步骤。在本发明的一个方面，细胞由磷酸盐缓冲盐水(PBS)洗涤。在可选的方面，洗涤溶液缺少钙并且可以缺少镁或可以缺少许多或全部二价阳离子。在不存在钙的情况下，最初的活化步骤可导致活化放大。如本领域普通技术人员易于理解的，洗涤步骤可通过本领域技术人员已知的方法完成，例如，根据生产商的说明使用半自动“直流”离心机完成洗涤。在洗涤之后，细胞可重悬于多种生物相容性缓冲液或其他带有缓冲剂或不带有缓冲剂的盐水溶液中。可选地，可除去分离术(apheresis)样品的不想要的成分并且将细胞直接重悬于培养基中。

[0226] 一方面，T细胞通过溶解红细胞并且除去单核细胞从外周血淋巴细胞中分离出来，例如，通过PERCOLL™梯度离心或通过对流离心洗脱。T细胞的特定的亚群(例如，CD3+，CD28

+,CD4+,CD8+,CD45RA+,和CD45RO+T细胞)可进一步通过阳性或阴性选择技术分离。本领域技术人员可意识到的是,在本发明中可使用多轮选择。在一些方面,理想的是,进行选择步骤并且在活化和扩增过程中使用"未选择的细胞"。"未选择的细胞"还可以经受进一步的多轮选择。

[0227] 可使用定向于对阴性选择的细胞唯一的表面标记物的抗体的组合完成通过阴性选择对T细胞群的富集。一种方法是通过负磁性免疫粘附或流式细胞术进行细胞分类和/或选择,所述负磁性免疫粘附或流式细胞术使用定向于在阴性选择的细胞上存在的细胞表面标记物的单克隆抗体的混合物。例如,为了通过阴性选择富集CD4+细胞,单克隆抗体混合物通常包括CD14,CD20,CD11b,CD16,HLA-DR和CD8的抗体。在一些方面,理想的是富集或阳性选择调节性T细胞,该T细胞通常表达CD4+,CD25+,CD62Lhi,GITR+和FoxP3+。可选地,在一些方面,T调节性细胞由抗-CD25结合珠或其他类似的选择方法除去。

[0228] T细胞的活化和扩增

[0229] 无论在对T细胞进行基因修饰以表达理想的CAR之前或之后,总体上可使用如下美国专利和美国专利申请公开中所描述的方法活化和扩增T细胞:US6,352,694;US6,534,055;US6,905,680;US6,692,964;US5,858,358;US6,887,466;US6,905,681;US7,144,575;US7,067,318;US7,172,869;US7,232,566;US7,175,843;US5,883,223;US6,905,874;US6,797,514;US6,867,041;以及US20060121005。

[0230] 一旦确立了转染或转导的T细胞能够通过期望的调节并在期望的水平上表达作为表面膜蛋白的IL13CAR,就可以确定嵌合受体在宿主细胞中是否具有用于所需的信号诱导的功能性。随后,转导的T细胞被重新引入或给药于受治者以活化受治者体内的抗肿瘤反应。

[0231] 药物组合物

[0232] 再一方面,本发明公开的内容涉及促进将本文所述的转导的T细胞给药于有此需要的受治者的药物组合物。根据本文公开的内容的转导的T细胞与合适的载体或稀释剂可制成药物组合物或可制成用于体内给药的合适的植入物,所述合适的载体或稀释剂进一步可以是药学上可接受的。制备这种组合物或植入物的方法已在本领域进行描述(参见,例如,Remington'sPharmaceutical Sciences,16th Ed.,Mack,ed.(1980))。在合适的情况下,转导的T细胞被配制成半固体或液体形式的制剂,例如,胶囊、溶液、注射液、吸入剂或气溶胶,用于各自给药途径的常规方式。本领域已知的方式可用于防止或最小化组合物在到达靶组织或靶器官之前的释放和吸收,或确保组合物随时间释放。然而,理想的是使用不导致细胞表达嵌合受体的药学上可接受的形式。因此,理想的是,转导的T细胞可制成包含平衡盐溶液的药物组合物,所述平衡盐溶液优选地为Hanks平衡盐溶液或生理盐水。例如,采用生理学上可接受的载体或赋形剂配制组合物以制备药物组合物。载体和组合物可以是无菌的。制剂应当适于给药模式。

[0233] 合适的药学上可接受的载体包括但不限于:水,盐溶液(例如,NaCl),盐水,缓冲盐水,醇类,甘油,乙醇,阿拉伯树胶,植物油,苄基醇,聚乙二醇,明胶,诸如乳糖之类的糖类,直链淀粉或淀粉,葡萄糖,硬脂酸镁,滑石,硅酸,粘性石蜡,芳香油,脂肪酸酯,羟甲基纤维素,聚乙烯吡咯烷酮,等等,以及它们的组合。如果需要的话,药物制剂可与不与活性化合物发生有害反应的辅剂混合,所述辅剂例如润滑剂,防腐剂,稳定剂,润湿剂,乳化剂,用于影

响渗透压的盐，缓冲剂，着色、调味和/或芳香物质，等等。

[0234] 如果需要的话，组合物还可包含微量润湿剂或乳化剂或pH缓冲剂。组合物可以是液体溶液，悬浮液，乳液，片剂，药丸，胶囊，持续释放剂型或粉剂。组合物可与传统粘合剂和诸如甘油三酯之类的载体一同被配制成栓剂。口服制剂可包括标准载体，例如，药物级别的甘露醇，乳糖，淀粉，硬脂酸镁，聚乙烯吡咯烷酮，糖精钠，纤维素，碳酸镁，等等。

[0235] 组合物可根据常规步骤配制成适于给药于人体的药物组合物。例如，用于静脉内给药的组合物通常是无菌等渗水性缓冲剂中的溶液。在必要的情况下，组合物还可包括增溶剂和缓解注射部位的疼痛的局部麻醉剂。总体而言，单独提供各个成分或各个成分以单位剂量的形式混合在一起，例如，作为密封包装内的干的冻干粉末或无水浓缩物，所述密封包装例如安瓶或显示活性化合物的量的小袋。在通过输注给药组合物的情况下，所述组合物可分散于含有无菌药用级水、盐水或葡萄糖/水的输注瓶中。在通过注射给药组合物的情况下，可提供用于注射的无菌水或盐水的安瓶，从而在给药之前可将各个成分混合。

[0236] 这些组合物的引入方法包括但不限于：颅内给药，髓内给药，皮内给药，肌肉内给药，腹膜内给药，眼内给药，静脉内给药，皮下给药，局部给药，口服给药和鼻内给药。其他合适的引入方法还可包括基因疗法(如下文描述的)，可再充电或可生物降解的装置，颗粒加速装置(“基因枪”)和缓慢释放聚合装置。本发明的药物组合物还可作为与其他化合物的联合治疗的一部分给药。

[0237] 对于局部施用而言，可使用包含与局部施用相容的载体并且具有优选地大于水的动态粘度的非喷雾形式，粘性至半固体或固体形式。合适的制剂包括但不限于：溶液，悬浮液，乳液，霜剂，软膏，粉末，灌肠剂，乳剂，溶胶，擦剂，药膏，气溶胶，等等，如果需要的话，对这些制剂进行灭菌或与辅剂混合，所述辅剂例如，防腐剂，稳定剂，润湿剂，缓冲剂或用于影响渗透压的盐，等等。化合物可并入化妆品制剂中。对于局部施用而言，喷雾气溶胶制剂也是合适的，其中，活性成分优选地与固体或液体惰性载体物质结合包装在挤压瓶中或与加压挥发性物质混合(通常为气体推进剂，例如，加压气体)。

[0238] 本文描述的化合物可配制成中性或盐形式。药学上可接受的盐包括由游离氨基形成的那些盐和由游离羧基形成的那些盐，所述由游离氨基形成的那些盐例如，源自盐酸，磷酸，醋酸，草酸，酒石酸等的那些盐，所述由游离羧基形成的那些盐例如，源自钠，钾，铵，钙，氢氧化铁，异丙基胺，三乙基胺，2-乙基氨基乙醇，组氨酸，普鲁卡因等的那些盐。

[0239] 试剂盒

[0240] 本文公开的内容还提供药物包装或试剂盒，所述药物包装或试剂盒包括填充有本文公开的药物组合物的成分中的一种或多种的一个或多个容器。任选地，这些容器可以关联有政府机构规定的形式的下述通知，该通知调节药物或生物产品的生产、使用或销售，该通知反映生产机构批准销售用于人类给药。所述包装或试剂盒可以贴有关于给药模式、药物给药顺序(例如，分开给药，顺序给药或同时给药)等等的信息。所述包装或试剂盒还可包括提醒患者进行治疗的工具。所述包装或试剂盒可以是单个单位剂量的联合治疗或可以是多个单位剂量。具体而言，化合物可被分开，以任何组合混合在一起，存在于单个小瓶中或片剂中。优选地是，将化合物组装在泡罩包装中或其他分配工具中。对于本发明的目的而言，单位剂量是指依赖于每种化合物的单独的药效学的并且在标准时间过程中以FDA批准的剂量给药的剂量。

[0241] 治疗方法

[0242] 另一方面，本文公开的内容涉及治疗有此需要的个体体内的恶性肿瘤的方法，所述方法包括给药表达IL13CAR和MGMT蛋白质的一种或多种T细胞，IL13CAR包含IL13Rα2蛋白质的一种或多种配体(例如，抗体)。在特定的方面，本文公开的内容涉及治疗有此需要的个体体内的脑癌的方法，所述方法包括给药一种或多种T细胞，该一种或多种T细胞带有并表达编码如下蛋白质的核酸序列，所述蛋白质包含SEQ ID NO:26、SEQ ID NO:36或SEQ ID NO:37(配体)、SEQ ID NO:28(TM)、SEQ ID NO:29和SEQ ID NO:30(CD28和CD3-ζ信号传导结构域)，并且任选地还包含SEQ ID NO:27(铰链)。在另一实施方式中，核酸序列包含SEQ ID NO:1(IL13 CAR-P140KMGMT)，SEQ ID NO:2(IL-13(E13Y)CAR-P140KMGMT)，SEQ ID NO:3(IL-13(E13K R109K)CAR-P140KMGMT)或其组合。一方面，T细胞是自体T细胞或人白细胞抗原(HLA)-相合细胞。另一方面，脑癌是高度恶性胶质瘤，例如，高度恶性胶质瘤是多形性胶质母细胞瘤(GBM)、间质性星形细胞瘤或儿童胶质瘤。在一种实施方式中，本文公开的方法用于治疗与不利的IL13Rα2表达有关的癌症。

[0243] 已被证明具有过表达IL13Rα2的细胞的其他癌症包括但不限于：乳腺癌，胰腺癌，头颈癌，卵巢癌和结肠直肠癌。在另一实施方式中，癌症是已经转移的癌症。因此，本文还涉及通过向受治者给药一种或多种T细胞来治疗这些癌症中的一种或多种的方法，所述一种或多种T细胞由上述IL13CAR-MGMT构建体中的一种或多种转导。

[0244] 因为T细胞表达CAR并且表达突变的MGMT，该突变的MGMT产生针对MGMT过表达的耐药性的保护或产生针对MGMT变体(例如，P140K)的耐药性的保护，所以，治疗脑癌的方法可进一步包括向个体(脑癌患者)顺序或同时给药一种或多种化疗剂。换言之，修饰的T细胞在给药化疗剂之前，给药化疗剂过程中或给药化疗剂之后给药。化疗剂的实例包括替莫唑胺(TMZ)，1,3-双(2-氯乙基)-1-亚硝基脲(BCNU或卡莫司汀)，福莫司汀(fotemustine)和洛莫司汀。在特定的方面，一种或多种T细胞表达包含SEQ ID NO:1(IL13 CAR-P140KMGMT)、SEQ ID NO:2(IL-13(E13Y)CAR-P140KMGMT)、SEQ ID NO:3(IL-13(E13K R109K)CAR-P140KMGMT)或其组合的核酸序列并且将一种或多种化疗剂同时给药至个体。在特定的方面，个体是哺乳动物，例如，人类或其他灵长类动物，或者啮齿类动物，例如，小鼠或大鼠。

[0245] 下文实施例4和实施例5部分举例说明由编码并表达IL13CAR-MGMT嵌合体转导的T细胞的效力。实施例4显示了与由编码IL13CAR但不共表达P140KMGMT蛋白质的载体转导的T细胞相比，由编码IL13CAR-2A-P140KMGMT蛋白质的载体转导的分离的T细胞在暴露于TMZ时具有提高的耐药性(活力增强)(例如，参见图5)。因此，本文涉及增强由IL13CAR-MGMT构建体转导的免疫细胞的活力的方法，IL13CAR-MGMT构建体例如本文所述的构建体。在示例性的实施方式中，提供下述T细胞作为治疗诊断患有脑癌的受治者的方法，该T细胞由包含SEQ ID NO:1、SEQ ID NO:2或SEQ ID NO:3的核酸序列的逆转录病毒转导，所述受治者顺序或同时接受TMZ治疗。

