COSTIMULATORY CHIMERIC ANTIGEN RECEPTOR T CELLS TARGETING IL13Ra2

FIGURE 1

AA

IL13(E13Y)-zetakine

huIL-13
(E13Y)

huCD4 tm

huCD3 ζ cyt

hu4F

IL13(EQ)BB

huIL-13
(E13Y)

L235E
N297Q

huCD4 tm

huαβ γ

huCD3 ζ cyt

4-1BB cyt
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Costimulatory Chimeric Antigen Receptor T Cells Targeting IL13Ra2

BACKGROUND

[001] Tumor-specific T cell based immunotherapies, including therapies employing engineered T cells, have been investigated for anti-tumor treatment. In some cases the T cells used in such therapies do not remain active in vivo for a long enough period. In some cases, the tumor-specificity of the T cells is relatively low. Therefore, there is a need in the art for tumor-specific cancer therapies with longer term anti-tumor functioning.

[002] Malignant gliomas (MG), which include anaplastic astrocytoma (AA-grade III) and glioblastoma (GBM-grade IV), have an incidence rate of approximately 20,000 new cases diagnosed annually in the United States. According to the American Brain Tumor Association total prevalence of individuals living with a malignant brain tumor, based on United States 2010 census data, is roughly 140,000 persons. Although MG is a rare disease, it is highly aggressive and heterogeneous with respect to its malignant behavior and nearly uniformly lethal. Current standard-of-care therapies for high-grade MG yield only short term benefits, and these brain tumors are virtually incurable. Indeed, even with modern surgical and radiotherapeutic techniques, which often exacerbate the already severe morbidities imposed by location in the central nervous system (CNS), the 5-year survival rates are quite low. Furthermore, for the majority of patients who relapse with disease, there are few therapeutic options. Thus, there is a significant need for more effective therapies, particularly for those patients that have recurred/progressed following frontline therapies, and participation of this patient population in clinical trials is warranted.

[003] Adoptive T cell therapy (ACT) utilizing chimeric antigen receptor (CAR) engineered T cells may provide a safe and effective way to reduce recurrence rates of MG, since CAR T cells can be engineered to specifically recognize antigenically-distinct tumor populations (Cartellieri et al. 2010 JBiomed Biotechnol 2010:956304; Ahmed et

SUMMARY

[004] Described herein are chimeric transmembrane immunoreceptors (chimeric antigen receptors or "CARs") which comprise an extracellular domain, a transmembrane region and an intracellular signaling domain. The extracellular domain is made up of an IL-13 ligand that binds interleukin-13Ra2 (IL13Ra2) and, optionally, a spacer, comprising, for example a portion human Fc domain. The transmembrane portion includes a CD4 transmembrane domain, a CD8 transmembrane domain, a CD28 transmembrane domain, a CD3 transmembrane domain or a 4IBB transmembrane domain. The intracellular signaling domain includes the signaling domain from the zeta chain of the human CD3 complex (CD3ζ) and one or more costimulatory domains, e.g., a 4-1BB costimulatory domain. The extracellular domain enables the CAR, when expressed on the surface of a T cell, to direct T cell activity to those cells expressing IL13Ra2, a receptor expressed on the surface of tumor cells, including glioma. Importantly, the IL13Ra2 binding portion of the CAR includes an amino acid modification, such as an E13Y mutation, that increases binding specificity. The inclusion of a costimulatory domain, such as the 4-1BB (CD137) costimulatory domain in series with CD3ζ in the intracellular region enables the T cell to receive co-stimulatory signals. T cells, for example, patient-specific, autologous T cells can be engineered to express the CARs described herein and the engineered cells can be expanded and used in ACT. Various T cell subsets can be used. In addition, the CAR can be expressed in other immune cells such as NK cells. Where a patient is treated with an
immune cell expressing a CAR described herein the cell can be an autologous or allogenic T cell. In some cases the cells used are CD4+ and CD8+ central memory T cells (TCM), which are CD45RO+CD62L+, and the use of such cells can improve long-term persistence of the cells after adoptive transfer compared to the use of other types of patient-specific T cells.

[005] Described herein is a nucleic acid molecule encoding a chimeric antigen receptor (CAR) consisting of: human IL-13 or a variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications; a transmembrane domain selected from: a CD4 transmembrane domain or variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications, a CD8 transmembrane domain or variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications, a CD28 transmembrane domain or variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications, and a CD3ζ signaling domain of a variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications.

[006] In various embodiments the costimulatory domain is selected from the group consisting of: a CD28 costimulatory domain or variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications, a 4-IBB costimulatory domain or variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications and an OX40 costimulatory domain or variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications. In certain embodiments, a 4IBB costimulatory domain or variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications in present.

[007] Additional embodiment the CAR comprises: a variant of a human IL13 having 1-10 amino acid modification that increase binding specificity for IL13Ra2 versus IL13Ral; the human IL-13 or variant thereof is an IL-13 variant comprising the amino acid sequence of SEQ ID NO:3 with 1 to 5 amino acid modifications, provided that the amino acid at position 11 of SEQ ID NO:3 other than E; two different costimulatory domains selected from the group consisting of: a CD28 costimulatory domain or a variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications, a 4IBB costimulatory domain
or a variant thereof having 1-10 (e.g., 1 or 2) amino acid modifications and an OX40
costimulatory domain or a variant thereof having 1-10 (e.g., 1 or 2) amino acid
modifications; two different costimulatory domains selected from the group consisting of:
a CD28 costimulatory domain or a variant thereof having 1-2 amino acid modifications, a
4IBB costimulatory domain or a variant thereof having 1-2 amino acid modifications and
an OX40 costimulatory domain or a variant thereof having 1-2 amino acid modifications;
human IL-13 or a variant thereof having 1-2 amino acid modifications; a transmembrane
domain selected from: a CD4 transmembrane domain or variant thereof having 1-2 amino
acid modifications, a CD8 transmembrane domain or variant thereof having 1-2 amino
acid modifications, a CD28 transmembrane domain or a variant thereof having 1-2 amino
acid modifications, and a CD3ζ transmembrane domain or a variant thereof having 1-2
amino acid modifications; a costimulatory domain; and CD3ζ signaling domain of a
variant thereof having 1-2 amino acid modifications; a spacer region located between the
IL-13 or variant thereof and the transmembrane domain (e.g., the spacer region comprises
an amino acid sequence selected from the group consisting of SEQ ID NO: 4, 14-20, 50
and 52); the spacer comprises an IgG hinge region; the spacer region comprises 10-150
amino acids; the 4-IBB signaling domain comprises the amino acid sequence of SEQ ID
NO:6; the CD3ζ signaling domain comprises the amino acid sequence of SEQ ID NO:7;
and a linker of 3 to 15 amino acids that is located between the costimulatory domain and
the CD3ζ signaling domain or variant thereof. In certain embodiments where there are
two costimulatory domains, one is an 4-IBB costimulatory domain and the other a
costimulatory domain selected from: CD28 and CD28gg

[008] In some embodiments: nucleic acid molecule expresses a polypeptide comprising
an amino acid sequence selected from SEQ ID NOs: 10, 31-48 and 52; the chimeric
antigen receptor comprises a IL-13/IgG4/CD4t/41-BB region comprising the amino acid
of SEQ ID NO:1 1 and a CD3ζ signaling domain comprising the amino acid sequence of
SEQ ID NO:7; and the chimeric antigen receptor comprises the amino acid sequence of
SEQ ID NOs: 10, 31-48 and 52.

[009] Also disclosed is a population of human T cells transduced by a vector
comprising an expression cassette encoding a chimeric antigen receptor, wherein
chimeric antigen receptor comprises: human IL-13 or a variant thereof having 1-10 amino acid modifications; a transmembrane domain selected from: a CD4 transmembrane domain or variant thereof having 1-10 amino acid modifications, a CD8 transmembrane domain or variant thereof having 1-10 amino acid modifications, a CD28 transmembrane domain or a variant thereof having 1-10 amino acid modifications, and a CD3ζ transmembrane domain or a variant thereof having 1-10 amino acid modifications; a costimulatory domain; and CD3ζ signaling domain of a variant thereof having 1-10 amino acid modifications. In various embodiments: the population of human T cells comprise a vector expressing a chimeric antigen receptor comprising an amino acid sequence selected from SEQ ID NOs: 10, 31-48 and 52; the population of human T cells are comprises of central memory T cells (Tcm cells) (e.g., at least 20%, 30%, 40%, 50%, 60%, 70%, 80% of the cells are Tcm cells; at least 15%, 20%, 25%, 30%, 35% of the Tcm cells are CD4+ and at least 15%, 20%, 25%, 30%, 35% of the Tcm cells are CD8+ cells).

[0010] Also described is a method of treating cancer in a patient comprising administering a population of autologous or allogeneic human T cells (e.g., autologous or allogenic T cells comprising Tcm cells, e.g., at least 20%, 30%, 40%, 50%, 60%, 70%, 80% of the cells are Tcm cells; at least 15%, 20%, 25%, 30%, 35% of the Tcm cells are CD4+ and at least 15%, 20%, 25%, 30%, 35% of the Tcm cells are CD8+ cells) transduced by a vector comprising an expression cassette encoding a chimeric antigen receptor, wherein chimeric antigen receptor comprises an amino acid sequence selected from SEQ ID NOs: 10, 31-48 and 52. In various embodiments: the population of human T cells comprise central memory T cells; the cancer is glioblastoma; and the transduced human T cells where prepared by a method comprising obtaining T cells from the patient, treating the T cells to isolate central memory T cells, and transducing at least a portion of the central memory cells to with a viral vector comprising an expression cassette encoding a chimeric antigen receptor, wherein chimeric antigen receptor comprises an amino acid sequence selected from SEQ ID NOs: 10, 31-48 and 52.

[0011] Also described is: a nucleic acid molecule encoding an polypeptide comprising an amino acid sequence that is at least 95% identical to an amino acid sequence selected
from SEQ ID NO: 10 and SEQ ID NOs: 10, 31-48 and 52; a nucleic acid molecule encoding an polypeptide comprising an amino acid sequence that is identical to an amino acid sequence selected from SEQ ID NO: 10, 31-48 and 52 except for the presence of no more than 5 amino acid substitutions, deletions or insertions; a nucleic acid molecule encoding an polypeptide comprising an amino acid sequence that is identical to an amino acid sequence selected from SEQ ID NO: 10 and SEQ ID NOs: 10, 31-48 and 52 except for the presence of no more than 5 amino acid substitutions; and a nucleic acid molecule encoding an polypeptide comprising an amino acid sequence that is identical to an amino acid sequence selected from SEQ ID NO: 10 and SEQ ID NOs: 10, 31-48 and 52 except for the presence of no more than 2 amino acid substitutions.

[0012] Certain CAR described herein, for example, the IL13(EQ)BE^ CAR and the IL13(EQ)CD28- BBζ CAR, have certain beneficial characteristics compared to certain other IL13-targeted CAR. For example, they have improved selectivity for IL13Ra, elicit lower Th2 cytokine production, particularly lower IL13 production.

[0013] T cells expressing a CAR targeting IL13Ra2 can be useful in treatment of cancers such as glioblastoma, as well as other cancer that expresses IL13Ra2 which include but are not limited to medulloblastoma, breast cancer, head and neck cancer, kidney cancer, ovarian cancer and Kaposi’s sarcoma. Thus, this disclosure includes methods for treating cancer using T cells expressing a CAR described herein.

[0014] This disclosure also nucleic acid molecules that encode any of the CARs described herein (e.g., vectors that include a nucleic acid sequence encoding one of the CARs) and isolated T lymphocytes that express any of the CARs described herein.

[0015] The CAR described herein can include a spacer region located between the IL13 domain and the transmembrane domain. A variety of different spacers can be used. Some of them include at least portion of a human Fc region, for example a hinge portion of a human Fc region or a CH3 domain or variants thereof. Table 1 below provides various spacers that can be used in the CARs described herein.