[0246] 其他研究表明本文公开的IL13CAR-MGMT构建体还在修饰T细胞方面有效，所述T细胞可给药于受治者并且可提高受治者的存活率。如实施例5所示以及图6举例说明的，采用TMZ和下列T细胞治疗注射有U251MG胶质瘤细胞的小鼠，该T细胞由编码IL13CAR(不带有MGMT)的构建体转导或由IL13CAR-A2-P140KMGMT构建体转导。虽然在不存在P140KMGMT的条件下IL13CAR的表达使存活率相对于没有给药CAR T细胞而言得到提高，但是给药TMZ和由

编码IL13CAR-A2-P140KMGMT嵌合体的核酸序列转导的T细胞的动物具有最高的存活率(图6)。因此,本文涉及用于治疗诊断患有脑癌的受治者的方法,所述方法包括向受治者给药表达本文所述的IL13CAR-MGMT蛋白质的免疫细胞。

[0247] T细胞和/或化疗剂可使用任何合适的给药途径给药于个体。合适的给药途径的实例包括但不限于:颅内递送,髓内递送,皮内递送,肌肉内递送,腹膜内递送,眼内递送,静脉内递送,皮下递送,局部递送,口服和鼻内递送。

[0248] 一方面,所述方法还包括从所述个体获取一种或多种T细胞并且将本发明的嵌合核酸序列(例如,包含SEQ ID NO:1(IL13 CAR-P140KMGMT)、SEQ ID NO:2(IL-13(E13Y)CAR-P140KMGMT)、SEQ ID NO:3(IL-13(E13KR109K)CAR-P140KMGMT)或其组合的核酸序列)引入至所述T细胞。自个体获取T细胞的方法是本领域已知的并且包括,例如,血浆分离术。在一些方面,CAR T细胞在实验室中生长(扩增),直至其数量,例如,为数十亿。扩增的CAR T细胞群随后可融合至患者体内。在融合之后,T细胞在患者体内繁殖并在其工程化受体的指导下识别并杀伤将抗原固定在其表面上的癌细胞。

[0249] 以治疗有效量(即,足以治疗疾病的量,例如,通过缓解与疾病有关的症状,预防或延缓疾病的发作,和/或减轻疾病症状的严重性或频率)给药表达本文所述的IL13CAR和突变的MGMT的宿主细胞。在治疗特定个体的失调或病症方面的治疗有效量取决于疾病的症状和严重性,并且可通过标准临床技术确定。此外,体外或体内分析可任选地用于帮助识别最优剂量范围。待在制剂中使用的精确剂量还取决于给药途径以及疾病或失调的严重性,并且应当根据执业医生的判断和每个患者的情况来决定。有效剂量可从剂量响应曲线进行外推得到,所述剂量响应曲线来自体外或动物模型测试系统。

[0250] 理想地,有效量或足够数量的分离的转染T细胞或修饰T细胞存在于组合物中并且被引入至受治者体内,从而建立长期、特异性、抗肿瘤的响应,以相对于不进行治疗的情况下减小肿瘤尺寸或消除肿瘤生长或再生长。理想地,在与不存在修饰的T细胞的相同条件相比,给药于受治者的修饰的T细胞的量使肿瘤尺寸减小10%,20%,30%,40%,50%,60%,70%,80%,90%,95%,98%,或100%。

[0251] 因此,给药的修饰的T细胞的量应当考虑给药途径并且应当为引入足够数量的转导的T细胞以实现期望的治疗反应的量。而且,包括在本文所述的组合物中的每种活性药剂的量(例如,相对于待接触的每个细胞的量或相对于体重的量)可在不同的应用中而发生改变。总体而言,修饰的T细胞的浓度理想地应当足以在正在进行治疗的受治者体内提供约$1x10^6$个至约$1x10^9$个转导的T细胞,甚至更理想地,约$1x10^7$个至约$5x10^8$个转导T细胞,高于(例如,大于$5x10^8$个细胞)或低于(例如,$1x10^7$个细胞)上述任何合适的量也可使用。剂量方案可基于已为人知的基于细胞的疗法(参见,例如,Topalian and Rosenberg(1987)Acta Haematol.78 Suppl 1:75-6;美国专利US4,690,915)或可使用可选的连续输注方案。

[0252] 实施例

[0253] 用于替莫唑胺-耐药性胶质母细胞瘤免疫疗法的IL13CAR-P140KMGMT

[0254] 实施例1.表达质粒构建

[0255] 如图1A和图1B的IL13(WT)CAR构建体举例说明的,将编码IL13(WT)CAR构建体的cDNA、编码IL13(E13Y)CAR构建体的cDNA和编码IL13(E13K.R109K)CAR构建体的cDNA插入MFG逆转录病毒载体的BamHl和Notl克隆位点,以生成宿主质粒IL13(WT)CAR-pMFG、IL13

(E13Y)CAR-pMFG和IL13(E13K.R109K)CAR-pMFG质粒(参见,Kong et al.,(Clin Cancer Res,2012,18(21):5949-5960))。为了生成具有IL13CAR和P140K.MGMT cDNA序列的单顺反子转录子,通过Genscript USA(Piscataway,NJ)合成具有5'Notl和3'Eagl末端的2A-P140KMGMT片段。将2.3Kb片段克隆至pUC57克隆载体,用于确定序列。一旦确定了序列,将en bloc转移至IL13CAR-pMFG逆转录病毒载体的3'Notl位点,从而生成IL13(WT)CAR-2A-P140K.MGMT-pMFG、IL13(E13Y)CAR-2A-P140K.MGMT-pMFG、和IL13(E13K.R109K)CAR-2A-P140K.MGMT-pMFG质粒中的每一个。图2A和图2B举例说明了IL13(E13K.R109K)CAR和IL13(E13K.R109K)CAR-2A-P140K.MGMT构建体以及pMFG质粒构建体。

[0256] 实施例2.逆转录病毒颗粒的产生

[0257] 使用"乒乓"方法生成下述MFG逆转录病毒颗粒,该MFG逆转录病毒颗粒包含编码实施例1所述的IL13(E13K.R109K)CAR和IL13(E13K.R109K)CAR-2A-P140KMGMT构建体的构建体。来自实施例1的每个宿主质粒首先被转染至phoenix-eco细胞以生成嗜亲性逆转录病毒。转染效率通过IL13表达的流式细胞术测量。保存培养物上清液并将其用于转导双嗜性编码病毒的小鼠成纤维细胞系PG13(ATCC,Manassas,VA)。测试转导的PG13细胞的IL13并且通过荧光活化的细胞分类器富集IL13阳性细胞(图3A至图3B)。MGMT在IL13富集的细胞中的过表达还通过细胞溶解产物的western印迹分析测试(图3C)。

[0258] 如图3A和图3B所示,转导的细胞表达IL13并且被富集,从而大约97%的细胞表达IL13(E13K.R109K)CAR-2A-P140KMGMT构建体。图3C进一步显示了P140KMGMT在由IL13(E13K.R109K)CAR-2A-P140KMGMT转染的细胞中的表达相对于在未转染的细胞或仅由IL13CAR构建体转染的细胞中的表达提高。

[0259] 富集的细胞在组织培养条件下扩增以收获含有高滴度编码CAR的双嗜性逆转录病毒的培养物上清液。

[0260] 实施例3.人T细胞的基因修饰

[0261] 根据实施例2的方法生成的包含IL13(E13K.R109K)CAR和IL13(E13K.R109K)CAR-2A-P140KMGMT构建体的逆转录病毒颗粒用于转导人T细胞。从血液过滤器废弃物中分离人PBMC(Rhode Island Blood Center,Providence,RI)。在存在OKT3(10μg/ml)和IL2(3000U/ml)的条件下培养PBMC 36小时至48小时以富集T细胞群。在存在鱼精蛋白和IL2的条件下,在室温条件在重组人纤维蛋白片段包被的板中,采用含有培养物上清液的逆转录病毒离心转染富集的T细胞1小时。该步骤在随后的24小时内重复3次。3轮感染之后,使细胞在含有逆转录病毒的培养基中再生长24小时,随后转移至含有10%胎牛血清、抗生素和IL2的新鲜的RPMI-1640培养基中,用于进一步的实验。由IL13(E13K.R109K)CAR(不含P140KMGMT)成功转导的T细胞和未转导的T细胞在全部实验中用作对照。如流式细胞术检测细胞表面上的IL13所测量的(数据未显示),由含有IL13(E13K.R109K)CAR-2A-P140KMGMT构建体的逆转录病毒颗粒转染的大约20%至25%的T细胞对于IL13(E13K.R109K)CAR-2A-P140KMGMT是阳性的。IL13(E13K.R109K)CAR-2A-P140KMGMT的转导效率为约69.2%,其中,未转导的T细胞用作对照。

[0262] 实施例4.转导的T细胞中的替莫唑胺耐药性

[0263] 由上文所述的IL13(E13K.R109K)CAR(图1A)或IL13(E13K.R109K)CAR-2A-P140KMGMT(图2A)转导的T细胞通过浓度增加的替莫唑胺(TMZ:0-1000μM)分别孵育48小时。

每24小时更换培养基并且补充新鲜的TMZ。在用TMZ处理之后，通过台盼(Trypan)蓝排除法和Annexin V/7AAD染色法分析细胞的活力。通过流式细胞术测量Annexin V/7-AAD阴性细胞的频率，并且结果代表细胞活力。构建存活率曲线以外推活力和TMZ活性浓度。如图4所示，由IL13(E13K.R109K)CAR-2A-P140KMGMT转导的T细胞在暴露于TMZ之后相对于IL13(E13K.R109K)CAR转导的T细胞更好地存活。这个观察结果显示出：通过表达P140KMGMT的CAR对T细胞进行基因修饰会对修饰的T细胞产生化学保护。

[0264] 实施例5.IL13CAR-2A-P140KMGMT的功能特征

[0265] 转导的细胞的免疫调节功能也通过测量与胶质瘤细胞共培养时的转导的细胞分泌的细胞因子IL2和IFNγ来分析。已被IL13(E13K.R109K)CAR-2A-P140KMGMT逆转录病毒转导的T细胞在常规组织培养条件下(使用具有5％血清和IL2(3000U/ml)的RPMI1640培养基和200μM TMZ)采用200μM TMZ或不采用200μM TMZ培养48小时至72小时。接下来，采用U251MG胶质瘤细胞培养细胞72小时(如本文关于U251MG共培养所描述的那样)。通过ELISA测试培养物上清液的细胞因子分泌。

[0266] 白介素-2(IL2)是T细胞活力和增殖的标记物，同时干扰素-γ(IFNγ)是细胞毒性T细胞的功能的标记物。如图5A和图5B所示，转导的细胞在不存在TMZ或存在TMZ的条件下分泌IL2和IFNγ这两者。而且，TMZ的存在不会显著降低转导细胞的细胞因子分泌。TMZ耐药性的T细胞能够在暴露于TMZ之后维持其正常的细胞毒性功能，这说明这些基因修饰确实使得这些细胞对TMZ诱导的白细胞减少性细胞毒性具有耐受性。

[0267] 实施例6.IL13CAR-2A-MGMT的体内效力

[0268] 在小鼠体内，使用如实施例2所述生成的病毒颗粒，测试IL13E13K.R109K-2A-P140KMGMT构建体的体内效力。向50只无胸腺裸鼠皮下(左侧)注射U251MG胶质瘤细胞(40只小鼠，I-IV组)或PBS(10只小鼠)。胶质瘤植入之后4天，三组小鼠(I组，II组和III组，10只小鼠/组)通过口服给药TMZ(通过口服管饲，64mg/kg/天)进行治疗持续4天。在胶质瘤植入后第5天，对已口服给药TMZ进行治疗的三组小鼠进行如下治疗：I组：肿瘤内注射IL13E13K.R109KCAR-2A-P140KMGMT构建体(TMZ耐药性)；II组：肿瘤内注射不含MGMT的IL13IL13E13K.R109K构建体(TMZ敏感性)；III组：仅采用PBS治疗(没有注射T细胞)。IV组没有接受T细胞治疗或TMZ治疗。监测小鼠可以看到的肿瘤尺寸、行为改变以及发病率，直至注射T细胞之后90天，这时根据IACUC限制的要求宰杀小鼠。

[0269] 根据小鼠实验结果绘制的存活率曲线显示，与由TMZ敏感性的不含MGMT的IL13(E13K.R109K)CAR T细胞和TMZ治疗的II组动物的61天的中值存活期和14％的存活率相比，由IL13(E13K.R109K)CAR-2A-P140KMGMT T细胞和TMZ治疗的带有肿瘤的小鼠(I组)具有73天的中值存活期和40％动物的存活率。没有接受治疗的带有肿瘤的小鼠(IV组)具有29天的中值存活期。没有接受T细胞只接受了口服TMZ治疗的III组带有肿瘤的动物表现出20％的存活率，但是也表现出仅36天的较低的中值存活期，这被认为是TMZ治疗的背景抗肿瘤效果(图9和图10)。该观察结果表明接受3G TMZ耐药性CAR的I组的动物在通过CAR免疫治疗和TMZ化疗的协同作用消除肿瘤方面最有效。

[0270] 本文引用的所有专利、公开申请和参考文献的教导的全部内容通过引用并入本文。

[0271] 虽然已参考本发明的实施方式对本发明进行了具体展示和描述，但是本领域技术

人员应当理解的是,在不背离所附的权利要求书涵盖的本发明的范围的条件下,可在形式和细节上作出各种不同的改变。

序列表

<110> 罗杰威廉姆斯医院(Roger Williams Hospital)

<120> 治疗癌症的方法和组合物

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aagaaacttt ttaaggaggg acagttcaac cctaggaagc ccaccacgac gccagcgccg 480
cgaccaccaa caccggcgcc caccatcgcg tcgcagcccc tgtccctgcg cccagaggcg 540
tgccggccag cggcgggggg cgcagtgcac acgaggggcc tggacttcgc ccaattgctc 600
tgctacctgc tggatggaat cctcttcatc tatggtgtca ttctcactgc cttgttcctg 660
agagtggtta acttctgggt gaggagtaag aggagcaggc tcctgcacag tgactacatg 720
aacatgactc cccgccgccc cgggcccacc cgcaagcatt accagcccta tgccccacca 780
cgcgacttcg cagcctatcg ctccacgcgt aagttcagca ggagcgcaga cgcccccgcg 840
taccagcagg gccagaacca gctctataac gagctcaatc taggacgaag agaggagtac 900
gatgttttgg acaagagacg tggccgggac cctgagatgg ggggaaagcc gagaaggaag 960
aaccctcagg aaggcctgta caatgaactg cagaaagata agatggcgga ggcctacagt 1020
gagattggga tgaaaggcga gcgccggagg ggcaaggggc acgatggcct ttaccagggt 1080
ctcagtacag ccaccaagga cacctacgac gcccttcaca tgcaggccct gcccccctcgc 1140
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ggaaagggca ctagcgccgc tgatgctgtg gaagtgccag ctccagctgc tgtgctggga 1380

ggacctgagc cactgatgca gtgcaccgcc tggctgaacg cttacttcca tcagcctgaa 1440
gccatcgagg aatttcccgt gcctgccctg caccatccag tgttccagca ggagagtttt 1500
acaaggcagg tgctgtggaa gctgctgaaa gtggtgaagt tcggggaagt gatttcctac 1560
cagcagctgg ctgctctggc tggaaaccca aaagctgctc gggccgtggg aggagctatg 1620
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gtggggaact attctggggg actggccgtg aaagaatggc tgctggctca cgagggacat 1740
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gctggagcta cctcaggaag cccacctgcc ggccggaatt gacggccg 1848

<210> 4
<211> 606
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体
<400> 4

Met His Pro Leu Leu Asn Pro Leu Leu Leu Ala Leu Gly Leu Met Ala
1 5 10 15
Leu Leu Leu Thr Thr Val Ile Ala Leu Thr Cys Leu Gly Gly Phe Ala
20 25 30
Ser Pro Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Glu Leu Ile Glu
35 40 45
Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly
50 55 60
Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala
65 70 75 80
Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr
85 90 95
Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln
100 105 110
Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe
115 120 125
Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Arg Glu Gly Gln
130 135 140
Phe Asn Pro Arg Lys Pro Thr Thr Thr Pro Ala Pro Arg Pro Pro Thr
145 150 155 160
Pro Ala Pro Thr Ile Ala Ser Gln Pro Leu Ser Leu Arg Pro Glu Ala
165 170 175
Cys Arg Pro Ala Ala Gly Gly Ala Val His Thr Arg Gly Leu Asp Phe
180 185 190

Ala Gln Leu Leu Cys Tyr Leu Leu Asp Gly Ile Leu Phe Ile Tyr Gly
195 200 205
Val Ile Leu Thr Ala Leu Phe Leu Arg Val Val Thr Phe Trp Val Arg
210 215 220
Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn Met Thr Pro
225 230 235 240
Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr Ala Pro Pro
245 250 255
Arg Asp Phe Ala Ala Tyr Arg Ser Thr Arg Lys Phe Ser Arg Ser Ala
260 265 270
Asp Ala Pro Ala Tyr Gln Gln Gly Gln Asn Gln Leu Tyr Asn Glu Leu
275 280 285
Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Asp Lys Arg Arg Gly
290 295 300
Arg Asp Pro Glu Met Gly Gly Lys Pro Arg Arg Lys Asn Pro Gln Glu
305 310 315 320
Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser
325 330 335
Glu Ile Gly Met Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly
340 345 350
Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr Asp Ala Leu
355 360 365
His Met Gln Ala Leu Pro Pro Arg Gln Pro Ala Ala Ala Glu Gly Arg
370 375 380
Gly Ser Leu Leu Thr Cys Gly Asp Val Glu Glu Asn Pro Gly Pro Met
385 390 395 400
Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu Gly
405 410 415
Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys Leu
420 425 430
Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala Pro
435 440 445
Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala Trp
450 455 460
Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro Val
465 470 475 480
Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg Gln
485 490 495
Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile Ser

500 505 510

Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg Ala
515 520 525

Val Gly Gly Ala Met Arg Gly Asn Pro Val Lys Ile Leu Ile Pro Cys
530 535 540

His Arg Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser Gly Gly
545 550 555 560

Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg Leu Gly
565 570 575

Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu Lys
580 585 590

Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
595 600 605

<210> 5

<211> 606

<212> PRT

<213> 人工序列

<220>

<223> 合成构建体

<400> 5

Met His Pro Leu Leu Asn Pro Leu Leu Leu Ala Leu Gly Leu Met Ala
1 5 10 15

Leu Leu Leu Thr Thr Val Ile Ala Leu Thr Cys Leu Gly Gly Phe Ala
20 25 30

Ser Pro Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Tyr Leu Ile Glu
35 40 45

Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly
50 55 60

Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala
65 70 75 80

Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr
85 90 95

Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln
100 105 110

Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe
115 120 125

Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Arg Glu Gly Gln
130 135 140

Phe Asn Pro Arg Lys Pro Thr Thr Thr Pro Ala Pro Arg Pro Pro Thr

145 150 155 160
Pro Ala Pro Thr Ile Ala Ser Gln Pro Leu Ser Leu Arg Pro Glu Ala
165 170 175
Cys Arg Pro Ala Ala Gly Gly Ala Val His Thr Arg Gly Leu Asp Phe
180 185 190
Ala Gln Leu Leu Cys Tyr Leu Leu Asp Gly Ile Leu Phe Ile Tyr Gly
195 200 205
Val Ile Leu Thr Ala Leu Phe Leu Arg Val Val Thr Phe Trp Val Arg
210 215 220
Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn Met Thr Pro
225 230 235 240
Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr Ala Pro Pro
245 250 255
Arg Asp Phe Ala Ala Tyr Arg Ser Thr Arg Lys Phe Ser Arg Ser Ala
260 265 270
Asp Ala Pro Ala Tyr Gln Gln Gly Gln Asn Gln Leu Tyr Asn Glu Leu
275 280 285
Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Asp Lys Arg Arg Gly
290 295 300
Arg Asp Pro Glu Met Gly Gly Lys Pro Arg Arg Lys Asn Pro Gln Glu
305 310 315 320
Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser
325 330 335
Glu Ile Gly Met Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly
340 345 350
Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr Asp Ala Leu
355 360 365
His Met Gln Ala Leu Pro Pro Arg Gln Pro Ala Ala Ala Glu Gly Arg
370 375 380
Gly Ser Leu Leu Thr Cys Gly Asp Val Glu Glu Asn Pro Gly Pro Met
385 390 395 400
Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu Gly
405 410 415
Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys Leu
420 425 430
Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala Pro
435 440 445
Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala Trp
450 455 460

Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro Val
465 470 475 480
Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg Gln
485 490 495
Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile Ser
500 505 510
Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg Ala
515 520 525
Val Gly Gly Ala Met Arg Gly Asn Pro Val Lys Ile Leu Ile Pro Cys
530 535 540
His Arg Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser Gly Gly
545 550 555 560
Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg Leu Gly
565 570 575
Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu Lys
580 585 590
Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
595 600 605

<210> 6
<211> 606
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体
<400> 6

Met His Pro Leu Leu Asn Pro Leu Leu Leu Ala Leu Gly Leu Met Ala
1 5 10 15
Leu Leu Leu Thr Thr Val Ile Ala Leu Thr Cys Leu Gly Gly Phe Ala
20 25 30
Ser Pro Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Lys Leu Ile Glu
35 40 45
Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly
50 55 60
Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala
65 70 75 80
Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr
85 90 95
Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln
100 105 110

Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe
115 120 125
Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Lys Glu Gly Gln
130 135 140
Phe Asn Pro Arg Lys Pro Thr Thr Thr Pro Ala Pro Arg Pro Pro Thr
145 150 155 160
Pro Ala Pro Thr Ile Ala Ser Gln Pro Leu Ser Leu Arg Pro Glu Ala
165 170 175
Cys Arg Pro Ala Ala Gly Gly Ala Val His Thr Arg Gly Leu Asp Phe
180 185 190
Ala Gln Leu Leu Cys Tyr Leu Leu Asp Gly Ile Leu Phe Ile Tyr Gly
195 200 205
Val Ile Leu Thr Ala Leu Phe Leu Arg Val Val Thr Phe Trp Val Arg
210 215 220
Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn Met Thr Pro
225 230 235 240
Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr Ala Pro Pro
245 250 255
Arg Asp Phe Ala Ala Tyr Arg Ser Thr Arg Lys Phe Ser Arg Ser Ala
260 265 270
Asp Ala Pro Ala Tyr Gln Gln Gly Gln Asn Gln Leu Tyr Asn Glu Leu
275 280 285
Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Asp Lys Arg Arg Gly
290 295 300
Arg Asp Pro Glu Met Gly Gly Lys Pro Arg Arg Lys Asn Pro Gln Glu
305 310 315 320
Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser
325 330 335
Glu Ile Gly Met Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly
340 345 350
Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr Asp Ala Leu
355 360 365
His Met Gln Ala Leu Pro Pro Arg Gln Pro Ala Ala Ala Glu Gly Arg
370 375 380
Gly Ser Leu Leu Thr Cys Gly Asp Val Glu Glu Asn Pro Gly Pro Met
385 390 395 400
Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu Gly
405 410 415
Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys Leu

420 425 430

Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala Pro

435 440 445

Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala Trp

450 455 460

Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro Val

465 470 475 480

Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg Gln

485 490 495

Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile Ser

500 505 510

Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg Ala

515 520 525

Val Gly Gly Ala Met Arg Gly Asn Pro Val Lys Ile Leu Ile Pro Cys

530 535 540

His Arg Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser Gly Gly

545 550 555 560

Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg Leu Gly

565 570 575

Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu Lys

580 585 590

Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn

595 600 605

<210> 7

<211> 6

<212> DNA

<213> 人工序列

<220>

<223> 合成构建体

<400> 7

ggatcc 6

<210> 8

<211> 6

<212> DNA

<213> 人工序列

<220>

<223> 合成构建体

<400> 8

gccacc 6

<210> 9
<211> 96
<212> DNA
<213> 智人
<400> 9
atgcatccgc tcctcaatcc tctcctgttg gcactgggcc tcatggcgct tttgttgacc 60
acggtcattg ctctcacttg ccttggcggc tttgcc 96
<210> 10
<211> 342
<212> DNA
<213> 智人
<400> 10
tccccaggcc ctgtgcctcc ctctacagcc ctcagggagc tcattgagga gctggtcaac 60
atcacccaga accagaaggc tccgctctgc aatggcagca tggtatggag catcaacctg 120
acagctggca tgtactgtgc agccctggaa tccctgatca acgtgtcagg ctgcagtgcc 180
atcgagaaga cccagaggat gctgagcgga ttctgcccgc acaaggtctc agctgggcag 240
ttttccagct tgcatgtccg agacaccaaa atcgaggtgg cccagtttgt aaaggacctg 300
ctcttacatt taaagaaact ttttcgcgag ggacagttca ac 342
<210> 11
<211> 6
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 11
cctagg 6
<210> 12
<211> 135
<212> DNA
<213> 智人
<400> 12
aagcccacca cgacgccagc gccgcgacca ccaacaccgg cgcccaccat cgcgtcgcag 60
cccctgtccc tgcgcccaga ggcgtgccgg ccagcggcgg ggggcgcagt gcacacgagg 120
gggctggact tcgcc 135
<210> 13
<211> 6
<212> DNA
<213> 人工序列
<220>