Table 1: Examples of Spacers
<table>
<thead>
<tr>
<th>Name</th>
<th>Length</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>a3</td>
<td>3 aa</td>
<td>AAA</td>
</tr>
<tr>
<td>linker</td>
<td>10 aa</td>
<td>GGGSSGGGSG (SEQ ID NO:14)</td>
</tr>
<tr>
<td>IgG4 hinge (S→P) (S228P)</td>
<td>12 aa</td>
<td>ESKYGPPCPACP (SEQ ID NO:15)</td>
</tr>
<tr>
<td>IgG4 hinge</td>
<td>12 aa</td>
<td>ESKYGPPCPACP (SEQ ID NO:52)</td>
</tr>
<tr>
<td>IgG4 hinge + linker</td>
<td>22 aa</td>
<td>ESKYGPPCPACP (SEQ ID NO:16)</td>
</tr>
<tr>
<td>CD28 hinge</td>
<td>39 aa</td>
<td>IEVMYPPPYLDNEKSNGTIHHVKGKHL CPSPLFPGPSK (SEQ ID NO:17)</td>
</tr>
<tr>
<td>CD8 hinge-48aa</td>
<td>48 aa</td>
<td>AKPTTTPAPRPPTPAPTIASQPLSLRPE ACRPAAGGAVHTRGLDFACD (SEQ ID NO:18)</td>
</tr>
<tr>
<td>CD8 hinge-45aa</td>
<td>45 aa</td>
<td>TTTPAPRPPTPAPTIASQPLSLRPEACR PAAGGAVHTRGLDFACD (SEQ ID NO:19)</td>
</tr>
<tr>
<td>IgG4(HL-CH3)</td>
<td>129 aa</td>
<td>ESKYGPPCPACP (SEQ ID NO:20)</td>
</tr>
<tr>
<td>IgG4(L235E, N297Q)</td>
<td>229 aa</td>
<td>ESKYGPPCPACP (SEQ ID NO:4)</td>
</tr>
<tr>
<td>IgG4(S228P, L235E, N297Q)</td>
<td>229 aa</td>
<td>ESKYGPPCPACP (SEQ ID NO:4)</td>
</tr>
</tbody>
</table>
Some spacer regions include all or part of an immunoglobulin (e.g., IgGl, IgG2, IgG3, IgG4) hinge region, i.e., the sequence that falls between the CH1 and CH2 domains of an immunoglobulin, e.g., an IgG4 Fc hinge or a CD8 hinge. Some spacer regions include an immunoglobulin CH3 domain or both a CH3 domain and a CH2 domain. The immunoglobulin derived sequences can include one or more amino acid modifications, for example, 1, 2, 3, 4 or 5 substitutions, e.g., substitutions that reduce off-target binding.

[0016] An "amino acid modification" refers to an amino acid substitution, insertion, and/or deletion in a protein or peptide sequence. An "amino acid substitution" or "substitution" refers to replacement of an amino acid at a particular position in a parent peptide or protein sequence with another amino acid. A substitution can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. The following are examples of various groupings of amino acids: 1) Amino acids with nonpolar R groups: Alanine, Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan, Methionine; 2) Amino acids with uncharged polar R groups: Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine, Glutamine; 3) Amino acids with charged polar R groups (negatively charged at pH 6.0): Aspartic acid, Glutamic acid; 4) Basic amino acids (positively charged at pH
Lysine, Arginine, Histidine (at pH 6.0). Another grouping may be those amino acids with phenyl groups: Phenylalanine, Tryptophan, and Tyrosine.

[0017] In certain embodiments, the spacer is derived from an IgG1, IgG2, IgG3, or IgG4 that includes one or more amino acid residues substituted with an amino acid residue different from that present in an unmodified spacer. The one or more substituted amino acid residues are selected from, but not limited to, one or more amino acid residues at positions 220, 226, 228, 229, 230, 233, 234, 235, 234, 237, 238, 239, 243, 247, 267, 268, 280, 290, 292, 297, 298, 299, 300, 305, 309, 218, 326, 330, 331, 332, 333, 334, 336, 339, or a combination thereof. In this numbering scheme, described in greater detail below, the first amino acid in the IgG4(L235E,N297Q) spacer in Table 1 is 219 and the first amino acid in the IgG4(FL-HCH3) spacer in Table 1 is 219 as is the first amino acid in the IgG hinge sequence and the IgG4 hinge linker (HL) sequence in Table 1.


[0019] In certain embodiments, the modified spacer is derived from IgG4 region that includes one or more amino acid residues substituted with an amino acid residue different from that present in an unmodified region. The one or more substituted amino acid residues are selected from, but not limited to, one or more amino acid residues at positions 220, 226, 228, 229, 230, 233, 234, 235, 234, 237, 238, 239, 243, 247, 267, 268, 280, 290, 292, 297, 298, 299, 300, 305, 309, 218, 326, 330, 331, 332, 333, 334, 336, 339, or a combination thereof.

[0020] In some embodiments, the modified spacer is derived from an IgG4 region that includes, but is not limited to, one or more of the following amino acid residue

[0021] For amino acid positions in immunoglobulin discussed herein, numbering is according to the EU index or EU numbering scheme (Kabat et al. 1991 Sequences of Proteins of Immunological Interest, 5th Ed., United States Public Health Service, National Institutes of Health, Bethesda, hereby entirely incorporated by reference). The EU index or EU index as in Kabat or EU numbering scheme refers to the numbering of the EU antibody (Edelman et al. 1969 Proc Natl Acad Sci USA 63:78-85).

[0022] A variety of transmembrane domains can be used in CAR directed against IL13Ra2. Table 2 includes examples of suitable transmembrane domains. Where a spacer domain is present, the transmembrane domain is located carboxy terminal to the spacer domain.

Table 2: Examples of Transmembrane Domains

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession</th>
<th>Length</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3z</td>
<td>J04132.1</td>
<td>21 aa</td>
<td>LCYLLDGILFYGVLTALFL (SEQ ID NO:21)</td>
</tr>
<tr>
<td>CD28</td>
<td>NM_006139</td>
<td>27aa</td>
<td>FWVLLVYGGVLACSLVTUAFIVFWV (SEQ ID NO:22)</td>
</tr>
<tr>
<td>CD28(M)</td>
<td>NM_006139</td>
<td>28aa</td>
<td>MFWVLLVYGGVLACSLVTUAFIVFWV (SEQ ID NO:22)</td>
</tr>
<tr>
<td>CD4</td>
<td>M35160</td>
<td>22aa</td>
<td>MALLVGLGAGGLLFIGLGIFF (SEQ ID NO:5)</td>
</tr>
<tr>
<td>CD8tm</td>
<td>NM_001768</td>
<td>21aa</td>
<td>IYIWAPLAGTCGVLLLMLVIT (SEQ ID NO:23)</td>
</tr>
</tbody>
</table>
Many of the CAR described herein include one or more (e.g., two) costimulatory domains. The costimulatory domain(s) are located between the transmembrane domain and the CD3ζ signaling domain. Table 3 includes examples of suitable costimulatory domains together with the sequence of the CD3ζ signaling domain.

**Table 3: Examples of Costimulatory Domains**

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession</th>
<th>Length</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3ζ</td>
<td>J04132.1</td>
<td>113 aa</td>
<td>RVKFSRSADAPAYQQGQNQLYNELNGLREEYDVLDKRRGRDPPEMGKPRKKNPQEGLYNELQDKMAEAYSEIGMKGERRGKGDHGLYQGLSTATKDYDALHMQALPPR</td>
</tr>
<tr>
<td>CD28</td>
<td>NM_006139</td>
<td>42 aa</td>
<td>RSKRSRLHSYMDNMTPRPQGPPRTKHQPYAPRDFAYRS (SEQ ID NO: 27)</td>
</tr>
<tr>
<td>CD28gg*</td>
<td>NM_006139</td>
<td>42 aa</td>
<td>RSKRSRGGSYDMNTPQPQGPPRTKHQPYAPRDFAYRS (SEQ ID NO:28)</td>
</tr>
<tr>
<td>41BB</td>
<td>NM_001561</td>
<td>42 aa</td>
<td>KRGKRLLYIFKQPFMRPVTTQEDGCSCRFPEEEEGLCE (SEQ ID NO:29)</td>
</tr>
<tr>
<td>OX40</td>
<td></td>
<td>42 aa</td>
<td>ALYLLRRDQRLPPDAAKPPGGSFRTPIQEEQADAHSTLAKI (SEQ ID NO:30)</td>
</tr>
</tbody>
</table>

**DESCRIPTION OF DRAWINGS**

[0023] **Figure 1** is a schematic depiction of IL13(E13Y)-zetakine CAR (Left) composed of the IL13Ra2-specific human IL-13 variant (huIL-13(E13Y)), human IgG4 Fc spacer (huIg4Fc), human CD4 transmembrane (huCD4 tm), and human CD3ζ chain cytoplasmic
 portions as indicated. Also depicted is a IL13(EQ)BE^ CAR which is the same as the IL13(E13Y)-zetakine with the exception of the two point mutations, L235E and N297Q indicated in red, that are located in the CH2 domain of the IgG4 spacer, and the addition of a costimulatory 4-IBB cytoplasmic domain (4-IBB cyt).

[0024] Figures 2A-C depict certain vectors an open reading frames. A is a diagram of the cDNA open reading frame of the 2670 nucleotide IL13(EQ)BBZ-T2ACD19t construct, where the IL13Ra2-specific ligand IL13(E13Y), IgG4(EQ) Fc hinge, CD4 transmembrane, 4-IBB cytoplasmic signaling, three-glycine linker, and CD3ζ cytoplasmic signaling domains of the IL13(EQ)BBZ CAR, as well as the T2A ribosome skip and truncated CD19 sequences are indicated. The human GM-CSF receptor alpha and CD19 signal sequences that drive surface expression of the IL13(EQ)BBζ CAR and CD19t are also indicated. B is a diagram of the sequences flanked by long terminal repeats (indicated by 'R') that will integrate into the host genome. C is a map of the IL13(EQ)BBZ-T2A-CD19t_epHIV7 plasmid.

[0025] Figure 3 depicts the construction of pHIV7.

[0026] Figure 4 depicts the elements of pHIV7.

[0027] Figure 5 depicts a production scheme for IL13(EQ)BBζ/CD19t+ TCM.

[0028] Figures 6A-C depicts the results of flow cytometric analysis of surface transgene and T cell marker expression. IL13(EQ)BBζ/CD19t+ TCM HD006.5 and HD187.1 were co-stained with anti-IL13-PE and anti-CD8-FITC to detect CD8+ CAR+ and CD4+ (i.e., CD8 negative) CAR+ cells (A), or anti-CD 19-PE and anti-CD4-FITC to detect CD4+ CD19t+ and CD8+ (i.e., CD4 negative) CAR+ cells (B). IL13(EQ)BBζ/CD19t+ TCM HD006.5 and HD187.1 stained with fluorochromeconjugatedanti-CD3, TCR, CD4, CD8, CD62L and CD28 (grey histograms) or isotype controls (black histograms) (C). In all cases the percentages based on viable lymphocytes (DAPI negative) stained above isotype.

[0029] Figures 7A-B depict the in vitro functional characterization of IL13Ra2-specific effector function of IL13(EQ)BBZ+ TCM. IL13(EQ)BBZ/CD19t+ TCM HD006.5 and
HD187.1 were used as effectors in a 6-hour $^{51}$Cr release assay using a 10:1 E:T ratio based on CD19t expression. The IL13Ra2-positive tumor targets were K562 engineered to express IL13Ra2 (K562-IL13Ra2) and primary glioma line PBT030-2, and the IL13Ra2-negative tumor target control was K562 parental line (A).

IL13(EQ)BBZ/CD19t+ TCM HD006.5 and HD187.1 were evaluated for antigen-dependent cytokine production following overnight co-culture at a 10:1 E:T ratio with IL13Ra2-positive and negative targets. Cytokine levels were measured using the Bioplex Pro Human Cytokine TH1/TH2 Assay kit and INF-γ are reported (B).

[0030] Figures 8A-C depict the result of studies demonstrating the regression of established glioma tumor xenografts after adoptive transfer of IL13(EQ)BBζ/CD19t+ TCM. EGFP-ffLuc+ PBT030-2 tumor cells ($1 \times 10^5$) were stereotactically implanted into the right forebrain of NSG mice. On day 5, mice received either 2x10 $^6$ IL13(E%BBζ/CD19t+ TCM (1.1x10 $^6$ CAR+; n=6), 2x10 $^6$ mock TCM (no CAR; n=6) or PBS (n=6). Representative mice from each group showing relative tumor burden using Xenogen Living Image (A). Quantification of ffLuc flux (photons/sec) shows that IL13(E%BBζ/CD19t+ TCM induce tumor regression as compared to mock-transduced TCM and PBS (#p<0.02, *p<0.001, repeated measures ANOVA) (B). Kaplan Meier survival curve (n=6 per group) demonstrating significantly improved survival (p=0.0008; log-rank test) for mice treated with IL13(E%BBζ/CD19t+ TCM (C).