<223> 合成构建体
<400> 13
caattg 6
<210> 14
<211> 69
<212> DNA
<213> 智人
<400> 14
ctctgctacc tgctggatgg aatcctcttc atctatggtg tcattctcac tgccttgttc 60
ctgagagtg 69
<210> 15
<211> 6
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 15
gttaac 6
<210> 16
<211> 132
<212> DNA
<213> 智人
<400> 16
ttctgggtga ggagtaagag gagcaggctc ctgcacagtg actacatgaa catgactccc 60
cgccgccccg ggcccacccg caagcattac cagccctatg ccccaccacg cgacttcgca 120
gcctatcgct cc 132
<210> 17
<211> 6
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 17
acgcgt 6
<210> 18
<211> 330
<212> DNA
<213> 智人
<400> 18

aagttcagca ggagcgcaga cgccccgcg taccagcagg gccagaacca gctctataac 60
gagctcaatc taggacgaag agaggagtac gatgttttgg acaagagacg tggccgggac 120
cctgagatgg ggggaaagcc gagaaggaag aaccctcagg aaggcctgta caatgaactg 180
cagaaagata agatggcgga ggcctacagt gagattggga tgaaaggcga gcgccggagg 240
ggcaaggggc acgatggcct ttaccagggt ctcagtacag ccaccaagga cacctacgac 300
gcccttcaca tgcaggccct gcccccctcgc 330

<210> 19
<211> 3
<212> DNA
<213> 智人
<400> 19
taa 3

<210> 20
<211> 15
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 20
cagccagcgg ccgca 15

<210> 21
<211> 54
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 21
gagggcagag gaagtcttct aacatgcggt gacgtggagg agaatcccgg ccct 54

<210> 22
<211> 621
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 22
atggacaaag attgcgagat gaagcggacc acactggact ccccccctggg caaactggag 60
ctgtctggct gtgaacaggg gctgcacgag atcaaactgc tgggaaaggg cactagcgcc 120
gctgatgctg tggaagtgcc agctccagct gctgtgctgg gaggacctga gccactgatg 180
cagtgcaccg cctggctgaa cgcttacttc catcagcctg aagccatcga ggaatttccc 240

gtgcctgccc tgcaccatcc agtgttccag caggagagtt ttacaaggca ggtgctgtgg 300
aagctgctga aagtggtgaa gttcggggaa gtgatttcct accagcagct ggctgctctg 360
gctggaaacc caaaagctgc tcgggccgtg ggaggagcta tgagaggcaa tccagtgaaa 420
atcctgattc cctgccacag ggtggtgtgt agctccggag ctgtggggaa ctattctggg 480
ggactggccg tgaaagaatg gctgctggct cacgagggac ataggctggg aaagcctggc 540
ctgggagggt ctagtggact ggctggagct tggctgaagg gagctggagc tacctcagga 600
agcccacctg ccggccggaa t 621

<210> 23
<211> 3
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 23
tga 3

<210> 24
<211> 6
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 24
cggccg 6

<210> 25
<211> 32
<212> PRT
<213> 智人
<400> 25

Met	His	Pro	Leu	Leu	Asn	Pro	Leu	Leu	Leu	Ala	Leu	Gly	Leu	Met	Ala
1				5				10					15		
Leu	Leu	Leu	Thr	Thr	Val	Ile	Ala	Leu	Thr	Cys	Leu	Gly	Gly	Phe	Ala
			20					25				30			

<210> 26
<211> 114
<212> PRT
<213> 智人
<400> 26

Ser	Pro	Gly	Pro	Val	Pro	Pro	Ser	Thr	Ala	Leu	Arg	Glu	Leu	Ile	Glu
1				5				10					15		

Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly
20 25 30
Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala
35 40 45
Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr
50 55 60
Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln
65 70 75 80
Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe
85 90 95
Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Arg Glu Gly Gln
100 105 110
Phe Asn

<210> 27
<211> 45
<212> PRT
<213> 智人
<400> 27
Lys Pro Thr Thr Thr Pro Ala Pro Arg Pro Pro Thr Pro Ala Pro Thr
1 5 10 15
Ile Ala Ser Gln Pro Leu Ser Leu Arg Pro Glu Ala Cys Arg Pro Ala
20 25 30
Ala Gly Gly Ala Val His Thr Arg Gly Leu Asp Phe Ala
35 40 45

<210> 28
<211> 23
<212> PRT
<213> 智人
<400> 28
Leu Cys Tyr Leu Leu Asp Gly Ile Leu Phe Ile Tyr Gly Val Ile Leu
1 5 10 15
Thr Ala Leu Phe Leu Arg Val
20

<210> 29
<211> 44
<212> PRT
<213> 智人
<400> 29
Phe Trp Val Arg Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met

1 5 10 15
Asn Met Thr Pro Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro
20 25 30
Tyr Ala Pro Pro Arg Asp Phe Ala Ala Tyr Arg Ser
35 40
<210> 30
<211> 110
<212> PRT
<213> 智人
<400> 30
Lys Phe Ser Arg Ser Ala Asp Ala Pro Ala Tyr Gln Gln Gly Gln Asn
1 5 10 15
Gln Leu Tyr Asn Glu Leu Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val
20 25 30
Leu Asp Lys Arg Arg Gly Arg Asp Pro Glu Met Gly Gly Lys Pro Arg
35 40 45
Arg Lys Asn Pro Gln Glu Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys
50 55 60
Met Ala Glu Ala Tyr Ser Glu Ile Gly Met Lys Gly Glu Arg Arg Arg
65 70 75 80
Gly Lys Gly His Asp Gly Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys
85 90 95
Asp Thr Tyr Asp Ala Leu His Met Gln Ala Leu Pro Pro Arg
100 105 110
<210> 31
<211> 5
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体
<400> 31
Gln Pro Ala Ala Ala
1 5
<210> 32
<211> 18
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体

<400> 32

Glu Gly Arg Gly Ser Leu Leu Thr Cys Gly Asp Val Glu Glu Asn Pro
1 5 10 15

Gly Pro

<210> 33

<211> 207

<212> PRT

<213> 人工序列

<220>

<223> 合成构建体

<400> 33

Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu
1 5 10 15

Gly Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys
20 25 30

Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala
35 40 45

Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala
50 55 60

Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro
65 70 75 80

Val Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg
85 90 95

Gln Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile
100 105 110

Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg
115 120 125

Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Lys Ile Leu Ile Pro
130 135 140

Cys His Arg Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser Gly
145 150 155 160

Gly Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg Leu
165 170 175

Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu
180 185 190

Lys Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
195 200 205

<210> 34

<211> 342

<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 34
tccccaggcc ctgtgcctcc ctctacagcc ctcaggtacc tcattgagga gctggtcaac 60
atcacccaga accagaaggc tccgctctgc aatggcagca tggtatggag catcaacctg 120
acagctggca tgtactgtgc agccctggaa tccctgatca acgtgtcagg ctgcagtgcc 180
atcgagaaga cccagaggat gctgagcgga ttctgcccgc acaaggtctc agctgggcag 240
ttttccagct tgcatgtccg agacaccaaa atcgaggtgg cccagtttgt aaaggacctg 300
ctcttacatt taaagaaact ttttcgcgag ggacagttca ac 342
<210> 35
<211> 342
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 35
tccccaggcc ctgtgcctcc ctctacagcc ctcaggaagc tcattgagga gctggtcaac 60
atcacccaga accagaaggc tccgctctgc aatggcagca tggtatggag catcaacctg 120
acagctggca tgtactgtgc agccctggaa tccctgatca acgtgtcagg ctgcagtgcc 180
atcgagaaga cccagaggat gctgagcgga ttctgcccgc acaaggtctc agctgggcag 240
ttttccagct tgcatgtccg agacaccaaa atcgaggtgg cccagtttgt aaaggacctg 300
ctcttacatt taaagaaact ttttaaggag ggacagttca ac 342
<210> 36
<211> 114
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体
<400> 36
Ser Pro Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Tyr Leu Ile Glu
1 5 10 15
Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly
20 25 30
Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala
35 40 45
Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr
50 55 60

Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln
65 70 75 80
Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe
85 90 95
Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Arg Glu Gly Gln
100 105 110
Phe Asn

<210> 37
<211> 114
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体
<400> 37

Ser Pro Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Lys Leu Ile Glu
1 5 10 15
Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly
20 25 30
Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala
35 40 45
Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr
50 55 60
Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln
65 70 75 80
Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe
85 90 95
Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Lys Glu Gly Gln
100 105 110
Phe Asn

<210> 38
<211> 207
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体
<400> 38

Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu
1 5 10 15
Gly Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys

20 25 30

Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala
35 40 45

Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala
50 55 60

Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro
65 70 75 80

Val Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg
85 90 95

Gln Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile
100 105 110

Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg
115 120 125

Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Pro Ile Leu Ile Pro
130 135 140

Cys His Arg Val Val Cys Ser Ser Gly Ala Val Ala Asn Tyr Ser Gly
145 150 155 160

Gly Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg Leu
165 170 175

Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu
180 185 190

Lys Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
195 200 205

<210> 39

<211> 207

<212> PRT

<213> 人工序列

<220>

<223> 合成构建体

<400> 39

Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu
1 5 10 15

Gly Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys
20 25 30

Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala
35 40 45

Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala
50 55 60

Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro

65 70 75 80
Val Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg
85 90 95
Gln Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile
100 105 110
Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg
115 120 125
Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Pro Ile Leu Ile Pro
130 135 140
Cys His Arg Val Val Cys Ser His Gly Gly Val Gly Asn His Ser Ser
145 150 155 160
Gly Val Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg Leu
165 170 175
Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu
180 185 190
Lys Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
195 200 205

<210> 40
<211> 207
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体
<400> 40

Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu
1 5 10 15
Gly Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys
20 25 30
Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala
35 40 45
Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala
50 55 60
Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro
65 70 75 80
Val Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg
85 90 95
Gln Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile
100 105 110
Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg

115 120 125
Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Pro Ile Leu Ile Pro
130 135 140
Cys His Arg Val Val Tyr Ser Ser Gly Gly Val Gly Asn Phe Ser Gly
145 150 155 160
Gly Pro Ala Val Arg Glu Trp Leu Leu Ala His Glu Gly His Arg Leu
165 170 175
Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu
180 185 190
Lys Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
195 200 205

<210> 41
<211> 207
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体
<400> 41
Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu
1 5 10 15
Gly Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys
20 25 30
Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala
35 40 45
Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala
50 55 60
Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro
65 70 75 80
Val Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg
85 90 95
Gln Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile
100 105 110
Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg
115 120 125
Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Pro Ile Leu Ile Pro
130 135 140
Cys His Arg Val Val Cys Ser Ser Gly Ala Val Gly Thr His Ser Gly
145 150 155 160
Gly Leu Ala Val Lys Glu Trp Leu Leu Ser His Glu Gly His Arg Leu

165 170 175
Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu
180 185 190
Lys Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
195 200 205

<210> 42
<211> 6
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 42
ccatgg 6

<210> 43
<211> 238
<212> PRT
<213> 智人
<400> 43
Met Leu Gly Gln Pro Ala Pro Leu Glu Arg Phe Ala Ser Arg Arg Pro
1 5 10 15
Gln Val Leu Ala Val Arg Thr Val Cys Asp Leu Val Leu Gly Lys Met
20 25 30
Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu Gly
35 40 45
Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys Leu
50 55 60
Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala Pro
65 70 75 80
Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala Trp
85 90 95
Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro Val
100 105 110
Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg Gln
115 120 125
Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile Ser
130 135 140
Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg Ala
145 150 155 160
Val Gly Gly Ala Met Arg Gly Asn Pro Val Lys Ile Leu Ile Pro Cys

165 170 175
His Arg Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser Gly Gly
180 185 190
Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg Leu Gly
195 200 205
Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu Lys
210 215 220
Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
225 230 235

<210> 44
<211> 380
<212> PRT
<213> 智人
<400> 44

Met Ala Phe Val Cys Leu Ala Ile Gly Cys Leu Tyr Thr Phe Leu Ile
1 5 10 15
Ser Thr Thr Phe Gly Cys Thr Ser Ser Ser Asp Thr Glu Ile Lys Val
20 25 30
Asn Pro Pro Gln Asp Phe Glu Ile Val Asp Pro Gly Tyr Leu Gly Tyr
35 40 45
Leu Tyr Leu Gln Trp Gln Pro Pro Leu Ser Leu Asp His Phe Lys Glu
50 55 60
Cys Thr Val Glu Tyr Glu Leu Lys Tyr Arg Asn Ile Gly Ser Glu Thr
65 70 75 80
Trp Lys Thr Ile Ile Thr Lys Asn Leu His Tyr Lys Asp Gly Phe Asp
85 90 95
Leu Asn Lys Gly Ile Glu Ala Lys Ile His Thr Leu Leu Pro Trp Gln
100 105 110
Cys Thr Asn Gly Ser Glu Val Gln Ser Ser Trp Ala Glu Thr Thr Tyr
115 120 125
Trp Ile Ser Pro Gln Gly Ile Pro Glu Thr Lys Val Gln Asp Met Asp
130 135 140
Cys Val Tyr Tyr Asn Trp Gln Tyr Leu Leu Cys Ser Trp Lys Pro Gly
145 150 155 160
Ile Gly Val Leu Leu Asp Thr Asn Tyr Asn Leu Phe Tyr Trp Tyr Glu
165 170 175
Gly Leu Asp His Ala Leu Gln Cys Val Asp Tyr Ile Lys Ala Asp Gly
180 185 190
Gln Asn Ile Gly Cys Arg Phe Pro Tyr Leu Glu Ala Ser Asp Tyr Lys

195 200 205

Asp Phe Tyr Ile Cys Val Asn Gly Ser Ser Glu Asn Lys Pro Ile Arg
210 215 220

Ser Ser Tyr Phe Thr Phe Gln Leu Gln Asn Ile Val Lys Pro Leu Pro
225 230 235 240

Pro Val Tyr Leu Thr Phe Thr Arg Glu Ser Ser Cys Glu Ile Lys Leu
245 250 255

Lys Trp Ser Ile Pro Leu Gly Pro Ile Pro Ala Arg Cys Phe Asp Tyr
260 265 270

Glu Ile Glu Ile Arg Glu Asp Asp Thr Thr Leu Val Thr Ala Thr Val
275 280 285

Glu Asn Glu Thr Tyr Thr Leu Lys Thr Thr Asn Glu Thr Arg Gln Leu
290 295 300

Cys Phe Val Val Arg Ser Lys Val Asn Ile Tyr Cys Ser Asp Asp Gly
305 310 315 320

Ile Trp Ser Glu Trp Ser Asp Lys Gln Cys Trp Glu Gly Glu Asp Leu
325 330 335

Ser Lys Lys Thr Leu Leu Arg Phe Trp Leu Pro Phe Gly Phe Ile Leu
340 345 350

Ile Leu Val Ile Phe Val Thr Gly Leu Leu Leu Arg Lys Pro Asn Thr
355 360 365

Tyr Pro Lys Met Ile Pro Glu Phe Phe Cys Asp Thr
370 375 380

<210> 45

<211> 608

<212> PRT

<213> 人工序列

<220>

<223> 合成构建体

<400> 45

Met His Pro Leu Leu Asn Pro Leu Leu Leu Ala Leu Gly Leu Met Ala
1 5 10 15

Leu Leu Leu Thr Thr Val Ile Ala Leu Thr Cys Leu Gly Gly Phe Ala
20 25 30

Ser Pro Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Glu Leu Ile Glu
35 40 45

Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly
50 55 60

Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala

65 70 75 80
Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr
85 90 95
Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln
100 105 110
Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe
115 120 125
Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Arg Glu Gly Gln
130 135 140
Phe Asn Pro Arg Lys Pro Thr Thr Thr Pro Ala Pro Arg Pro Pro Thr
145 150 155 160
Pro Ala Pro Thr Ile Ala Ser Gln Pro Leu Ser Leu Arg Pro Glu Ala
165 170 175
Cys Arg Pro Ala Ala Gly Gly Ala Val His Thr Arg Gly Leu Asp Phe
180 185 190
Ala Gln Leu Leu Cys Tyr Leu Leu Asp Gly Ile Leu Phe Ile Tyr Gly
195 200 205
Val Ile Leu Thr Ala Leu Phe Leu Arg Val Val Thr Phe Trp Val Arg
210 215 220
Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn Met Thr Pro
225 230 235 240
Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr Ala Pro Pro
245 250 255
Arg Asp Phe Ala Ala Tyr Arg Ser Thr Arg Lys Phe Ser Arg Ser Ala
260 265 270
Asp Ala Pro Ala Tyr Gln Gln Gly Gln Asn Gln Leu Tyr Asn Glu Leu
275 280 285
Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Asp Lys Arg Arg Gly
290 295 300
Arg Asp Pro Glu Met Gly Gly Lys Pro Arg Arg Lys Asn Pro Gln Glu
305 310 315 320
Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser
325 330 335
Glu Ile Gly Met Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly
340 345 350
Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr Asp Ala Leu
355 360 365
His Met Gln Ala Leu Pro Pro Arg Gln Pro Ala Ala Ala Glu Gly Arg
370 375 380

Gly Ser Leu Leu Thr Cys Gly Asp Val Glu Glu Asn Pro Gly Pro Pro
385 390 395 400
Trp Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro
405 410 415
Leu Gly Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile
420 425 430
Lys Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro
435 440 445
Ala Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr
450 455 460
Ala Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe
465 470 475 480
Pro Val Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr
485 490 495
Arg Gln Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val
500 505 510
Ile Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala
515 520 525
Arg Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Lys Ile Leu Ile
530 535 540
Pro Cys His Arg Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser
545 550 555 560
Gly Gly Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg
565 570 575
Leu Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp
580 585 590
Leu Lys Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
595 600 605

<210> 46
<211> 608
<212> PRT
<213> 人工序列
<220>
<223> 合成构建体
<400> 46

Met His Pro Leu Leu Asn Pro Leu Leu Leu Ala Leu Gly Leu Met Ala
1 5 10 15
Leu Leu Leu Thr Thr Val Ile Ala Leu Thr Cys Leu Gly Gly Phe Ala
20 25 30

Ser Pro Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Tyr Leu Ile Glu
35 40 45
Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly
50 55 60
Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala
65 70 75 80
Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr
85 90 95
Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln
100 105 110
Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe
115 120 125
Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Arg Glu Gly Gln
130 135 140
Phe Asn Pro Arg Lys Pro Thr Thr Thr Pro Ala Pro Arg Pro Pro Thr
145 150 155 160
Pro Ala Pro Thr Ile Ala Ser Gln Pro Leu Ser Leu Arg Pro Glu Ala
165 170 175
Cys Arg Pro Ala Ala Gly Gly Ala Val His Thr Arg Gly Leu Asp Phe
180 185 190
Ala Gln Leu Leu Cys Tyr Leu Leu Asp Gly Ile Leu Phe Ile Tyr Gly
195 200 205
Val Ile Leu Thr Ala Leu Phe Leu Arg Val Val Thr Phe Trp Val Arg
210 215 220
Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn Met Thr Pro
225 230 235 240
Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr Ala Pro Pro
245 250 255
Arg Asp Phe Ala Ala Tyr Arg Ser Thr Arg Lys Phe Ser Arg Ser Ala
260 265 270
Asp Ala Pro Ala Tyr Gln Gln Gly Gln Asn Gln Leu Tyr Asn Glu Leu
275 280 285
Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Asp Lys Arg Arg Gly
290 295 300
Arg Asp Pro Glu Met Gly Gly Lys Pro Arg Arg Lys Asn Pro Gln Glu
305 310 315 320
Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser
325 330 335
Glu Ile Gly Met Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly

340 345 350

Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr Asp Ala Leu
355 360 365

His Met Gln Ala Leu Pro Pro Arg Gln Pro Ala Ala Ala Glu Gly Arg
370 375 380

Gly Ser Leu Leu Thr Cys Gly Asp Val Glu Glu Asn Pro Gly Pro Pro
385 390 395 400

Trp Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro
405 410 415

Leu Gly Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile
420 425 430

Lys Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro
435 440 445

Ala Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr
450 455 460

Ala Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe
465 470 475 480

Pro Val Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr
485 490 495

Arg Gln Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val
500 505 510

Ile Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala
515 520 525

Arg Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Lys Ile Leu Ile
530 535 540

Pro Cys His Arg Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser
545 550 555 560

Gly Gly Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg
565 570 575

Leu Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp
580 585 590

Leu Lys Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
595 600 605

<210> 47

<211> 608

<212> PRT

<213> 人工序列

<220>

<223> 合成构建体

<400> 47

Met His Pro Leu Leu Asn Pro Leu Leu Leu Ala Leu Gly Leu Met Ala
1 5 10 15
Leu Leu Leu Thr Thr Val Ile Ala Leu Thr Cys Leu Gly Gly Phe Ala
20 25 30
Ser Pro Gly Pro Val Pro Pro Ser Thr Ala Leu Arg Lys Leu Ile Glu
35 40 45
Glu Leu Val Asn Ile Thr Gln Asn Gln Lys Ala Pro Leu Cys Asn Gly
50 55 60
Ser Met Val Trp Ser Ile Asn Leu Thr Ala Gly Met Tyr Cys Ala Ala
65 70 75 80
Leu Glu Ser Leu Ile Asn Val Ser Gly Cys Ser Ala Ile Glu Lys Thr
85 90 95
Gln Arg Met Leu Ser Gly Phe Cys Pro His Lys Val Ser Ala Gly Gln
100 105 110
Phe Ser Ser Leu His Val Arg Asp Thr Lys Ile Glu Val Ala Gln Phe
115 120 125
Val Lys Asp Leu Leu Leu His Leu Lys Lys Leu Phe Lys Glu Gly Gln
130 135 140
Phe Asn Pro Arg Lys Pro Thr Thr Thr Pro Ala Pro Arg Pro Pro Thr
145 150 155 160
Pro Ala Pro Thr Ile Ala Ser Gln Pro Leu Ser Leu Arg Pro Glu Ala
165 170 175
Cys Arg Pro Ala Ala Gly Gly Ala Val His Thr Arg Gly Leu Asp Phe
180 185 190
Ala Gln Leu Leu Cys Tyr Leu Leu Asp Gly Ile Leu Phe Ile Tyr Gly
195 200 205
Val Ile Leu Thr Ala Leu Phe Leu Arg Val Val Thr Phe Trp Val Arg
210 215 220
Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn Met Thr Pro
225 230 235 240
Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr Ala Pro Pro
245 250 255
Arg Asp Phe Ala Ala Tyr Arg Ser Thr Arg Lys Phe Ser Arg Ser Ala
260 265 270
Asp Ala Pro Ala Tyr Gln Gln Gly Gln Asn Gln Leu Tyr Asn Glu Leu
275 280 285
Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Asp Lys Arg Arg Gly
290 295 300

Arg Asp Pro Glu Met Gly Gly Lys Pro Arg Arg Lys Asn Pro Gln Glu
305 310 315 320
Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser
325 330 335
Glu Ile Gly Met Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly
340 345 350
Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr Asp Ala Leu
355 360 365
His Met Gln Ala Leu Pro Pro Arg Gln Pro Ala Ala Ala Glu Gly Arg
370 375 380
Gly Ser Leu Leu Thr Cys Gly Asp Val Glu Glu Asn Pro Gly Pro Pro
385 390 395 400
Trp Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro
405 410 415
Leu Gly Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile
420 425 430
Lys Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro
435 440 445
Ala Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr
450 455 460
Ala Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe
465 470 475 480
Pro Val Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr
485 490 495
Arg Gln Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val
500 505 510
Ile Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala
515 520 525
Arg Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Lys Ile Leu Ile
530 535 540
Pro Cys His Arg Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser
545 550 555 560
Gly Gly Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg
565 570 575
Leu Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp
580 585 590
Leu Lys Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
595 600 605

<210> 48

<211> 621
<212> DNA
<213> 人工序列
<220>
<223> 合成构建体
<400> 48
atggacaaag attgcgagat gaagcggacc acactggact ccccccctggg caaactggag 60
ctgtctggct gtgaacaggg gctgcacgag atcaaactgc tgggaaaggg cactagcgcc 120
gctgatgctg tggaagtgcc agctccagct gctgtgctgg gaggacctga gccactgatg 180
cagtgcaccg cctggctgaa cgcttacttc catcagcctg aagccatcga ggaatttccc 240
gtgcctgccc tgcaccatcc agtgttccag caggagagtt ttacaaggca ggtgctgtgg 300
aagctgctga aagtggtgaa gttcggggaa gtgatttcct accagcagct ggctgctctg 360
gctggaaacc caaaagctgc tcgggccgtg ggaggagcta tgagaggcaa tccagtgcca 420
atcctgattc cctgccacag ggtggtgtgt agctccggag ctgtggggaa ctattctggg 480
ggactggccg tgaaagaatg gctgctggct cacgagggac ataggctggg aaagcctggc 540
ctgggagggt ctagtggact ggctggagct tggctgaagg gagctggagc tacctcagga 600
agcccacctg ccggccggaa t 621
<210> 49
<211> 207
<212> PRT
<213> 智人
<400> 49
Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu
1 5 10 15
Gly Lys Leu Glu Leu Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys
20 25 30
Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp Ala Val Glu Val Pro Ala
35 40 45
Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr Ala
50 55 60
Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro
65 70 75 80
Val Pro Ala Leu His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg
85 90 95
Gln Val Leu Trp Lys Leu Leu Lys Val Val Lys Phe Gly Glu Val Ile
100 105 110
Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala Ala Arg
115 120 125
Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Pro Ile Leu Ile Pro

130 135 140

Cys His Arg Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser Gly
145 150 155 160
Gly Leu Ala Val Lys Glu Trp Leu Leu Ala His Glu Gly His Arg Leu
165 170 175
Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly Ala Trp Leu
180 185 190
Lys Gly Ala Gly Ala Thr Ser Gly Ser Pro Pro Ala Gly Arg Asn
195 200 205