[0031] Figures 9A-C depict the results of studies comparing ant-tumor efficacy of IL13(EQ)BBZ TCM and IL13-zetakine CTL clones. EGFP-ffLuc+ PBT030-2 TSs (lx10 $^5$) were stereotactically implanted into the right forebrain of NSG mice. On day 8, mice received either 1.6x10 $^6$ mock TCM (no CAR), 1.0x10 $^6$ CAR+ IL13(EQ)BBζ TCM (1.6x10 $^6$ total T cells; 63% CAR), 1.0x10$^6$ IL13-zetakine CD8+ CTL cl. 2D7 (clonal CAR+), or no treatment (n=6 per group). Representative mice from each group showing relative tumor burden using Xenogen Living Image (A). Linear regression lines of natural log of ffLuc flux (photons/sec) over time, P-values are for group by time interaction comparisons (B). Kaplan Meier survival analysis (n=6 per group) demonstrate significantly improved survival (p=0.02; log-rank test) for mice treated with IL13(EQ)BBζ TCM as compared to IL13-zetakine CD8+ CTL cl. 2D7 (C).
Figures 1OA-C depict the results of studies comparing ant-tumor efficacy of IL13(EQ)BE^ TCM and IL13-zetakine CTL clones. EGFP-ffLuc+ PBT030-2 TSs (1x10^5) were stereotactically implanted into the right forebrain of NSG mice. On day 8, mice received either 1.3x10^6 mock TCM (no CAR; n=6), 1.0, 0.3 or O.1x10^6 CAR+ IL13(EQ)BBC TCM (78% CAR+; n=6-7), 1.0, 0.3 or O.1x10^6 IL13-zetakine CD8+ CTL cl. 2D7 (clonal CAR+; n=6-7), or no treatment (n=5). Xenogen imaging of representative mice from each group showing relative tumor burden (A). Linear regression lines of natural log of ffLuc flux (photons/sec) shows that IL13(EF%BBζ TCM achieve superior tumor regression as compared to first-generation IL13-zetakine CTL cl. 2D7, mock TCM and tumor only (B). Average flux per group at day 27 post tumor injection demonstrating that the O.1x10^6 IL13(E%BBζ TCM dose outperforms the ten-fold higher 1.0x10^6 dose of IL13-zetakine CD8+ CTL cl. 2D7 (p = 0.043; Welch two sample t-test) (C).

Figure 11 depicts the results of studies demonstrating IL13(E%BBζ TCM display improved persistence compared IL13-zetakine CTL clones. CD3 immunohistochemistry evaluating T cell persistence at the tumor site 7-days post T cell infusion. Significant numbers of T cells are detected for IL13(E%BBζ TCM (top panel). By contrast, very few viable CD3+ IL13-zetakine T cells are detected (bottom panel).

Figures 12A-D depict the results of experiments comparing route of CAR+ T cell delivery (i.e. versus i.v.) for large established tumors. EGFP-ffLuc+ PBT030-2 TSs (1x10^5) were implanted into the right forebrain of NSG mice. On days 19 and 26, mice were injected i.v. through the tail vein with either 5x10^6 CAR+ IL13(EQ)BBζ + Tcm (11.8x10^6 total cells; n=4), or mock Tcm (11.8x10^6 cells; n=4). Alternatively, on days 19, 22, 26 and 29 mice were injected i.e. with either 1x10^6 CAR+ IL13(EQ)BBζ + Tcm (2.4x10^6 total cells; n=4), or mock Tcm (2.4x10^6 cells; n=5). Average ffLuc flux (photons/sec) over time shows that i.e. delivered IL13(EQ)BBζ Tcm mediates tumor regression of day 19 tumors. By comparison, i.v. delivered T cells do not shown reduction in tumor burden as compared to untreated or mock Tcm controls (A). Kaplan Meier survival curve demonstrates improved survival for mice treated i.e. IL13(EQ)BBZ Tcm as compared to mice treated with i.v. administered CAR+ Tcm (p = 0.0003 log rank test) (B). Representative H&E and CD3 IHC of mice treated i.v. (C) versus i.e. (D) with...
IL13(EQ)BBZ+ Tcm. CD3+ T cells were only detected in the i.e. treated group, with no CD3+ cells detected in the tumor or surrounding brain parenchyma for i.v. treated mice.

Figures 13A-B depict the results of studies showing that CAR+ T cell injected intracranially, either intratumoral (i.c.t.) or intraventricular (i.c.v.), can traffic to tumors on the opposite hemisphere. EGFP-fLuc+ PBT030-2 TSs (1×105) were stereotactically implanted into the right and left forebrains of NSG mice. On day 6, mice were injected i.e. at the right tumor site with 1.0x10⁶ II,13(EQ)BBζ+ Tcm (1.6x10⁶ total cells; 63% CAR; n=4). Schematic of multifocal glioma experimental model (A). CD3 IHC showing T cells infiltrating both the right and left tumor sites (B).

Figures 14A-C depict the results of a series of studies evaluating costimulatory domains of IL13Ra2-specific CAR. Schematic of IL13Ra2-specific CAR constructs comparing various intracellular endo/signaling domains, including the first generation CD3ζ CAR lacking costimulation, versus second generation CARs incorporating either 4-1BB or CD28, versus a third generation CAR containing both CD28 and 41BB. All CAR cassettes also contain the T2A ribosomal skip and truncated CD19 (CD19t) sequences as a marker for transduced cells (A). CD4 and CD8 TCM were lentivirally transduced and CAR-expressing T cells were immunomagnetically enriched via anti-CD4. CD19 and IL13 (i.e., CAR) expression levels as measured by flow cytometry (B). Stability of each CAR construct was determined by dividing the CAR (IL13) mean florescence intensity (MFI) by that of the transduction marker (CD19t) (C). The 4-IBB containing CARs demonstrated the lowest expression levels as compared to the CD19t transduction marker.

Figures 15A-B depict the results of studies demonstrating that IL13Ra2-specific CAR containing the 4-IBB costimulatory domain produce less Th1 and Th2 cytokines. The ability of the indicated mock-transduced or CAR-expressing T cells to kill IL13Ra2-expressing PBT030-2 tumor cell targets was determined in a 4-hour 51Cr-release assay at the indicated effector:target ratios. Mean % chromium release ± S.D. of triplicate wells are depicted (A). As expected, mock-transduced T cells did not efficiently lyse the targets. In contrast, all CAR-expressing T cells lysed the tumor cells in a similar manner.
The indicated mock-transduced or CAR-expressing T cells were co-cultured overnight with IL13Ra2-expressing PBT030-2 tumor cells at a 10:1 ratio and supernatants were analyzed for IL-13 and IFN-γ levels by cytometric bead array (B). Means + S.D. of triplicate wells are depicted. Interestingly, T cells expressing the zeta, 41BB-zeta or CD28-41BB-zeta CARs exhibited lower antigen-stimulated cytokine production than T cells expressing the CD28-zeta CAR.

[0038] Figures 16A-C depict the results of a series of studies of the in vivo efficacy of IL13Ra2-specific CARs. NSG mice received an intracranial injection of ffLuc+ PBT030-2 tumor cells on day 0, and were randomized into 6 groups (n = 9-10 mice per group) for i.e. treatment with either PBS (Tumor Only), mock-transduced T cells or T cells expressing the indicated IL13Ra2-specific CAR on day 8. Quantitative bioluminescence imaging was then carried out to monitor tumor growth over time. Bioluminescence images for representative mice in each group (A). Mean + S.E. of total flux levels of luciferase activity over time in each group (B). Flux levels for each mouse at Day 27. All groups treated with IL13Ra2-specific CAR T cells, except those treated with T cells expressing the CD28-CAR, show statistically-significant reduction in tumor volume compared to mice treated with mock-transduced T cells (C).

[0039] Figure 17 depicts the amino acid sequence of II.13(EQ. BBζ/CD19f+ (SEQ ID NO:10).

[0040] Figure 18 depicts a sequence comparison of IL13^41 BBζ[IL13{EQ}4 IBBζ T2A-CD19t_epHIV7; pF02630] (SEQ ID NO: 12) and CD19Rop_epHIV7 (pJ01683) (SEQ ID NO: 13).

[0041] Figure 19 depicts the amino acid sequence of IL13(EmY)-CD8h3-CD8tm2-41BB Zeta (SEQ ID NO:31 with GMSCFRα signal peptide; SEQ ID NO:39 without GMSCFRα signal peptide).

[0042] Figure 20 depicts the amino acid sequence of IL13(EmY)-CD8h3-CD28tm-CD28gg-41BB-Zeta (SEQ ID NO:32 with GMSCFRα signal peptide; SEQ ID NO:40 without GMSCFRα signal peptide).
[0043] **Figure 21** depicts the amino acid sequence of IL13(EmY)-IgG4(HL-CH3)-CD4tm-41BB-Zeta (SEQ ID NO:33 with GMSCFRa signal peptide; SEQ ID NO:41 without GMSCFRa signal peptide).

[0044] **Figure 22** depicts the amino acid sequence of IL13(EmY)-IgG4(L235E,N297Q)-CD8tm-41BB-Zeta (SEQ ID NO:34 with GMSCFRa signal peptide; SEQ ID NO:42 without GMSCFRa signal peptide).

[0045] **Figure 23** depicts the amino acid sequence of IL13(EmY)-Linker-CD28tm-CD28gg-41BB-Zeta (SEQ ID NO:35 with GMSCFRa signal peptide; SEQ ID NO:43 without GMSCFRa signal peptide).

[0046] **Figure 24** depicts the amino acid sequence of IL13(EmY)-HL-CD28m-CD28gg-41BB-Zeta (SEQ ID NO:36 with GMSCFRa signal peptide; SEQ ID NO:44 without GMSCFRa signal peptide).

[0047] **Figure 25** depicts the amino acid sequence of IL13(EmY)-IgG4(HL-CH3)-CD28tm-CD28gg-41BB-Zeta (SEQ ID NO:37 with GMSCFRa signal peptide; SEQ ID NO:45 without GMSCFRa signal peptide).

[0048] **Figure 26** depicts the amino acid sequence of IL13(EmY)-IgG4(L235E,N297Q)-CD28tm-CD28gg-41BB-Zeta (SEQ ID NO:38 with GMSCFRa signal peptide; SEQ ID NO:46 without GMSCFRa signal peptide).

[0049] **Figure 27** depicts the amino acid sequence of IL13(EmY)-CD8h3-CD8tm-41BB Zeta (SEQ ID NO:47 with GMSCFRa signal peptide; SEQ ID NO:48 without GMSCFRa signal peptide).

[0050]

**DETAILED DESCRIPTION**

[0051] Described below is the structure, construction and characterization of various IL13Ra2-specific chimeric antigen receptors. A chimeric antigen (CAR) is a recombinant
biomolecule that contains, at a minimum, an extracellular recognition domain, a transmembrane region, and an intracellular signaling domain. The term "antigen," therefore, is not limited to molecules that bind antibodies, but to any molecule that can bind specifically to a target. For example, a CAR can include a ligand that specifically binds a cell surface receptor. The extracellular recognition domain (also referred to as the extracellular domain or simply by the recognition element which it contains) comprises a recognition element that specifically binds to a molecule present on the cell surface of a target cell. The transmembrane region anchors the CAR in the membrane. The intracellular signaling domain comprises the signaling domain from the zeta chain of the human CD3 complex and optionally comprises one or more costimulatory signaling domains. CARs can both to bind antigen and transduce T cell activation, independent of MHC restriction. Thus, CARs are "universal" immunoreceptors which can treat a population of patients with antigen-positive tumors irrespective of their HLA genotype. Adoptive immunotherapy using T lymphocytes that express a tumor-specific CAR can be a powerful therapeutic strategy for the treatment of cancer.

[0052] One IL13Ra2-specific CAR described herein is referred to as IL13(EQ)BE^A. This CAR includes a variety of important features including: a IL13a2 ligand having an amino acid change that improves specificity of binding to IL13a2; the domain of CD137 (4-IBB) in series with CD3ζ to provide beneficial costimulation; and an IgG4 Fc region that is mutated at two sites within the CH2 region (L235E; N297Q) in a manner that reduces binding by Fc receptors (FcRs). Other CAR described herein contain a second costimulatory domain.

[0053] In some cases the CAR described herein, including the IL13(EQ)BBζ CAR can be produced using a vector in which the CAR open reading frame is followed by a T2A ribosome skip sequence and a truncated CD19 (CD19t), which lacks the cytoplasmic signaling tail (truncated at amino acid 323). In this arrangement, co-expression of CD19t provides an inert, non-immunogenic surface marker that allows for accurate measurement of gene modified cells, and enables positive selection of gene-modified cells, as well as efficient cell tracking and/or imaging of the therapeutic T cells in vivo following adoptive transfer. Co-expression of CD19t provides a marker for immunological targeting of the
transduced cells in vivo using clinically available antibodies and/or immunotoxin reagents to selectively delete the therapeutic cells, and thereby functioning as a suicide switch.

[0054] Gliomas, express IL13 receptors, and in particular, high-affinity IL13 receptors. However, unlike the IL13 receptor, glioma cells overexpress a unique IL13Ra2 chain capable of binding IL13 independently of the requirement for IL4RP or yc44. Like its homolog IL4, IL13 has pleotropic immunoregulatory activity outside the CNS. Both IL13 and IL4 stimulate IgE production by B lymphocytes and suppress pro-inflammatory cytokine production by macrophages.

[0055] Detailed studies using autoradiography with radiolabeled IL13 have demonstrated abundant IL13 binding on nearly all malignant glioma tissues studied. This binding is highly homogeneous within tumor sections and in single cell analysis. However, molecular probe analysis specific for IL13Ra2 mRNA did not detect expression of the glioma-specific receptor by normal brain elements and autoradiography with radiolabeled IL13 also could not detect specific IL13 binding in the normal CNS. These studies suggest that the shared IL13Ra/IL4p/yc receptor is not expressed detectably in the normal CNS. Therefore, IL13Ra2 is a very specific cell-surface target for glioma and is a suitable target for a CAR designed for treatment of a glioma.