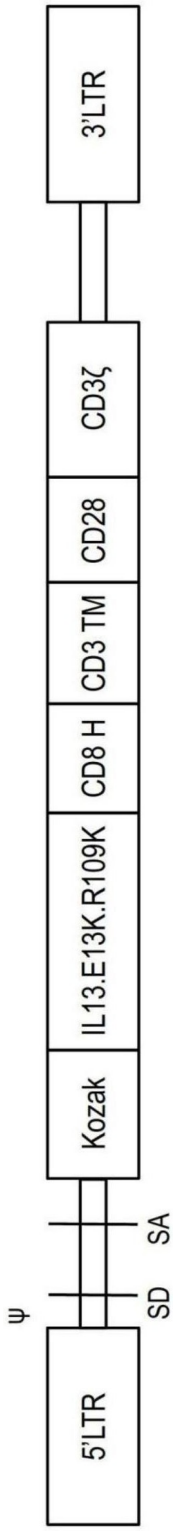

图1A

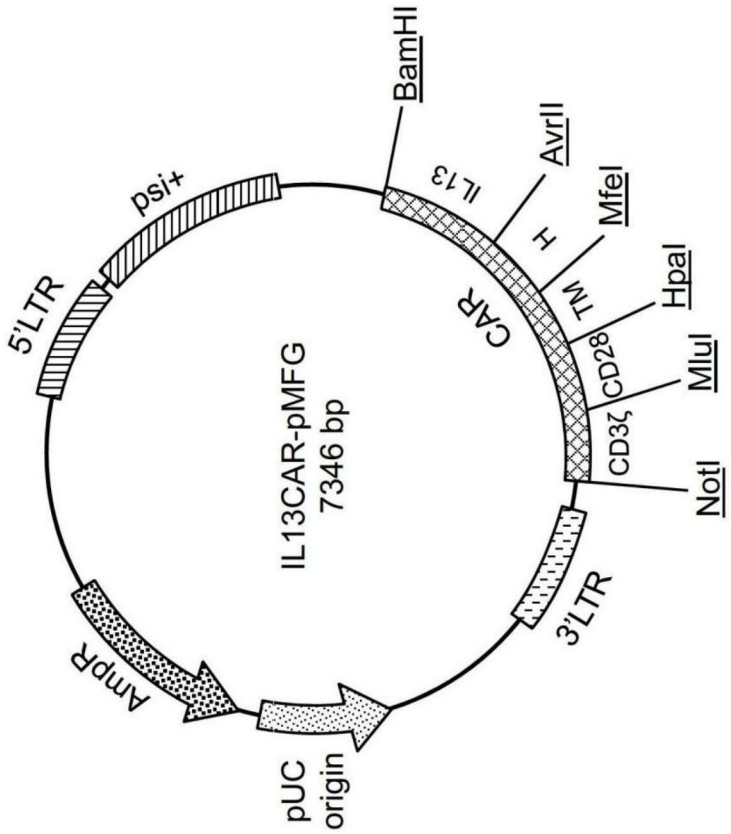
BamHI
AvrII
MfeI
HpaI
MluI
NotI
IL13
H
TM
CAR
CD28
CD3ζ
psi+
5'LTR
IL13CAR-pMFG
7346 bp
AmpR
pUC
origin
3'LTR

图1B

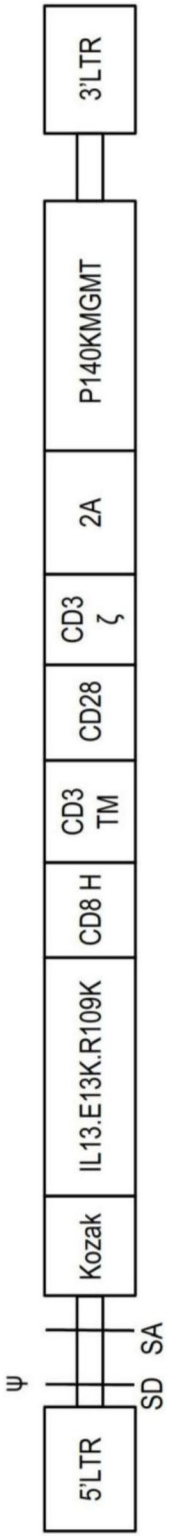
5'LTR
Ψ
SD
SA
Kozak
IL13.E13K.R109K
CD8 H
CD3 TM
CD28
CD3 ζ
2A
P140KMGMT
3'LTR

图2A

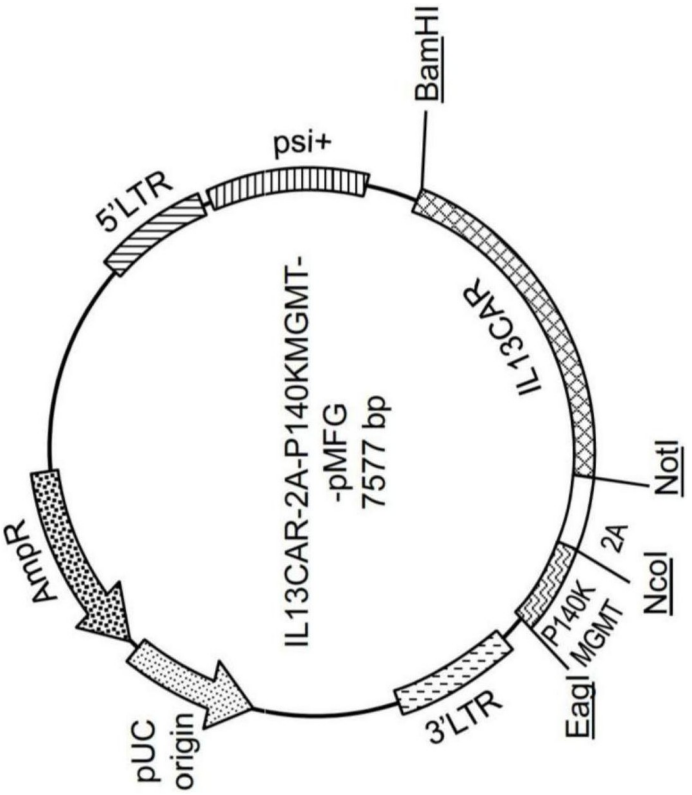
BamHI
psi+
5'LTR
IL13CAR
IL13CAR-2A-P140KMGMT-
-pMFG
7577 bp
NotI
2A
NcoI
P140K
MGMT
EagI
3'LTR
AmpR
pUC
origin

图2B

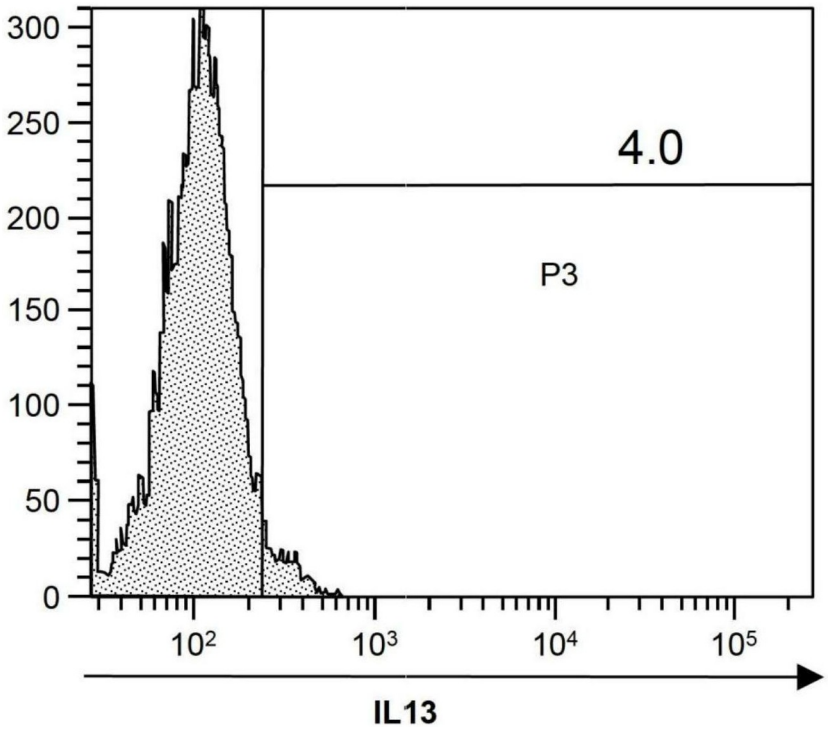
300
250
200
150
100
50
0
4.0
P3
10²
10³
10⁴
10⁵
IL13

图3A

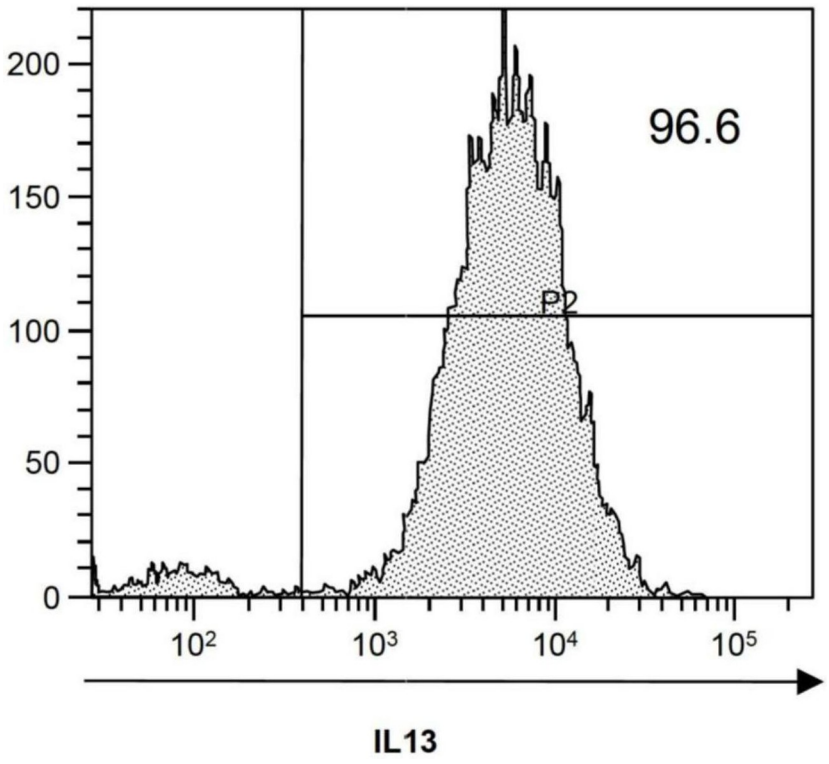
200
150
100
50
0
96.6
P2
10^2
10^3
10^4
10^5
IL13

图3B

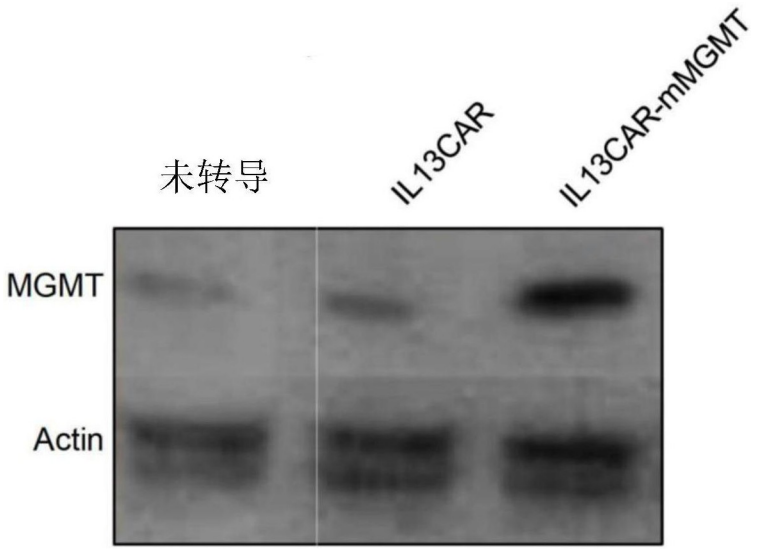
未转导
IL13CAR
IL13CAR-mMGMT
MGMT
Actin

图3C

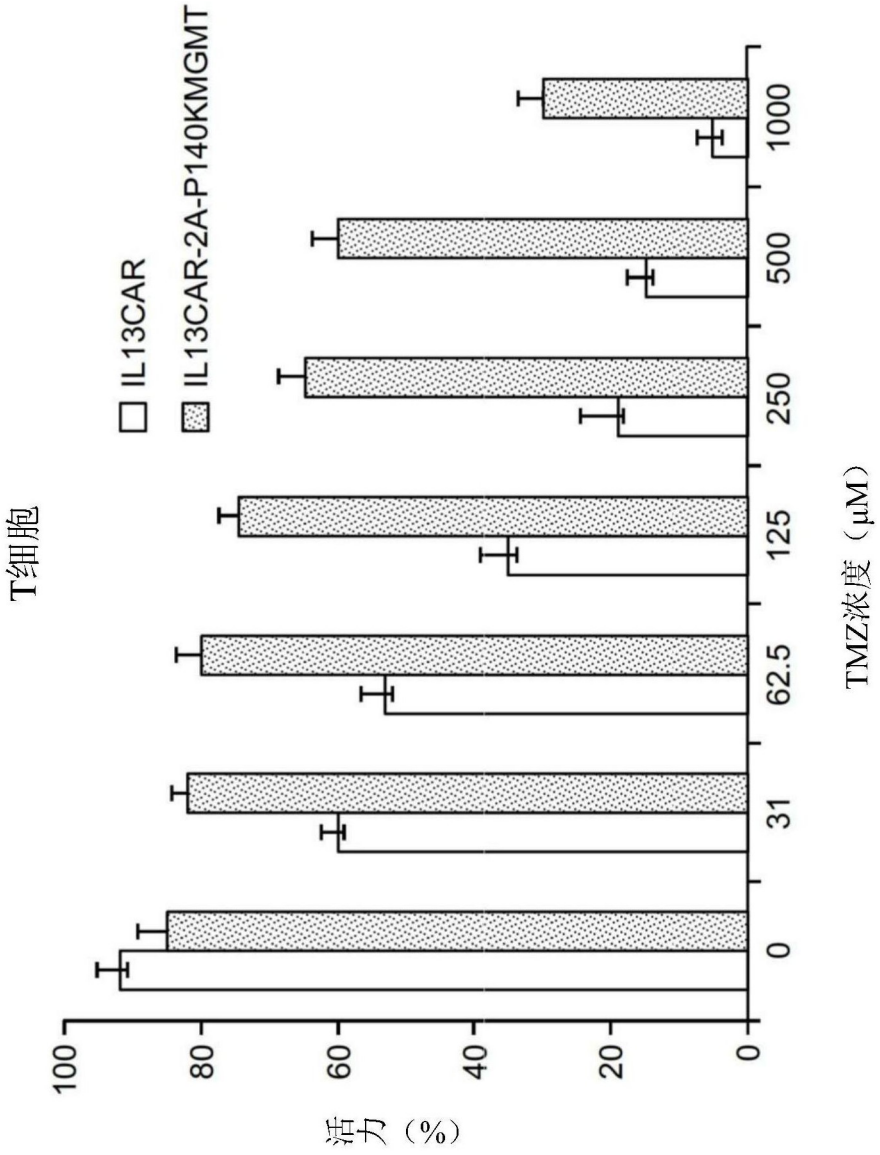
T细胞
IL13CAR
IL13CAR-2A-P140KMGMT
活力（%）
100
80
60
40
20
0
0
31
62.5
125
250
500
1000
TMZ浓度（μM）