[0056] Binding of IL13-based therapeutic molecules to the broadly expressed IL13/Ra/IL4p/yc receptor complex, however, has the potential of mediating undesired toxicities to normal tissues outside the CNS, and thus limits the systemic administration of these agents. An amino acid substitution in the IL13 alpha helix A at amino acid 13 of tyrosine for the native glutamic acid selectively reduces the affinity of IL13 to the IL13Ra/IL4p/yc receptor. Binding of this mutant (termed IL13(E13Y)) to IL13Ra2, however, was increased relative to wild-type IL13. Thus, this minimally altered IL13 analog simultaneously increases IL13's specificity and affinity for glioma cells. Therefore, CAR described herein include an IL13 containing a mutation (E to Y or E to some other amino acid such as K or R or L or V) at amino acid 13 (according to the numbering of Debinski et al. 1999 Clin Cancer Res 5:3143s). IL13 having the natural
sequence also may be used, however, and can be useful, particularly in situations where the modified T cells are to be locally administered, such as by injection directly into a tumor mass.

[0057] The CAR described herein can be produced by any means known in the art, though preferably it is produced using recombinant DNA techniques. Nucleic acids encoding the several regions of the chimeric receptor can be prepared and assembled into a complete coding sequence by standard techniques of molecular cloning known in the art (genomic library screening, PCR, primer-assisted ligation, site-directed mutagenesis, etc.) as is convenient. The resulting coding region is preferably inserted into an expression vector and used to transform a suitable expression host cell line, preferably a T lymphocyte cell line, and most preferably an autologous T lymphocyte cell line.

[0058] Various T cell subsets isolated from the patient, including unselected PBMC or enriched CD3 T cells or enriched CD3 or memory T cell subsets, can be transduced with a vector for CAR expression. Central memory T cells are one useful T cell subset. Central memory T cell can be isolated from peripheral blood mononuclear cells (PBMC) by selecting for CD45RO+/CD62L+ cells, using, for example, the CliniMACS® device to immunomagnetically select cells expressing the desired receptors. The cells enriched for central memory T cells can be activated with anti-CD3/CD28, transduced with, for example, a SIN lentiviral vector that directs the expression of an IL13Ra2-specific CAR (e.g., iIL13(EθBBζ) as well as a truncated human CD19 (CD19t), a non-immunogenic surface marker for both in vivo detection and potential ex vivo selection. The activated/genetically modified central memory T cells can be expanded in vitro with IL-2/IL-15 and then cryopreserved.

Example 1: Construction and Structure of an IL13Ra2-specific CAR

[0059] The structure of a useful IL13Ra2-specific CAR is described below. The codon optimized CAR sequence contains a membrane-tethered IL-13 ligand mutated at a single site (E13Y) to reduce potential binding to IL13Ra1, an IgG4 Fc spacer containing two mutations (L235E; N297Q) that greatly reduce Fc receptor-mediated recognition models,
a CD4 transmembrane domain, a costimulatory 4-IBB cytoplasmic signaling domain, and a CD3ζ cytoplasmic signaling domain. A T2A ribosome skip sequence separates this IL13(EQ)BBζ CAR sequence from CD19t, an inert, non-immunogenic cell surface detection/selection marker. This T2A linkage results in the coordinate expression of both IL13(EQ)BBζ and CD19t from a single transcript. Figure 1A is a schematic drawing of the 2670 nucleotide open reading frame encoding the IL13(EQ)BBZ-T2ACD19t construct. In this drawing, the IL13Ra2-specific ligand IL13(E13Y), IgG4(EQ) Fc, CD4 transmembrane, 4-IBB cytoplasmic signaling, three-glycine linker, and CD3ζ cytoplasmic signaling domains of the IL13(EQ)BBZ CAR, as well as the T2A ribosome skip and truncated CD19 sequences are all indicated. The human GM-CSF receptor alpha and CD19 signal sequences that drive surface expression of the IL13(EQ)BBZ CAR and CD19t are also indicated. Thus, the IL13(EQ)BBZ-T2ACD19t construct includes a IL13Ra2-specific, hinge-optimized, costimulatory chimeric immunoreceptor sequence (designated IL13(EQ)BBZ), a ribosome-skip T2A sequence, and a CD19t sequence.

The IL13(EQ)BBZ sequence was generated by fusion of the human GM-CSF receptor alpha leader peptide with IL13(E13Y) ligand 5 L235E/N297Q-modified IgG4 Fc hinge (where the double mutation interferes with FcR recognition), CD4 transmembrane, 4-IBB cytoplasmic signaling domain, and CD3ζ cytoplasmic signaling domain sequences. This sequence was synthesized de novo after codon optimization. The T2A sequence was obtained from digestion of a T2A-containing plasmid. The CD19t sequence was obtained from that spanning the leader peptide sequence to the transmembrane components (i.e., basepairs 1-972) of a CD19-containing plasmid. All three fragments, 1) IL13(EQ)BBZ, 2) T2A, and 3) CD19t, were cloned into the multiple cloning site of the epHIV7 lentiviral vector. When transfected into appropriate cells, the vector integrates the sequence depicted schematically in Figure 1B into the host cells genome. Figure 1C provides a schematic drawing of the 9515 basepair IL13(EQ)BBZ-T2A-CD19t _epHIV7 plasmid itself.

As shown schematically in Figure 2, IL13(EQ)BBZ CAR differs in several important respects from a previously described IL13Ra2-specific CAR referred to as IL13(E13Y)-zetakine (Brown et al. 2012 Clinical Cancer Research 18:2199). The
IL13(E13Y)-zetakine is composed of the IL13Ra2-specific human IL-13 mutein (huIL-13(E13Y)), human IgG4 Fc spacer (huy4Fc), human CD4 transmembrane (huCD4 tm), and human CD3ζ chain cytoplasmic (huCD3ζ cyt) portions as indicated. In contrast, the IL13(EQ)BE^ has two point mutations, L235E and N297Q that are located in the CH2 domain of the IgG4 spacer, and a costimulatory 4-IBB cytoplasmic domain (4-IBB cyt).

Example 2: Construction and Structure of epHIV7 used for Expression of an IL13Ra2-specific CAR

[0062] The pHIV7 plasmid is the parent plasmid from which the clinical vector IL13(EQ)BBZ-T2A-CD19t_epHIV7 was derived in the T cell Therapeutics Research Laboratory (TCTRL) at City of Hope (COH). The epHIV7 vector used for expression of the CAR was produced from pHIV7 vector. Importantly, this vector uses the human EF1 promoter to drive expression of the CAR. Both the 5’ and 3’ sequences of the vector were derived from pv653RSN as previously derived from the HXBc2 provirus. The polypurine tract DNA flap sequences (cPPT) were derived from HIV-1 strain pNL4-3 from the NIH AIDS Reagent Repository. The woodchuck post-transcriptional regulatory element (WPRE) sequence was previously described.

[0063] Construction of pHIV7 is schematically depicted in Figure 3. Briefly, pv653RSN, containing 653 bp from gag-pol plus 5’ and 3’ long-terminal repeats (LTRs) with an intervening SL3-neomycin phosphotransferase gene (Neo), was subcloned into pBluescript, as follows: In Step 1, the sequences from 5’ LTR to rev-responsive element (RRE) made p5’HIV-l 51, and then the 5’ LTR was modified by removing sequences upstream of the TATA box, and ligated first to a CMV enhancer and then to the SV40 origin of replication (p5’HIV-2). In Step 2, after cloning the 3’ LTR into pBluescript to make p3’HIV-l, a 400-bp deletion in the 3’ LTR enhancer/promoter was made to remove cis-regulatory elements in HIV U3 and form p3’HIV-2. In Step 3, fragments isolated from the p5’HIV-3 and p3’HIV-2 were ligated to make HIV-3. In Step 4, the p3’HIV-2 was further modified by removing extra upstream HIV sequences to generate p3’HIV-3 and a 600-bp BamHI-Sall fragment containing WPRE was added to p3’HIV-3 to make the p3’HIV-4. In Step 5, the pHIV-3 RRE was reduced in size by PCR and ligated to a 5’
fragment from pHIV-3 (not shown) and to the p3'HIV-4, to make pHIV-6. In Step 6, a 190-bp BglII-BamHI fragment containing the cPPT DNA flap sequence from HIV-1 pNL4-3 (55) was amplified from pNL4-3 and placed between the RRE and the WPRE sequences in pHIV6 to make pHIV-7. This parent plasmid pHIV-7-GFP (GFP, green fluorescent protein) was used to package the parent vector using a four-plasmid system.

[0064] A packaging signal, psi $\psi$, is required for efficient packaging of viral genome into the vector. The RRE and WPRE enhance the RNA transcript transport and expression of the transgene. The flap sequence, in combination with WPRE, has been demonstrated to enhance the transduction efficiency of lentiviral vector in mammalian cells.

[0065] The helper functions, required for production of the viral vector, are divided into three separate plasmids to reduce the probability of generation of replication competent lentivirus via recombination: 1) pCgp encodes the gag/pol protein required for viral vector assembly; 2) pCMV-Rev2 encodes the Rev protein, which acts on the RRE sequence to assist in the transportation of the viral genome for efficient packaging; and 3) pCMV-G encodes the glycoprotein of the vesiculo-stomatitis virus (VSV), which is required for infectivity of the viral vector.

[0066] There is minimal DNA sequence homology between the pHIV7 encoded vector genome and the helper plasmids. The regions of homology include a packaging signal region of approximately 600 nucleotides, located in the gag/pol sequence of the pCgp helper plasmid; a CMV promoter sequence in all three helper plasmids; and a RRE sequence in the helper plasmid pCgp. It is highly improbable that replication competent recombinant virus could be generated due to the homology in these regions, as it would require multiple recombination events. Additionally, any resulting recombinants would be missing the functional LTR and tat sequences required for lentiviral replication.

[0067] The CMV promoter was replaced by the EFla-HTLV promoter (EFlp), and the new plasmid was named epHIV7 (Figure 4). The EFlp has 563 bp and was introduced into epHIV7 using Nrul and Nhel, after the CMV promoter was excised.
The lentiviral genome, excluding gag/pol and rev that are necessary for the pathogenicity of the wild-type virus and are required for productive infection of target cells, has been removed from this system. In addition, the IL13(EQ)BBZ-T2ACD19t_epHIV7 vector construct does not contain an intact 3’LTR promoter, so the resulting expressed and reverse transcribed DNA proviral genome in targeted cells will have inactive LTRs. As a result of this design, no HIV-I derived sequences will be transcribed from the provirus and only the therapeutic sequences will be expressed from their respective promoters. The removal of the LTR promoter activity in the SIN vector is expected to significantly reduce the possibility of unintentional activation of host genes (56). Table 4 summarizes the various regulator elements present in IL13(EQ)BBZ-T2ACD19t_epHIV7.

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<td>3’ U3 with deletion to generate SIN vector</td>
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Example 3: Production of Vectors for Transduction of Patient T Cells

[0069] For each plasmid (IL13(EQ)BBZ-T2A-CD19t_epHIV7; pCgp; pCMV-G; and pCMV-Rev2), a seed bank is generated, which is used to inoculate the fermenter to produce sufficient quantities of plasmid DNA. The plasmid DNA is tested for identity, sterility and endotoxin prior to its use in producing lentiviral vector.

[0070] Briefly, cells were expanded from the 293T working cell (WCB), which has been tested to confirm sterility and the absence of viral contamination. A vial of 293T cells from the 293T WCB was thawed. Cells were grown and expanded until sufficient numbers of cells existed to plate an appropriate number of 10 layer cell factories (CFs) for vector production and cell train maintenance. A single train of cells can be used for production.

[0071] The lentiviral vector was produced in sub-batches of up to 10 CFs. Two sub-batches can be produced in the same week leading to the production of approximately 20 L of lentiviral supernatant/week. The material produced from all sub-batches were pooled during the downstream processing phase, in order to produce one lot of product. 293T cells were plated in CFs in 293T medium (DMEM with 10% FBS). Factories were placed in a 37°C incubator and horizontally leveled in order to get an even distribution of the cells on all the layers of the CF. Two days later, cells were transfected with the four lentiviral plasmids described above using the CaP04 method, which involves a mixture of Tris:EDTA, 2M CaCl2, 2X HBS, and the four DNA plasmids. Day 3 after transfection, the supernatant containing secreted lentiviral vectors was collected, purified and concentrated. After the supernatant was removed from the CFs, End-of-Production Cells were collected from each CF. Cells were trypsinized from each factory and collected by centrifugation. Cells were resuspended in freezing medium and

<table>
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<th>Regulatory Elements and Genes</th>
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<tr>
<td>R</td>
<td>9507-86</td>
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cryopreserved. These cells were later used for replication-competent lentivirus (RCL) testing.