图4

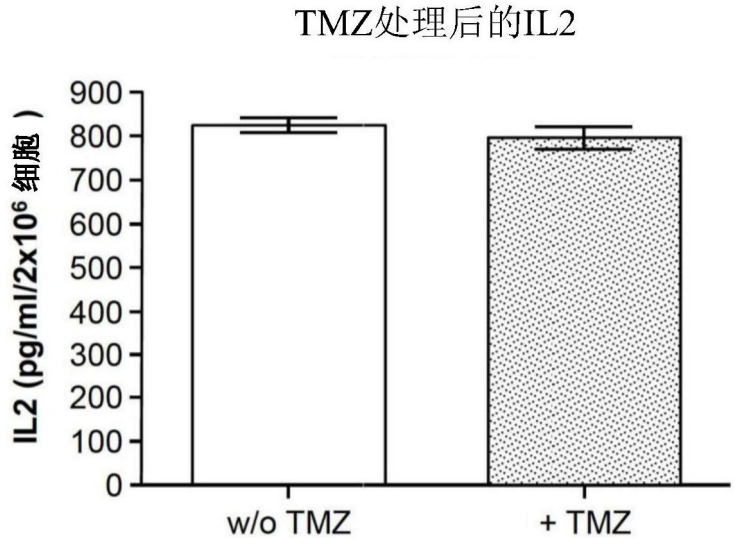
TMZ处理后的IL2
IL2 (pg/ml/2x10⁶ 细胞)
900
800
700
600
500
400
300
200
100
0
w/o TMZ
+ TMZ

图5A

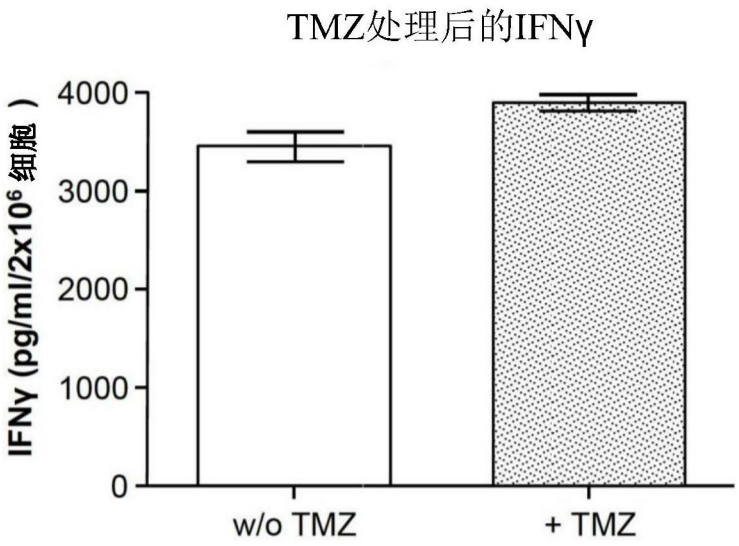
TMZ处理后的IFNγ
IFNγ (pg/ml/2x10⁶ 细胞)
4000
3000
2000
1000
0
w/o TMZ
+ TMZ

图5B

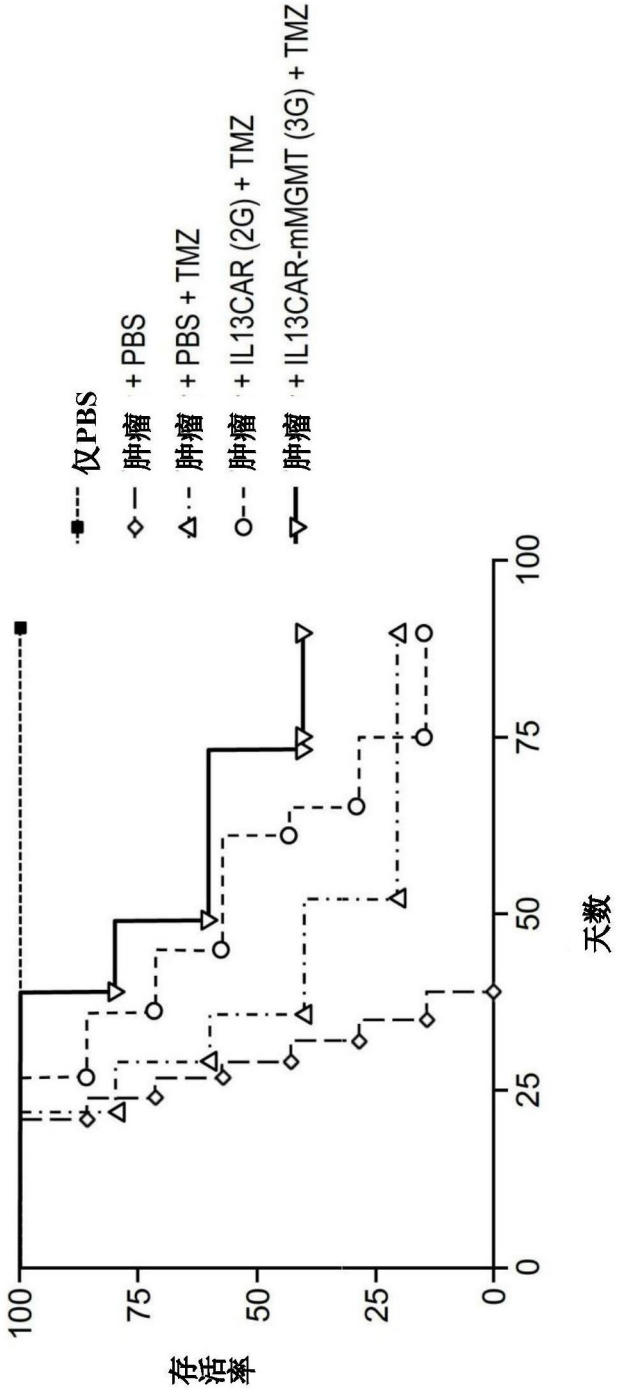
仅PBS
肿瘤 + PBS
肿瘤 + PBS + TMZ
肿瘤 + IL13CAR (2G) + TMZ
肿瘤 + IL13CAR-mMGMT (3G) + TMZ
存活率
100
75
50
25
0
0
25
50
75
100
天数

图6

IL13CAR-P140KMGMT的DNA序列 SEQ ID NO:1

GGA TCC GCC ACC **ATG** CAT CCG CTC CTC AAT CCT CTC CTG TTG GCA CTG GGC CTC ATG GCG CTT TTG TTG ACC ACG GTC ATT GCT CTC ACT	信号
TGC CTT GGC GGC TTT GCC TCC CCA GGC CCT GTG CCT CCC TCT ACA GCC CTC AGG GAG CTC ATT GAG GAG CTG GTC AAC ATC ACC CAG AAC CAG AAG GCT CCG CTC TGC AAT GGC AGC ATG GTA TGG AGC ATC AAC CTG ACA GCT GGC ATG TAC TGT GCA GCC CTG GAA TCC CTG ATC AAC GTG TCA GGC TGC AGT GCC ATC GAG AAG ACC CAG AGG ATG CTG AGC GGA TTC TGC CCG CAC AAG GTC TCA GCT GGG CAG TTT TCC AGC TTG CAT GTC CGA GAC ACC AAA ATC GAG GTG GCC CAG TTT GTA AAG GAC CTG CTC TTA CAT TTA AAG AAA CTT TTT CGC GAG GGA CAG TTC AAC	IL13(WT)
CCT AGG AAG CCC ACC ACG ACG CCA GCG CCG CGA CCA CCA ACA CCG GCG CCC ACC ATC GCG TCG CAG CCC CTG TCC CTG CGC CCA GAG GCG TGC CGG CCA GCG GCG GGG GGC GCA GTG CAC ACG AGG GGG CTG GAC TTC GCC	铰链
CAA TTG CTC TGC TAC CTG CTG GAT GGA ATC CTC TTC ATC TAT GGT GTC	TM
ATT CTC ACT GCC TTG TTC CTG AGA GTG GTT AAC TTC TGG GTG AGG AGT AAG AGG AGC AGG CTC CTG CAC AGT GAC TAC ATG AAC ATG ACT CCC CGC CGC CCC GGG CCC ACC CGC AAG CAT TAC CAG CCC TAT GCC CCA CCA CGC	CD28
GAC TTC GCA GCC TAT CGC TCC ACG CGT AAG TTC AGC AGG AGC GCA GAC GCC CCC GCG TAC CAG CAG GGC CAG AAC CAG CTC TAT AAC GAG CTC AAT CTA GGA CGA AGA GAG GAG TAC GAT GTT TTG GAC AAG AGA CGT GGC CGG GAC CCT GAG ATG GGG GGA AAG CCG AGA AGG AAG AAC CCT CAG GAA GGC CTG TAC AAT GAA CTG CAG AAA GAT AAG ATG GCG GAG GCC TAC AGT GAG ATT GGG ATG AAA GGC GAG CGC CGG AGG GGC AAG GGG CAC GAT GGC CTT TAC CAG GGT CTC AGT ACA GCC ACC AAG GAC ACC TAC GAC GCC CTT CAC	CD3ζ
ATG CAG GCC CTG CCC CCT CGC **TAA** CAG CCA GCG GCC GC A GAG GGC AGA GGA AGT CTT CTA ACA TGC GGT GAC GTG GAG GAG AAT CCC GGC CCT CCA	A2
TGG **ATG** GAC AAA GAT TGC GAG ATG AAG CGG ACC ACA CTG GAC TCC CCC CTG GGC AAA CTG GAG CTG TCT GGC TGT GAA CAG GGG CTG CAC GAG ATC AAA CTG CTG GGA AAG GGC ACT AGC GCC GCT GAT GCT GTG GAA GTG CCA GCT CCA GCT GCT GTG CTG GGA GGA CCT GAG CCA CTG ATG CAG TGC ACC GCC TGG CTG AAC GCT TAC TTC CAT CAG CCT GAA GCC ATC GAG GAA TTT CCC GTG CCT GCC CTG CAC CAT CCA GTG TTC CAG CAG GAG AGT TTT ACA AGG CAG GTG CTG TGG AAG CTG CTG AAA GTG GTG AAG TTC GGG GAA GTG ATT TCC TAC CAG CAG CTG GCT GCT CTG GCT GGA AAC CCA AAA GCT GCT CGG GCC GTG GGA GGA GCT ATG AGA GGC AAT CCA GTG AAA ATC CTG ATT CCC TGC CAC AGG GTG GTG TGT AGC TCC GGA GCT GTG GGG AAC TAT TCT GGG GGA CTG GCC GTG AAA GAA TGG CTG CTG GCT CAC GAG GGA CAT AGG CTG GGA AAG CCT GGC CTG GGA GGG TCT AGT GGA CTG GCT GGA GCT TGG CTG AAG GGA GCT GGA GCT ACC TCA GGA AGC CCA CCT GCC GGC CGG AAT	P140K MGMT
TGA CGG CCG	