[0072] To purify and formulate vectors crude supernatant was clarified by membrane filtration to remove the cell debris. The host cell DNA and residual plasmid DNA were degraded by endonuclease digestion (Benzonase®). The viral supernatant was clarified of cellular debris using a 0.45 μm filter. The clarified supernatant was collected into a pre-weighted container into which the Benzonase® is added (final concentration 50 U/mL). The endonuclease digestion for residual plasmid DNA and host genomic DNA as performed at 37°C for 6 h. The initial tangential flow ultrafiltration (TFF) concentration of the endonuclease-treated supernatant was used to remove residual low molecular weight components from the crude supernatant, while concentrating the virus ~20 fold. The clarified endonuclease-treated viral supernatant was circulated through a hollow fiber cartridge with a NMWCO of 500 kD at a flow rate designed to maintain the shear rate at -4,000 sec-1 or less, while maximizing the flux rate. Diafiltration of the nuclease-treated supernatant was initiated during the concentration process to sustain the cartridge performance. An 80% permeate replacement rate was established, using 4% lactose in PBS as the diafiltration buffer. The viral supernatant was brought to the target volume, representing a 20-fold concentration of the crude supernatant, and the diafiltration was continued for 4 additional exchange volumes, with the permeate replacement rate at 100%.

[0073] Further concentration of the viral product was accomplished by using a high speed centrifugation technique. Each sub-batch of the lentivirus was pelleted using a Sorvall RC-26 plus centrifuge at 6000 RPM (6,088 RCF) at 6oC for 16-20 h. The viral pellet from each sub-batch was then reconstituted in a 50 mL volume with 4% lactose in PBS. The reconstituted pellet in this buffer represents the final formulation for the virus preparation. The entire vector concentration process resulted in a 200-fold volume reduction, approximately. Following the completion of all of the sub-batches, the material was then placed at -80oC, while samples from each sub-batch were tested for sterility. Following confirmation of sample sterility, the sub-batches were rapidly thawed at 37oC with frequent agitation. The material was then pooled and manually aliquoted in the Class
II Type A/B3 biosafety cabinet in the viral vector suite. A fill configuration of 1 mL of the concentrated lentivirus in sterile USP class 6, externally threaded O-ring cryovials was used. Center for Applied Technology Development (CATD)'s Quality Systems (QS) at COH released all materials according to the Policies and Standard Operating Procedures for the CBG and in compliance with current Good Manufacturing Practices (cGMPs).

[0074] To ensure the purity of the lentiviral vector preparation, it was tested for residual host DNA contaminants, and the transfer of residual host and plasmid DNA. Among other tests, vector identity was evaluated by RT-PCR to ensure that the correct vector is present. All release criteria were met for the vector intended for use in this study.

Example 4: Preparation of T cells Suitable for Use in ACT

[0075] T lymphocytes are obtained from a patient by leukopheresis, and the appropriate allogenic or autologous T cell subset, for example, Central Memory T cells (TCM), are genetically altered to express the CAR, then administered back to the patient by any clinically acceptable means, to achieve anti-cancer therapy.

[0076] An outline of the manufacturing strategy for TCM is depicted in Figure 8 (Manufacturing schema for IL13(EQ)BBζ/CD19t+ TCM). Specifically, apheresis products obtained from consented research participants are ficollled, washed and incubated overnight. Cells are then depleted of monocyte, regulatory T cell and naïve T cell populations using GMP grade anti-CD14, anti-CD25 and anti-CD45RA reagents (Miltenyi Biotec) and the CliniMACSTM separation device. Following depletion, negative fraction cells are enriched for CD62L+ TCM cells using DREG56-biotin (COH clinical grade) and anti-biotin microbeads (Miltenyi Biotec) on the CliniMACSTM separation device.

[0077] Following enrichment, TCM cells are formulated in complete X-Vivo5 plus 50 IU/mL IL-2 and 0.5 ng/mL IL-15 and transferred to a Teflon cell culture bag, where they are stimulated with Dynal ClinExTM Vivo CD3/CD28 beads. Up to five days after stimulation, cells are transduced with IL13(EQ)BBZ-T2A-CD19t_epHIV7 lentiviral
vector at a multiplicity of infection (MOI) of 1.0 to 0.3. Cultures are maintained for up to 42 days with addition of complete X-Vivo5 and IL-2 and IL-15 cytokine as required for cell expansion (keeping cell density between 3x10^5 and 2x10^6 viable cells/mL, and cytokine supplementation every Monday, Wednesday and Friday of culture). Cells typically expand to approximately 10^9 cells under these conditions within 21 days. At the end of the culture period cells are harvested, washed twice and formulated in clinical grade cryopreservation medium (Cryostore CS5, BioLife Solutions).

[0078] On the day(s) of T cell infusion, the cryopreserved and released product is thawed, washed and formulated for re-infusion. The cryopreserved vials containing the released cell product are removed from liquid nitrogen storage, thawed, cooled and washed with a PBS/2% human serum albumin (HSA) Wash Buffer. After centrifugation, the supernatant is removed and the cells resuspended in a Preservative-Free Normal Saline (PFNS)/2% HSA infusion diluent. Samples are removed for quality control testing.

[0079] Two qualification runs on cells procured from healthy donors were performed using the manufacturing platform described above. Each preclinical qualification run product was assigned a human donor (HD) number - HD006.5 and HD187.1. Importantly, as shown in Table 5, these qualification runs expanded >80 fold within 28 days and the expanded cells expressed the IL13(EQ)BBy/CD19t transgenes.

**Table 5: Summary of Expression Data from Pre-clinical Qualification Run Product**

<table>
<thead>
<tr>
<th>Cell Product</th>
<th>CAR</th>
<th>CD19</th>
<th>CD4+</th>
<th>CD8+</th>
<th>Fold Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD006.5</td>
<td>20%</td>
<td>22%</td>
<td>24%</td>
<td>76%</td>
<td>84-fold (28 days)</td>
</tr>
<tr>
<td>Hd187.1</td>
<td>18%</td>
<td>25%</td>
<td>37%</td>
<td>63%</td>
<td>259-fold (28 days)</td>
</tr>
</tbody>
</table>

Example 5: Flow cytometric analysis of surface transgene and T cell marker expression in IL13(EQ)BBY/CD19t+TcM
The two preclinical qualification run products described in Example 4 were used in pre-clinical studies to as described below. **Figures 6A-C** depict the results of flow cytometric analysis of surface transgene and T cell marker expression. IL13(EQ)BBy/CD19t+ TCM HD006.5 and HD187.1 were co-stained with anti-IL13-PE and anti-CD8-FITC to detect CD8+ CAR+ and CD4+ (i.e., CD8 negative) CAR+ cells (Figure 6A), or anti-CD19-PE and anti-CD4-FITC to detect CD4+ CD19t+ and CD8+ (i.e., CD4 negative) CAR+ cells (Figure 6B). IL13(EQ)BBy/CD19t+ TCM HD006.5 and HD187.1 were stained with fluorochrome-conjugated anti-CD3, TCR, CD4, CD8, CD62L and CD28 (grey histograms) or isotype controls (black histograms). (Figure 6C). In each of Figures 6A-C, the percentages indicated are based on viable lymphocytes (DAPI negative) stained above isotype.

**Example 6: Effector Activity of IL13(EQ)BBy/CD19t+ TCM**

The effector activity of IL13(EQ)BBy/CD19t+ TCM was assessed and the results of this analysis are depicted in **Figures 7A-B**. Briefly, IL13(EQ)BBy/CD19t+ TCM HD006.5 and HD187.1 were used as effectors in a 6-hour 51Cr-release assay using a 10E:1T ratio based on CD19t expression. The IL13Ra2 -positive tumor targets were K562 engineered to express IL13Ra2 (K562-IL13Ra2) and primary glioma line PBT030-2, and the IL13Ra2 -negative tumor target control was the K562 parental line (Figure 7A). IL13(EQ)BBy/CD19t+ HD006.5 and HD187.1 were evaluated for antigen-dependent cytokine production following overnight co-culture at a 10E:1T ratio with the same IL13Ra2 -positive and negative targets as described in above. Cytokine levels were measured using the Bio-Plex Pro Human Cytokine TH1/TH2 Assay kit and INF-γ levels are depicted (Figure 7B).

**Example 7: In vivo Anti-tumor Activity of IL13(EQ)BBy/CD19t+ TCM**

The studies described below demonstrate that IL13(EQ)BBy/CD19t+ TCM exhibit anti-tumor efficacy in in vivo mouse models. Specifically, we have evaluated the anti-tumor potency of IL13(EQ)BBy/CD19t+ TCM against the IL13Ra2+ primary low-passage glioblastoma tumor sphere line PBT030-2, which has been engineered to express both EGFP and firefly luciferase (ffLuc) reporter genes (PBT030-2 EGFP:ffLuc) (6). A panel
of primary lines (PBT) from patient glioblastoma specimens grown as tumor spheres (TSs) in serum-free media. These expanded TS lines exhibit stem cell-like characteristics, including expression of stem cell markers, multilineage differentiation and capacity to initiate orthotopic tumors in immunocompromised mice (NSG) at low cell numbers. The PBT030-2 EGFP:ffLuc TS-initiated xenograft model (0.1x10^6 cells; 5 day engraftment) has been previously used to evaluate in vivo anti-tumor activity in NSG mice of IL13Ra2-specific CAR expressing T cells, whereby three injections of 2x10^6 cytolytic T lymphocytes (CTLs) over a course of 2 weeks were shown to reduce tumor growth. However, in those experiments the majority of the PBT030-2 tumors eventually recurred. By comparison, a single injection of IL13(EQ)BBy/CD19t+ TCM (1.1x10^6 CAR+ TCM; 2x10^6 total TCM) exhibited robust anti-tumor activity against PBT030-2 EGFP:flLuc TS-initiated tumors (0.1x10^6 cells; 5 day engraftment) as shown in Figures 8A-C. As compared to NSG mice treated with either PBS or mock transduced TCM (no CAR), IL13(EQ)BBy/CD19t+ TCM significantly reduce flLuc flux (p < 0.001 at >18-days) and significantly improve survival (p = 0.0008).

[0083] Briefly, EGFP-flLuc+ PBT030-2 tumor cells (1x10^6) were stereotactically implanted into the right forebrain of NSG mice. On day 5, mice received either 2x10^6 IL13(EQ)BBy/CD19t+ TCM (1.1x10^6 CAR+; n=6), 2x10^6 mock TCM (no CAR; n=6) or PBS (n=6). Figure 8A depicts representative mice from each group showing relative tumor burden using Xenogen Living Image. Quantification of flLuc flux (photons/sec) shows that IL13(EQ)BBζ/CD19t+ TCM induce tumor regression as compared to mock-transduced TCM and PBS (#p<0.02, *p<0.001, repeated measures ANOVA) (Figure 8B). As shown in Figure 8C, a Kaplan Meier survival curve (n=6 per group) demonstrates significantly improved survival (p=0.0008; log-rank test) for mice treated with IL13(EQ)BBy/CD19t+ TCM.

Example 8: Comparison of IL13(EQ)BB+ Tcm and Non-Tcm IL13-zetakine CD8+ CTL Clones in Antitumor Efficacy and T cell Persistence

[0084] The studies described below compare IL13(EQ)BBζ+ Tcm and a previously created IL13Ra2-specific human CD8+ CTLs (IL13-zetakine CD8+ CTL (described in

**[0085]** A panel of primary lines (PBT) from patient glioblastoma specimens grown as tumor spheres (TSs) in serum-free media was generated (Brown et al. 2012 Clin Cancer Res 18:2199; Brown et al. 2009 Cancer Res 69:8886). These expanded TS lines exhibit stem cell-like characteristics, including expression of stem cell markers, multi-lineage differentiation and capacity to initiate orthotopic tumors in immunocompromised mice (NSG) at low cell numbers. The IL13Ra2 primary low-passage glioblastoma TS line PBT030-2, which has been engineered to express both EGFP and firefly luciferase (ffLuc) reporter genes (PBT030-2 EGFP:ffLuc) (Brown et al. 2012 Clin Cancer Res 18:2199) was used for the experiments outlined below.

**[0086]** First, a single dose (1x10⁶ CAR T cells) of IL13(Eg4BBζ+ Tcm product was compared to IL13-zetakine CD8+ CTL clones evaluated against day 8 PBT030-2 EGFP:ffuc TS-initiated xenografts (O.1x10⁶ cells). While both IL13Ra2-specific CAR T cells (IL13-zetakine CTL and IL13(Eg4BBζ Tcm) demonstrated antitumor activity against established PBT030-2 tumors as compared to untreated and mock Tcm (CAR-negative) controls (Figures 9A and 9B), IL13(EQ)BBZ+ Tcm mediated significantly improved survival and durable tumor remission with mice living >150 days as compared to our first-generation IL13-zetakine CD8+ CTL clones (Figure 9C).

**[0087]** To further compare the therapeutic effectiveness of these two IL13Ra2-CAR T cell products, a dose titration of 1.0, 0.3 and O.1x10⁶ CAR T cells against day 8 PBT030-2 EGFP:ffuc TS-initiated tumors was performed (Figures 10A-C). The highest dose (1x10⁶) of IL13-zetakine CD8+ CTL cl. 2D7 mediated antitumor responses as measured by Xenogen flux in 3 of 6 animals (Figure 10C), but no significant antitumor responses were observed at lower CAR T cell doses. By comparison, injection of IL13(Eg4BBζ+ Tcm product mediated complete tumor regression in the majority of mice at all dose levels, including treatment with as few as O.1x10⁶ CAR T cells. These data demonstrate that IL13(Eg4BBζ+ Tcm is at least 10-fold more potent than IL13-zetakine CD8+ CTL.
clones in antitumor efficacy. The improved anti-tumor efficacy of is due to improved T cell persistence in the tumor microenvironment. Evaluation of CD3+ T cells 7-days post i.e. injection revealed significant numbers of IL13(EQ)BE^+ Tcm in the tumor microenvironment, whereas very few first-generation IL13-zeta CTLs were present (Figure 11).

**Example 9: Comparison of CAR T cell delivery route for treatment of large TS-initiated PBT tumors**

[0088] Described below are studies that compare the route of delivery, intravenous (i.v.) or intracranial (i.e.), on antitumor activity against invasive primary PBT lines. In pilot studies (data not shown), it was unexpectedly observed that i.v. administered IL13(E%BBζ+ Tcm provided no therapeutic benefit as compared to PBS for the treatment of small (day 5) PBT030-2 EGFP:ffLuc tumors. This is in contrast to the robust therapeutic efficacy observed with i.e. administered CAR+ T cells. Reasoning that day 5 PBT030-2 tumors may have been too small to recruit therapeutic T cells from the periphery, a comparison was made of i.v. versus i.e. delivery against larger day 19 PBT030-2 EGFP:ffLuc tumors. For these studies, PBT030-2 engrafted mice were treated with either two i.v. infusions (5 x 10^6 CAR+ Tcm; days 19 and 26) or four i.e. infusions (1 x 10^6 CAR+ Tcm; days 19, 22, 26 and 29) of IL13(EQ)BBZ+ Tcm, or mock Tcm (no CAR). Here too no therapeutic benefit as monitored by Xenogen imaging or Kaplan-Meier survival analysis for i.v. administered CAR+ T cells (Figures 12A and 12B). In contrast, potent antitumor activity was observed for i.e. administered IL13(E%BBζ+ Tcm (Figures 12A-B). Next, brains from a cohort of mice 7 days post T cell injection were harvested and evaluated for CD3+ human T cells by IHC. Surprisingly, for mice treated i.v. with either mock Tcm or IL13(E%BBζ Tcm there were no detectable CD3+ human T cells in the tumor or in others mouse brain regions where human T cells typically reside (i.e. the leptomeninges) (Figure 12C), suggesting a deficit in tumor tropism. This is in contrast to the significant number of T cells detected in the i.e. treated mice (Figure 12D).
[0089] Tumor derived cytokines, particularly MCP-1/CCL2, are important in recruiting T cells to the tumor. Thus, PBT030-2 tumor cells were evaluated and it was found that this line produces high levels of MCP-1/CCL2 comparable to U251T cells (data not shown), a glioma line previously shown to attract i.v. administered effector CD8+ T cells to i.e. engrafted tumors. Malignant gliomas are highly invasive tumors and are often multifocal in presentation. The studies described above establish that IL13BBZ tcm can eliminate infiltrated tumors such as PBT030-2, and mediate long-term durable antitumor activity. The capacity of intracranially delivered CAR T cells to traffic to multifocal disease was also examined. For this study PBT030-2 EGFP:flLuc TSs were implanted in both the left and right hemispheres (Figure 13A) and CAR+ T cells were injected only at the right tumor site. Encouragingly, for all mice evaluated (n=3) we detected T cells by CD3 IHC 7-days post T cell infusion both at the site of injection (i.e. right tumor), as well within the tumor on the left hemisphere (Figure 13B). These findings provide evidence that CAR+ T cells are able to traffic to and infiltrate tumor foci at distant sites. Similar findings were also observed in a second tumor model using the U251T glioma cell line (data not shown).

Example 10: Comparison of Costimulatory Domains

[0090] A series of studies were conducted to evaluate various costimulatory domains. The various CAR evaluated are depicted schematically in Figure 14A and included a first generation CD3ζ CAR lacking a costimulatory domain, two second generation CARs incorporating either a 4-IBB costimulatory domain or a CD28 costimulatory domain, and a third generation CAR containing both a CD28 costimulatory domain and 4IBB costimulatory domain. All CAR constructs also contain the T2A ribosomal skip sequence and a truncated CD19 (CD19t) sequence as a marker for transduced cells.

[0091] CD4 and CD8 tcm were lentivirally transduced and CAR-expressing T cells were immunomagnetically enriched via anti-CD19. CD19 and IL13 (i.e., CAR) expression levels as measured by flow cytometry. The results are shown in Figure 14B. Stability of each CAR construct was determined by dividing the CAR (IL13) mean fluorescence intensity (MFI) by that of the transduction marker (CD19t) (Figure 14C).
The two CAR including a 4-IBB costimulatory domain exhibited the lowest expression levels as compared to the CD19t transduction marker.

[0092] The ability of the indicated mock-transduced or CAR-expressing T cells to kill IL13Ra2-expressing PBT030-2 tumor cell targets was determined in a 4-hour 51Cr-release assay at the indicated effector:target ratios. The results of this study are in Figure 15A (mean % chromium release ± S.D. of triplicate wells are depicted). As expected, mock-transduced T cells did not efficiently lyse the targets. In contrast, all CAR-expressing T cells lysed the tumor cells in a similar manner. Figure 15B depicts the results of a study in which the indicated mock-transduced or CAR-expressing T cells were co-cultured overnight with IL13Ra2-expressing PBT030-2 tumor cells at a 10:1 ratio and supernatants were analyzed for IL-13 and IFN-γ levels by cytometric bead array. Interestingly, T cells expressing the zeta, 41BB-zeta or CD28-41BB-zeta CARs exhibited lower antigen-stimulated cytokine production than T cells expressing the CD28-zeta CAR.

[0093] The in vivo efficacy of the various CAR was examined as follows. Briefly, NSG mice received an intracranial injection of flLuc+ PBT030-2 tumor cells on day 0, and were randomized into 6 groups (n = 9-10 mice per group) for i.e. treatment with either PBS (Tumor Only), mock-transduced T cells or T cells expressing the indicated IL13Ra2-specific CAR on day 8. Quantitative bioluminescence imaging was then carried out to monitor tumor growth over time. Bioluminescence images for representative mice in each group (Figure 16A). Flux levels for each mouse at Day 27 (Figure 16B). All groups treated with IL13Ra2-specific CAR T cells, except those treated with T cells expressing the CD28-CAR, show statistically-significant reduction in tumor volume compared to mice treated with mock-transduced T cells (Figure 16C).

Example 11: Amino acid Sequence of IL13(EQ)BBC/CD19t

[0094] The complete amino acid sequence of IL13(Eβ4BBζ)CD19t is depicted in Figure 17. The entire sequence (SEQ ID NO:1) includes: a 22 amino acid GMCSF signal peptide (SEQ ID NO:2), a 112 amino acid IL-13 sequence (SEQ ID NO:3; amino acid substitution E13Y shown in bold); a 229 amino acid IgG4 sequence (SEQ ID NO:4; with
amino acid substitutions L235E and N297Q shown in bold); a 22 amino acid CD4 transmembrane sequence (SEQ ID NO:5); a 42 amino acid 4-1BB sequence (SEQ ID NO:6); a 3 amino acid Gly linker; a 112 amino acid CD3ζ sequence (SEQ ID NO:7); a 24 amino acid T2A sequence (SEQ ID NO:8); and a 323 amino acid CD19t sequence (SEQ ID NO:9).

[0095] The mature chimeric antigen receptor sequence (SEQ ID NO:10) includes: a 112 amino acid IL-13 sequence (SEQ ID NO:3; amino acid substitution E13Y shown in bold); a 229 amino acid IgG4 sequence (SEQ ID NO:4; with amino acid substitutions L235E and N297Q shown in bold); at 22 amino acid CD4 sequence (SEQ ID NO:5); a 42 amino acid 4-1BB sequence (SEQ ID NO:6); a 3 amino acid Gly linker; and a 112 amino acid CD3C sequence (SEQ ID NO:7). Within this CAR sequence (SEQ ID NO:10) is the IL-13/IgG4/CD4t/41-BB sequence (SEQ ID NO:11), which includes: a 112 amino acid IL-13 sequence (SEQ ID NO:3; amino acid substitution E13Y shown in bold); a 229 amino acid IgG4 sequence (SEQ ID NO:4; with amino acid substitutions L235E and N297Q shown in bold); at 22 amino acid CD4 sequence (SEQ ID NO:5); and a 42 amino acid 4-1BB sequence (SEQ ID NO:6). The IL13/IgG4/CD4t/41-BB sequence (SEQ ID NO:11) can be joined to the 112 amino acid CD3ζ sequence (SEQ ID NO:7) by a linker such as a Gly Gly Gly linker. The CAR sequence (SEQ ID NO:10) can be preceded by a 22 amino acid GMCSF signal peptide (SEQ ID NO:2).

[0096] Figure 18 depicts a comparison of the sequences of IL13[EQ]41BBC[IL13[EQ]41BBC T2A-CD19t_epHIV7; pF02630] (SEQ ID NO:12) and CD19Rop_epHIV7 (pJ01683) (SEQ ID NO:13).

Example 12: Amino acid Sequence of IL13(EQ)BBC/CD19t

[0097] Figures 19-26 depict the amino acid sequences of additional CAR directed against IL13Ra2 in each case the various domains are labelled except for the GlyGlyGly spacer located between certain intracellular domains. Each includes human IL13 with and Glu to Tyr (SEQ ID NO:3; amino acid substitution E13Y shown in highlighted). In the expression vector used to express these CAR, the amino acid sequence expressed can include a 24 amino acid T2A sequence (SEQ ID NO:8); and a 323 amino acid CD19t
sequence (SEQ ID NO:9) to permit coordinated expression of a truncated CD19 sequence on the surface of CAR-expressing cells.

[0098] A panel of CAR comprising human IL13(E13Y) domain, a CD28 tm domain, a CD28gg costimulatory domain, a 4-1BB costimulatory domain, and a CD3ζ domain CAR backbone and including either a HL (22 amino acids) spacer, a CD8 hinge (48 amino acids) spacer, IgG4-HL-CH3 (129 amino acids) spacer or a IgG4(EQ) (229 amino acids) spacer were tested for their ability to mediate IL13Ra2-specific killing as evaluated in a 72-hour co-culture assay. With the exception of HL (22 amino acids) which appeared to have poor CAR expression in this system, all were active.
WHAT IS CLAIMED IS:

1. A nucleic acid molecule encoding a chimeric antigen receptor, wherein the chimeric antigen receptor comprises: human IL-13 or a variant thereof having 1-10 amino acid modifications; a transmembrane domain selected from: a CD4 transmembrane domain or variant thereof having 1-10 amino acid modifications, a CD8 transmembrane domain or variant thereof having 1-10 amino acid modifications, a CD28 transmembrane domain or a variant thereof having 1-10 amino acid modifications; a CD3ζ transmembrane domain or a variant thereof having 1-10 amino acid modifications; a costimulatory domain; and CD3ζ signaling domain of a variant thereof having 1-10 amino acid modifications.

2. The nucleic acid molecule of claim 1 wherein the costimulatory domain is selected from the group consisting of: a CD28 costimulatory domain or a variant thereof having 1-10 amino acid modifications, a 4IBB costimulatory domain or a variant thereof having 1-10 amino acid modifications and an OX40 costimulatory domain or a variant thereof having 1-10 amino acid modifications.

3. The nucleic acid molecule of claim 1 comprising a variant of a human IL13 having 1-10 amino acid modification that increase binding specificity for IL13Ra2 versus IL13Ral.

4. The nucleic acid molecule of claim 1 wherein the human IL-13 or variant thereof is an IL-13 variant comprising the amino acid sequence of SEQ ID NO:3 with 1 to 5 amino acid modifications, provided that the amino acid at position 11 of SEQ ID NO:3 other than E.

5. The nucleic acid molecule of claim 2 wherein the chimeric antigen receptor comprises two different costimulatory domains selected from the group consisting of: a CD28 costimulatory domain or a variant thereof having 1-10 amino acid modifications, a 4IBB costimulatory domain or a variant thereof having 1-10 amino acid modifications and an OX40 costimulatory domain or a variant thereof having 1-10 amino acid modifications.
6. The nucleic acid molecule of claim 5 wherein the chimeric antigen receptor comprises two different costimulatory domains selected from the group consisting of: a CD28 costimulatory domain or a variant thereof having 1-2 amino acid modifications, a 4IBB costimulatory domain or a variant thereof having 1-2 amino acid modifications and an OX40 costimulatory domain or a variant thereof having 1-2 amino acid modifications.