图7A

IL13CAR-P140KMGMT的肽序列 SEQ ID NO:4

MHPLLNPLLLALGLMALLLTTVIALTCLGGFA SPGPVPPSTALRELIEELVNITQNQKAPL CNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVR DTKIEVAQFVKDLLLHLKKLFREGQFN PR KPTTTPAPRPPTPAPTIASQPLSLRPEAC	IL13 铰链
RPAAGGAVHTRGLDFA QL LCYLLDGILFIYGVILTALFLRV VN FWVRSKRSRLLHSDYM	TM/CD28
NMTPRRPGPTRKHYQPYAPPRDFAAYRS TR KFSRSADAPAYQQGQNQLYNELNLGR REEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK	CD3ζ
GHDGLYQGLSTATKDTYDALHMQALPPR **STOP** QP AAA EGRGSLLTCGDVEENPGP M	A2
DKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGKGTSAADAVEVPAPAAVLGGPEPL MQCTAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQL AALAGNPKAARAVGGAMRGNPV K ILIPCHRVVCSSGAVGNYSGGLAVKEWLLAHEGH RLGKPGLGGSSGLAGAWLKGAGATSGSPPAGRN **STOP**	P140K MGMT

图7B

IL13(E13Y)CAR-P140KMGMT的DNA序列 SEQ ID NO:2

GGA TCC GCC ACC **ATG** CAT CCG CTC CTC AAT CCT CTC CTG TTG GCA CTG GGC CTC ATG GCG CTT TTG TTG ACC ACG GTC ATT GCT CTC ACT	信号
TGC CTT GGC GGC TTT GCC TCC CCA GGC CCT GTG CCT CCC TCT ACA GCC CTC AGG TAC CTC ATT GAG GAG CTG GTC AAC ATC ACC CAG AAC CAG AAG GCT CCG CTC TGC AAT GGC AGC ATG GTA TGG AGC ATC AAC CTG ACA GCT GGC ATG TAC TGT GCA GCC CTG GAA TCC CTG ATC AAC GTG TCA GGC TGC AGT GCC ATC GAG AAG ACC CAG AGG ATG CTG AGC GGA TTC TGC CCG CAC AAG GTC TCA GCT GGG CAG TTT TCC AGC TTG CAT GTC CGA GAC ACC AAA ATC GAG GTG GCC CAG TTT GTA AAG GAC CTG CTC TTA CAT TTA AAG AAA CTT TTT CGC GAG GGA CAG TTC AAC	IL13(E13y)
CCT AGG AAG CCC ACC ACG ACG CCA GCG CCG CGA CCA CCA ACA CCG GCG CCC ACC ATC GCG TCG CAG CCC CTG TCC CTG CGC CCA GAG GCG TGC CGG CCA GCG GCG GGG GGC GCA GTG CAC ACG AGG GGG CTG GAC TTC GCC	铰链
CAA TTG CTC TGC TAC CTG CTG GAT GGA ATC CTC TTC ATC TAT GGT GTC	TM
ATT CTC ACT GCC TTG TTC CTG AGA GTG GTT AAC TTC TGG GTG AGG AGT AAG AGG AGC AGG CTC CTG CAC AGT GAC TAC ATG AAC ATG ACT CCC CGC CGC CCC GGG CCC ACC CGC AAG CAT TAC CAG CCC TAT GCC CCA CCA CGC	CD28
GAC TTC GCA GCC TAT CGC TCC ACG CGT AAG TTC AGC AGG AGC GCA GAC GCC CCC GCG TAC CAG CAG GGC CAG AAC CAG CTC TAT AAC GAG CTC AAT CTA GGA CGA AGA GAG GAG TAC GAT GTT TTG GAC AAG AGA CGT GGC CGG GAC CCT GAG ATG GGG GGA AAG CCG AGA AGG AAG AAC CCT CAG GAA GGC CTG TAC AAT GAA CTG CAG AAA GAT AAG ATG GCG GAG GCC TAC AGT GAG ATT GGG ATG AAA GGC GAG CGC CGG AGG GGC AAG GGG CAC GAT GGC CTT TAC CAG GGT CTC AGT ACA GCC ACC AAG GAC ACC TAC GAC GCC CTT CAC	CD3ζ
ATG CAG GCC CTG CCC CCT CGC **TAA** CAG CCA GCG GCC GC A GAG GGC AGA GGA AGT CTT CTA ACA TGC GGT GAC GTG GAG GAG AAT CCC GGC CCT CCA	A2
TGG **ATG** GAC AAA GAT TGC GAG ATG AAG CGG ACC ACA CTG GAC TCC CCC CTG GGC AAA CTG GAG CTG TCT GGC TGT GAA CAG GGG CTG CAC GAG ATC AAA CTG CTG GGA AAG GGC ACT AGC GCC GCT GAT GCT GTG GAA GTG CCA GCT CCA GCT GCT GTG CTG GGA GGA CCT GAG CCA CTG ATG CAG TGC ACC GCC TGG CTG AAC GCT TAC TTC CAT CAG CCT GAA GCC ATC GAG GAA TTT CCC GTG CCT GCC CTG CAC CAT CCA GTG TTC CAG CAG GAG AGT TTT ACA AGG CAG GTG CTG TGG AAG CTG CTG AAA GTG GTG AAG TTC GGG GAA GTG ATT TCC TAC CAG CAG CTG GCT GCT CTG GCT GGA AAC CCA AAA GCT GCT CGG GCC GTG GGA GGA GCT ATG AGA GGC AAT CCA GTG AAA ATC CTG ATT CCC TGC CAC AGG GTG GTG TGT AGC TCC GGA GCT GTG GGG AAC TAT TCT GGG GGA CTG GCC GTG AAA GAA TGG CTG CTG GCT CAC GAG GGA CAT AGG CTG GGA AAG CCT GGC CTG GGA GGG TCT AGT GGA CTG GCT GGA GCT TGG CTG AAG GGA GCT GGA GCT ACC TCA GGA AGC CCA CCT GCC GGC CGG AAT	P140K MGMT
TGA CGG CCG	

图8A

IL13(E13Y)CAR-P140KMGMT的肽序列 SEQ ID NO:5

MHPLLNPLLLALGLMALLLTTVIALTCLGGFA SPGPVPPSTALRYLIEELVNITQNQKAPL	IL13
CNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVR	(E13y)
DTKIEVAQFVKDLLLHLKKLFREGQFN PR KPTTTPAPRPPTPAPTIASQPLSLRPEAC	铰链
RPAAGGAVHTRGLDFA QL LCYLLDGILFIYGVILTALFLRV VN FWVRSKRSRLLHSDYM	TM/CD28
NMTPRRPGPTRKHYQPYAPPRDFAAYRS TR KFSRSADAPAYQQGQNQLYNELNLGR	CD3ζ
REEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK	
GHDGLYQGLSTATKDTYDALHMQALPPR **STOP** QP AAA EGRGSLLTCGDVEENPGP M	A2
DKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGKGTSAADAVEVPAPAAVLGGPEPL	P140K
MQCTAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQL	MGMT
AALAGNPKAARAVGGAMRGNPV K ILIPCHRVVCSSGAVGNYSGGLAVKEWLLAHEGH	
RLGKPGLGGSSGLAGAWLKGAGATSGSPPAGRN **STOP**	

图8B

IL13(E13K.R109K)CAR-P140KMGMT的DNA序列 SEQ ID NO:3

GGA TCC GCC ACC **ATG** CAT CCG CTC CTC AAT CCT CTC CTG TTG GCA CTG GGC CTC ATG GCG CTT TTG TTG ACC ACG GTC ATT GCT CTC ACT	信号
TGC CTT GGC GGC TTT GCC TCC CCA GGC CCT GTG CCT CCC TCT ACA GCC CTC AGG AAG CTC ATT GAG GAG CTG GTC AAC ATC ACC CAG AAC CAG AAG GCT CCG CTC TGC AAT GGC AGC ATG GTA TGG AGC ATC AAC CTG ACA GCT GGC ATG TAC TGT GCA GCC CTG GAA TCC CTG ATC AAC GTG TCA GGC TGC AGT GCC ATC GAG AAG ACC CAG AGG ATG CTG AGC GGA TTC TGC CCG CAC AAG GTC TCA GCT GGG CAG TTT TCC AGC TTG CAT GTC CGA GAC ACC AAA ATC GAG GTG GCC CAG TTT GTA AAG GAC CTG CTC TTA CAT TTA AAG AAA CTT TTT AAG GAG GGA CAG TTC AAC	IL13 (E13K R109K)
CCT AGG AAG CCC ACC ACG ACG CCA GCG CCG CGA CCA CCA ACA CCG GCG CCC ACC ATC GCG TCG CAG CCC CTG TCC CTG CGC CCA GAG GCG TGC CGG CCA GCG GCG GGG GGC GCA GTG CAC ACG AGG GGG CTG GAC TTC GCC	铰链
CAA TTG CTC TGC TAC CTG CTG GAT GGA ATC CTC TTC ATC TAT GGT GTC	TM
ATT CTC ACT GCC TTG TTC CTG AGA GTG GTT AAC TTC TGG GTG AGG AGT	
AAG AGG AGC AGG CTC CTG CAC AGT GAC TAC ATG AAC ATG ACT CCC CGC CGC CCC GGG CCC ACC CGC AAG CAT TAC CAG CCC TAT GCC CCA CCA CGC	CD28
GAC TTC GCA GCC TAT CGC TCC ACG CGT AAG TTC AGC AGG AGC GCA GAC GCC CCC GCG TAC CAG CAG GGC CAG AAC CAG CTC TAT AAC GAG CTC AAT CTA GGA CGA AGA GAG GAG TAC GAT GTT TTG GAC AAG AGA CGT GGC CGG GAC CCT GAG ATG GGG GGA AAG CCG AGA AGG AAG AAC CCT CAG GAA GGC CTG TAC AAT GAA CTG CAG AAA GAT AAG ATG GCG GAG GCC TAC AGT GAG ATT GGG ATG AAA GGC GAG CGC CGG AGG GGC AAG GGG CAC GAT GGC CTT TAC CAG GGT CTC AGT ACA GCC ACC AAG GAC ACC TAC GAC GCC CTT CAC	CD3ζ
ATG CAG GCC CTG CCC CCT CGC **TAA** CAG CCA GCG GCC GC A GAG GGC AGA GGA AGT CTT CTA ACA TGC GGT GAC GTG GAG GAG AAT CCC GGC CCT CCA	A2
TGG **ATG** GAC AAA GAT TGC GAG ATG AAG CGG ACC ACA CTG GAC TCC CCC CTG GGC AAA CTG GAG CTG TCT GGC TGT GAA CAG GGG CTG CAC GAG ATC AAA CTG CTG GGA AAG GGC ACT AGC GCC GCT GAT GCT GTG GAA GTG CCA GCT CCA GCT GCT GTG CTG GGA GGA CCT GAG CCA CTG ATG CAG TGC ACC GCC TGG CTG AAC GCT TAC TTC CAT CAG CCT GAA GCC ATC GAG GAA TTT CCC GTG CCT GCC CTG CAC CAT CCA GTG TTC CAG CAG GAG AGT TTT ACA AGG CAG GTG CTG TGG AAG CTG CTG AAA GTG GTG AAG TTC GGG GAA GTG ATT TCC TAC CAG CAG CTG GCT GCT CTG GCT GGA AAC CCA AAA GCT GCT CGG GCC GTG GGA GGA GCT ATG AGA GGC AAT CCA GTG AAA ATC CTG ATT CCC TGC CAC AGG GTG GTG TGT AGC TCC GGA GCT GTG GGG AAC TAT TCT GGG GGA CTG GCC GTG AAA GAA TGG CTG CTG GCT CAC GAG GGA CAT AGG CTG GGA AAG CCT GGC CTG GGA GGG TCT AGT GGA CTG GCT GGA GCT TGG CTG AAG GGA GCT GGA GCT ACC TCA GGA AGC CCA CCT GCC GGC CGG AAT	P140K MGMT
TGA CGG CCG	

图9A

IL13(E13K.R109K)CAR-P140KMGMT的肽序列 SEQ ID NO:6

MHPLLNPLLLALGLMALLLTTVIALTCLGGFA SPGPVPPSTALRKLIEELVNITQNQKAPL	IL13
CNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVR	(E13K R109K)
DTKIEVAQFVKDLLLHLKKLFKEGQFN PR KPTTTPAPRPPTPAPTIASQPLSLRPEAC	铰链
RPAAGGAVHTRGLDFA QL LCYLLDGILFIYGVILTALFLRV VN FWVRSKRSRLLHSDYM	TM/CD28
NMTPRRPGPTRKHYQPYAPPRDFAAYRS TR KFSRSADAPAYQQGQNQLYNELNLGR	CD3ζ
REEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK	
GHDGLYQGLSTATKDTYDALHMQALPPR **STOP** QP AAA EGRGSLLTCGDVEENPGP M	A2
DKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGKGTSAADAVEVPAPAAVLGGPEPL	P140K
MQCTAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQL	MGMT
AALAGNPKAARAVGGAMRGNPV K ILIPCHRVVCSSGAVGNYSGGLAVKEWLLAHEGH	
RLGKPGLGGSSGLAGAWLKGAGATSGSPPAGRN **STOP**	

图9B