7. The nucleic acid molecule of claim 1 wherein the chimeric antigen receptor comprises: human IL-13 or a variant thereof having 1-2 amino acid modifications; a transmembrane domain selected from: a CD4 transmembrane domain or variant thereof having 1-2 amino acid modifications, a CD8 transmembrane domain or variant thereof having 1-2 amino acid modifications, a CD28 transmembrane domain or a variant thereof having 1-2 amino acid modifications, and a CD3ζ transmembrane domain or a variant thereof having 1-2 amino acid modifications; a costimulatory domain; and CD3ζ signaling domain of a variant thereof having 1-2 amino acid modifications.

8. The nucleic acid molecule of claim 1 comprising a spacer region located between the IL-13 or variant thereof and the transmembrane domain.

9. The nucleic acid molecule of claim 6 wherein the spacer region comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 4, 14-20, 50 and 521.

10. The nucleic acid molecule of claim 6 wherein the spacer comprises an IgG hinge region.

11. The nucleic acid molecule of claim 6 wherein the spacer comprises 10-150 amino acids.

12. The nucleic acid molecule of claim 2 wherein the 4-IBB signaling domain comprises the amino acid sequence of SEQ ID NO:6.

13. The nucleic acid molecule of claim 1 wherein the CD3ζ signaling domain comprises the amino acid sequence of SEQ ID NO:7.
14. The nucleic acid molecule of claim 1 wherein a linker of 3 to 15 amino acids is located between the costimulatory domain and the CD3 ζ signaling domain or variant thereof.

15. The nucleic acid molecule of claim 1 wherein the nucleic acid molecule expresses a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 10, 31-48 and 52.

16. The nucleic acid molecule of claim 1 wherein the chimeric antigen receptor comprises a IL-13/IgG4/CD4t/41-BB region comprising the amino acid of SEQ ID NO: 11 and a CD3 ζ signaling domain comprising the amino acid sequence of SEQ ID NO:7.

17. The nucleic acid molecule of claim 14 wherein the chimeric antigen receptor comprises the amino acid sequence of SEQ ID NOs: 10, 31-48 and 52.

18. A population of human T cells transduced by a vector comprising an expression cassette encoding a chimeric antigen receptor, wherein chimeric antigen receptor comprises: human IL-13 or a variant thereof having 1-10 amino acid modifications; a transmembrane domain selected from: a CD4 transmembrane domain or variant thereof having 1-10 amino acid modifications, a CD8 transmembrane domain or variant thereof having 1-10 amino acid modifications, a CD28 transmembrane domain or a variant thereof having 1-10 amino acid modifications, and a CD3ζ transmembrane domain or a variant thereof having 1-10 amino acid modifications; a costimulatory domain; and CD3 ζ signaling domain of a variant thereof having 1-10 amino acid modifications.

19. A population of human T cells comprising a vector expressing a chimeric antigen receptor comprising an amino acid sequence selected from SEQ ID NOs: 10, 31-48 and 52.

20. The population of human T cells of claim 16 wherein the T cells are comprised of a population of central memory T cells.
21. A method of treating cancer in a patient comprising administering a population of autologous or allogeneic human T cells transduced by a vector comprising an expression cassette encoding a chimeric antigen receptor, wherein chimeric antigen receptor comprises an amino acid sequence selected from SEQ ID NOs: 10, 31-48 and 52.

22. The method of claim 19 wherein the population of human T cells comprise central memory T cells.

23. The method claim 19 wherein the cancer is glioblastoma.

24. The method of claim 20 wherein the transduced human T cells where prepared by a method comprising obtaining T cells from the patient, treating the T cells to isolate central memory T cells, and transducing at least a portion of the central memory cells to with a viral vector comprising an expression cassette encoding a chimeric antigen receptor, wherein chimeric antigen receptor comprises an amino acid sequence selected from SEQ ID NOs: 10, 31-48 and 52.

25. A nucleic acid molecule encoding an polypeptide comprising an amino acid sequence that is at least 95% identical to an amino acid sequence selected from SEQ ID NO:10 and SEQ ID NOs: 10, 31-48 and 52.

26. A nucleic acid molecule encoding an polypeptide comprising an amino acid sequence that is identical to an amino acid sequence selected from SEQ ID NO: 10, 31-48 and 52 except for the presence of no more than 5 amino acid substitutions, deletions or insertions.

27. A nucleic acid molecule encoding an polypeptide comprising an amino acid sequence that is identical to an amino acid sequence selected from SEQ ID NO: 10 and SEQ ID NOs: 10, 31-48 and 52 except for the presence of no more than 5 amino acid substitutions.

28. A nucleic acid molecule encoding an polypeptide comprising an amino acid sequence that is identical to an amino acid sequence selected from SEQ ID NO: 10 and
SEQ ID NOs: 10, 31-48 and 52 except for the presence of no more than 2 amino acid substitutions.
FIGURE 2

A

B

C

IL13(EQ)B8Z-T2ACD19t

IL13(EQ)B8Z-T2ACD19t

IL13(EQ)B8Z-T2ACD19t

3'}
FIGURE 3

Step 1 & 2  \( p^653RSN \)

\[
\begin{array}{cccccc}
SV & CMV & LTR & RRE & SL3 & Neo & LTR \\
\end{array}
\]

Step 3  \( pHIV-3 \)

\[
\begin{array}{cccccc}
SV & CMV & LTR & RRE & LTR & \triangle \\
\end{array}
\]

+ \( p^3HIV-4 \)

\[
\begin{array}{cccccc}
SV & CMV & LTR & RRE & WPRE & LTR & \triangle \\
\end{array}
\]

Step 4 & 5  \( pHIV-6 \)

\[
\begin{array}{cccccc}
SV & CMV & LTR & RRE & WPRE & LTR & \triangle \\
\end{array}
\]

+ \( cSPT Fesp \)

Step 6  \( pHIV-7 \)

\[
\begin{array}{cccccc}
SV & CMV & LTR & RRE & WPRE & LTR & \triangle \\
\end{array}
\]
FIGURE 5

Day - Bioprocess:

1 - Leukapheresis

2 - ClinMACSTM Selection; Option to Freeze Cells; CD3/CD28 Stimulation

Select for CD62L+ using biotin-Dreg56 and anti-biotin microbeads

Stimulate with CD3/CD28 Dynabeads®

Lentitransduce with IL13(EQ)BBZ-T2A-CD19t_epHIV7

Expand with IL-2/IL-15

IL13(EQ)BB(1/CD19t+ TCM in cGMP Vuflife Bag

2-5 - Lentiviral Transduction; Initiate Expansion

7+ - Dynabead® Removal

14+ - Cryopreservation
FIGURE 6

A

HD006.5
HD187.1

CD8-FITC
14%  11%
6%   7%

IL13-PE (CAR)

CD4-PE
9%   13%
5%   12%

CD19-PE

B

C

CD3  TCR  CD4  CD8  CD62L  CD28

HD006.5
100%  85%  24%  76%  80%   95%

HD187.1
100%  85%  97%  63%  85%   95%
FIGURE 7

A

% Specific Lysis

K562
K562-IL13Rα2
PBT030-2

HD006.5
HD187.1

> 9x
> 3x

B

INF-γ (pg/ml)

HD006.5
HD187.1
FIGURE 8

A

<table>
<thead>
<tr>
<th></th>
<th>D4</th>
<th>D8</th>
<th>D11</th>
<th>D14</th>
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<th>D26</th>
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<td><img src="image4" alt="Image" /></td>
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<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
</tr>
<tr>
<td>Mock Tcm</td>
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B

C

% Survival

![Graph](image22)
**FIGURE 9**
FIGURE 12

A

B

C

10x HE 10x CD3-DAB

D

10x HE 10x CD3-DAB
FIGURE 17

MLLLVTLLLCELPHAPFALLPGPVPPSTALRYLIEELVNITQONQKAPLCNGSMVWSINLTA

GMCSFRa signal peptide (22 aa) IL13 (112 aa)

YCAALESINVSGCIAEKTQRMLSGFCPHKVSAGQESSLHVREITKEVAQFVKDDLHLLKLF

REGRENESKYGPCCPCPACPAPEFEGGPSVFLFPKDPKDTLMISRTPEVTCVVDVQEDPEVQF

IgG4(L235E, N297Q in bold) (229 aa)

NWYVGDGEVHNAKTPREEQFQSTYRVSVLTLHQQDLNMGKEYKCVSNKGLPSSIEKTIS

KAKGQPREPQVYTLLPSQEMTKONQVSLTCLVKGYPSDIAVEWESNGQPENNYKTPPVL

DSDGSFLYSLTDKVQEGNVFSCSMHEALHNHYTQKSLSLGKMALIVLGGVAGLI

CD4tm (22 aa)

LFIGLGFFKRGRRKLLYIFKQPFMRPVQTQEDGCSRFPEEEEGGCELGGGRVKSRSADA

41BB (42 aa) Gly3 Zeta (112 aa)

PAYQQGQNYNLNLGRREEDVLDKRRGRDPEMGGKPRRNQEOGLYNELQDKMAE

AYSEIGMKAEERRRKGHDGLYQQLSTATKDTYDALHMQALPPRELLEGGGEGRSGSLTCDGV

T2A (24 aa)

FEENPGRMPPPRLLLFLTPEMVRPEEPVLVKEEDGNAVQLKGTSDGPTQQLTWSRE

CD19t (323 aa)
SPLKFLKLGLPGLGILMRIPLAIFFNVSQ MQGGFYLCQP PSEQ KAWQP GWT VNVE

GSGELFRWNVSDLGGGLGCGLKNSSEGPPSSP GSGLMP KLYVWAK DRPEIWE GEP PCV PR

DSLNSLSQDLTMAPGSTLWLSGVPD SVRGPL SWTHVHPKGPKSLLSL EKL DRPARD

MWVMTG LLLPRATAQDAGKYYCHRGNLT MSFHEITARPVLWHWLLRTGGWKVS AVT L

AYLIFCLCSLGILHQLR A L LVRRKR
FIGURE 18

Yellow highlighting indicates the IL-13 optimized codon region including the GMCSF signal sequence (IL13op).

Blue highlighting indicates the IgG4 optimized codon region (IgG4op[L235E, N297Q]).

Green highlighting indicates the two anticipated amino acid changes within the IgG4 hinge region (L235E and N297Q).

Turquoise highlighting indicates the CD4 transmembrane optimized codon region.

Yellow highlighting indicates the 41BB cytoplasmic signaling region (41BB cyto).

CTC highlighting indicates the 3 glycine linkers (g3).

Gray Highlighting indicates the CD3 zeta optimized codon region (zeta op).

Gray highlighting indicates the T2A sequence (T2A).

Gray highlighting Indicates the truncated CD19 sequence (CD19t).

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CD19Rop_1pHIV7  (1301) GTACCTCAACATCTTATTTAAGAAAGAACGTGGGAGTTGGGATAGCTGC
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CD19Rop_1pHIV7  (1401) TACAAAAACAAATTTAAAATTTATCTTTGCTGCTTATTACAGGGGAC
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Consensus     (1651) AAGTGATGCTGCTGATCGCTGCCTGCCTTTTTCCTGAGGATGGGGGAAGAC
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CD19Rop_1pHIV7  (1751) GCCCCGCAACAGCAGCTGAGATGCTGCTGCCTGCCTTTTTCCTGAGGATGGGGGAAGAC
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CD19Rop_1pHIV7  (1801) GCGCCGCCTACACCTGAGGGGCGGCGACAGCGGCTTGGACTGCGGCTTC

| CD19Rop_epHIV7 | (2501) AGCGGCAGGGGCAGACCCAGAACGAGGCGTGAACAGGAAAGCGGCCCAGACG | (2501) | 2551 | 2600 |
| IL13(EQ) 41BB2eta | (2334) ------------------------CCCAAGG------------------------ | | | |
| CD19Rop_epHIV7 | (2551) TGGCCTGTGGGCCCCGACACAGAGCTACTGACGAGCTGACCCAGGCGGCGG | (2551) | 2561 | 2650 |
| Consensus | (2551) CCGACGC | | | |
| IL13(EQ) 41BB2eta | (2341) --------GATGCTGCTCCGCCGCTTCTGC---------------CCCAACAGG | | | |
| CD19Rop_epHIV7 | (2601) GCTGTAGCCCTGCGCCGACCTACGGCCGTACGCTGACCCAGGCGGCGG | (2601) | 2651 | 2700 |
| Consensus | (2601) GA CTG CCG CT GC CCC | | | |
| IL13(EQ) 41BB2eta | (2369) -------------------------------------------------GTGTCGCCGGGAC--------AGTT | | | |
| CD19Rop_epHIV7 | (2651) AAGGCGCTGGAAATGGCTGGGGCGTACGCTGACCCAGGCGGCGG | (2651) | 2701 | 2750 |
| Consensus | (2651) G G C GC GAC A T AC | | | |
| IL13(EQ) 41BB2eta | (2386) CAGGACGCTTCGACGCTGGG-----------------------------ACACGAGA | | | |
| CD19Rop_epHIV7 | (2701) CAACAGGCTGGCAGACGACGACGAGCTGACCCAGGCGGCGG | (2701) | 2751 | 2800 |
| Consensus | (2701) CA CGC A G G GC G ACA | | | |
| IL13(EQ) 41BB2eta | (2415) TCGAGGTGGCCCCATCTGGAAGGCCCTGCTCGTC------------------C | | | |
| CD19Rop_epHIV7 | (2751) GCCAGGTGCTCTGTGACGACTGACGACGAGCTGACCCAGGCGGCGG | (2751) | 2801 | 2850 |
| Consensus | (2751) C AGT G CC T GA A CCGC C TCG GC C | | | |
| IL13(EQ) 41BB2eta | (2448) TGCACCTGTGAAA-----------------------------GCTGTCTCGG------GGA--- | | | |
| CD19Rop_epHIV7 | (2801) TACTACTGCGCAAGCAGTACTACTAGCTGACCGCGGACCTGACCCATGACTA | (2801) | 2851 | 2900 |
| Consensus | (2801) T C CTG AAG A GC G T C G G AA | | | |
| IL13(EQ) 41BB2eta | (2473) ---GGGCGGGTCTCAAC---------------- | | | |
| CD19Rop_epHIV7 | (2851) CTGGGGCCAGGCGACAGCAGCTGACGCTGACCCAGGGAGGCAATGACCGGCC | (2851) | 2901 | 2950 |
| Consensus | (2851) GGGCC G CA C GAGAGCAAGTACGGGCC | | | |
| IL13(EQ) 41BB2eta | (2502) | | | |
| CD19Rop_epHIV7 | (2901) CTCCTGCCCCCCTTGGCCTGGGCCCGAGTTCTGTCGCGGACCAGGCGG | (2901) | 2951 | 3000 |
| Consensus | (2901) CTCCTGCGCCCCCCTTGGCCTGGGCCCGAGTTCTGTCGCGGACCAGGCGG | | | |
| IL13(EQ) 41BB2eta | (2552) | | | |
| CD19Rop_epHIV7 | (2951) TTTCTGTTCCCCCAGGCGAAGAGCAGCTATGATGATCACGGGAGGACC | (2951) | 3001 | 3050 |
| Consensus | (2951) TTTCTGTTCCCCCAGGCGAAGAGCAGCTATGATGATCACGGGAGGACC | | | |
| IL13(EQ) 41BB2eta | (2602) | | | |
| CD19Rop_epHIV7 | (3001) GAGGTGAGCCTGCTGGTGGTGGTGCTGACGCTGACCCAGGGAGATCGTCCG | (3001) | 3051 | 3100 |
| Consensus | (3001) GAGGTGAGCCTGCTGGTGGTGGTGCTGACGCTGACCCAGGGAGATCGTCCG | | | |
| IL13(EQ) 41BB2eta | (2652) | | | |
| CD19Rop_epHIV7 | (3051) AGTTCACCTCTGTGAGCTGACGCGCAGGGGTGCTGACCCAGGACAGCAACCG | (3051) | 3101 | 3150 |
| Consensus | (3051) AGTTCACCTCTGTGAGCTGACGCGCAGGGGTGCTGACCCAGGACAGCAACCG | | | |
| IL13(EQ) 41BB2eta | (2702) | | | |
| CD19Rop_epHIV7 | (3101) GCCGGGAAAGGAGACATTCCACAGCTACCTACCGGGTTGGTGCTGAGTGCGACTGAC | (3101) | 3151 | 3200 |
| Consensus | (3101) GCCGGGAAAGGAGACATTCCACAGCTACCTACCGGGTTGGTGCTGAGTGCGACTGAC | | | |
| IL13(EQ) 41BB2eta | (2752) | | | |
| CD19Rop_epHIV7 | (3151) CCGGCTGCACCAGGACTGCGGGCTGAAACGCCCAAAAGSATCAACATGCAGGACGGTT | (3151) | 3201 | 3250 |
| Consensus | (3151) CCGGCTGCACCAGGACTGCGGGCTGAAACGCCCAAAAGSATCAACATGCAGGACGGTT | | | |
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Consensus (3201) CCAAAGGGCTCCAGCGAGATCGAGAAACCACATCGCAAGCCAG
3251 3300

IL13 (E3) 41BBZeta (2852)
CD19Rop_epHIV7 (3251) GGCCAGCGGCTGGAGGCCCCAGGTTGATACCCCTGCCCCTGCCAGGAAGAGA
Consensus (3251) GGCCAGCGGCTGGAGGCCCCAGGTTGATACCCCTGCCCCTGCCAGGAAGAGA
3301 3350

IL13 (E3) 41BBZeta (2902)
CD19Rop_epHIV7 (3301) GATGACCAAAATACAGGTCTCCTGTACCTGCTGTTAGGCTTTCTACC
Consensus (3301) GATGACCAAAATACAGGTCTCCTGTACCTGCTGTTAGGCTTTCTACC
3331 3400

IL13 (E3) 41BBZeta (2952)
CD19Rop_epHIV7 (3351) CCAGCGACATCGCCGCTGTTGAGGGAGGAGAACCGGAGAGACCAACAC
Consensus (3351) CCAGCGACATCGCCGCTGTTGAGGGAGGAGAACCGGAGAGACCAACAC
3401 3450

IL13 (E3) 41BBZeta (3002)
CD19Rop_epHIV7 (3401) TACAGACACACCCCGCTGCTGAGCAGAGAGCCGGCTCTCTTCTGCTTA
Consensus (3401) TACAGACACACCCCGCTGCTGAGCAGAGAGCCGGCTCTCTTCTGCTTA
3451 3500

IL13 (E3) 41BBZeta (3052)
CD19Rop_epHIV7 (3451) CAGCGAGCTGAGCTGGAGGAGGAGAACCGGAGAGACCAACAC
Consensus (3451) CAGCGAGCTGAGCTGGAGGAGGAGAACCGGAGAGACCAACAC
3501 3550

IL13 (E3) 41BBZeta (3102)
CD19Rop_epHIV7 (3501) GCTGACCGGTAGTGACAGAGAAGGAGAACCGGAGAGACCAACAC
Consensus (3501) GCTGACCGGTAGTGACAGAGAAGGAGAACCGGAGAGACCAACAC
3551 3600

IL13 (E3) 41BBZeta (3152)
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Consensus (3601) CCGGCTGCTGTCTTATCGGCGCGCTGCTTCTTTCTTTTTCT
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Consensus (3651) C--------------------------C
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Consensus (3701) -----------------------------------------------
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CD19Rop_epHIV7 (3850) -----------------------------------------------
Consensus (3850) -----------------------------------------------
3900 3950

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CD19Rop_epHIV7 (3900) -----------------------------------------------
Consensus (3900) -----------------------------------------------
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CD19Rop_epHIV7 (3766) TGAAGATGGCGGCAAGCCTTCGGCAGAAGAAAGAGAAGGCTGTATA
Consensus (3901) TGAAGATGGCGGCAAGCCTTCGGCAGAAGAAAGAGAAGGCTGTATA
3951 3950

IL13 (EQ) 41BBZeta (3552) ACGAATCTGCAAGAAGAAAGAAATGAGCCGGCAGCTACAGCAGATGCGCATG
CD19Rop_epHIV7 (3816) ACGAATCTGCAAGAAGAAAGAAATGAGCCGGCAGCTACAGCAGATGCGCATG
Consensus (3951) ACGAATCTGCAAGAAGAAAGAAATGAGCCGGCAGCTACAGCAGATGCGCATG
4001 4050

IL13 (EQ) 41BBZeta (3602) AAGGGCGAGCGGCGGGGCAAGGGCCAGACGAGCTGTATCGGGCCT
CD19Rop_epHIV7 (3866) AAGGGCGAGCGGCGGGGCAAGGGCCAGACGAGCTGTATCGGGCCT
Consensus (4001) AAGGGCGAGCGGCGGGGCAAGGGCCAGACGAGCTGTATCGGGCCT
4051 4100

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CD19Rop_epHIV7 (3916) GACGACCTAAGGACAGGAGTAACAGCTACAGCAGATGCGCATG
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**SUBSTITUTE SHEET (RULE 26)**
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CD19Rop_epHIV7 (8219) GCCCAACGCACCCCCGCGCCATTGACGTAATAATGACGTATGTTCCCATCG
Consensus (9401) GCCCAACGACCCCCGCGCCATTGACGTAATAATGACGTATGTTCCCATCG 9451 9500

IL13 (EQ) 41BBZeta (9052) TAAAGCCCAATAGGACTTTCTCCATTGACTGCTAATGGTGGAAATTTTCCCG
CD19Rop_epHIV7 (8269) TAAAGCCCAATAGGACTTTCTCCATTGACTGCTAATGGTGGAAATTTTCCCG
Consensus (9451) TAAAGCCCAATAGGACTTTCTCCATTGACTGCTAATGGTGGAAATTTTCCCG 9501 9550

IL13 (EQ) 41BBZeta (9102) TAAAACTGCCACCTGGCAGTACATCAACACGTAAATGACGTATGTTCCCATCGC
CD19Rop_epHIV7 (8319) TAAAACTGCCACCTGGCAGTACATCAACACGTAAATGACGTATGTTCCCATCGC
Consensus (9501) TAAAACTGCCACCTGGCAGTACATCAACACGTAAATGACGTATGTTCCCATCGC 9551 9600

IL13 (EQ) 41BBZeta (9152) CTTATTTGAGCTCTGCAATGCGTTAAAATGACGTATGTTCCCATCGC
CD19Rop_epHIV7 (8369) CTTATTTGAGCTCTGCAATGCGTTAAAATGACGTATGTTCCCATCGC
Consensus (9551) CTTATTTGAGCTCTGCAATGCGTTAAAATGACGTATGTTCCCATCGC 9601 9650

IL13 (EQ) 41BBZeta (9202) ACATGACCTTTATGGGATCTTCTACTGAGCAGTACTACTGATTAGTC
CD19Rop_epHIV7 (8419) ACATGACCTTTATGGGATCTTCTACTGAGCAGTACTACTGATTAGTC
Consensus (9601) ACATGACCTTTATGGGATCTTCTACTGAGCAGTACTACTGATTAGTC 9651 9700

IL13 (EQ) 41BBZeta (9252) ATGCCTTACTACACAGTGGGATTCCCAGTGCTAGCTCAATTGACGTATGTTCCCATCGC
CD19Rop_epHIV7 (8469) ATGCCTTACTACACAGTGGGATTCCCAGTGCTAGCTCAATTGACGTATGTTCCCATCGC
Consensus (9651) ATGCCTTACTACACAGTGGGATTCCCAGTGCTAGCTCAATTGACGTATGTTCCCATCGC 9701 9750

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CD19Rop_epHIV7 (8519) ATAGAGCTTAATGAACCCCGCTGTTCCACCCCTACATTGAGCTCA
Consensus (9701) ATAGAGCTTAATGAACCCCGCTGTTCCACCCCTACATTGAGCTCA 9751 9800

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CD19Rop_epHIV7 (8569) ATGGGAGTTTTGTTGGCAACAAAATATACACGGGACTTTCTCAAAATGTGC
Consensus (9751) ATGGGAGTTTTGTTGGCAACAAAATATACACGGGACTTTCTCAAAATGTGC 9801 9850

IL13 (EQ) 41BBZeta (9402) AACAAATCTCGCCCGATAGCAGCAATGCGGCTAGGCTAGGCTAGGCATTTC
CD19Rop_epHIV7 (8619) AACAAATCTCGCCCGATAGCAGCAATGCGGCTAGGCTAGGCTAGGCATTTC
Consensus (9801) AACAAATCTCGCCCGATAGCAGCAATGCGGCTAGGCTAGGCTAGGCATTTC 9851 9900

IL13 (EQ) 41BBZeta (9452) GAGAGTGTCGGGAAGCCCCTAGCTCTAGTCTGTACTTACACGCTGCTTTTTGCTCGT
CD19Rop_epHIV7 (8669) GAGAGTGTCGGGAAGCCCCTAGCTCTAGTCTGTACTTACACGCTGCTTTTTGCTCGT
Consensus (9851) GAGAGTGTCGGGAAGCCCCTAGCTCTAGTCTGTACTTACACGCTGCTTTTTGCTCGT 9901 9914

IL13 (EQ) 41BBZeta (9502) ACTGGGTTCCTCTCTG
CD19Rop_epHIV7 (8719) ACTGGGTTCCTCTCTG
Consensus (9901) ACTGGGTTCCTCTCTG
FIGURE 19

IL13(EmY)-CD8h3-CD8tm2-41BB-Zeta

MLLVTSLLLCELPHPAFLLIPGPVPPSTALRYLEELVNITQNKAPLNGSMVWSINLTAGM

GMCSFRa signal peptide

YCAALESLINVSGCSAEKTQRMLSGFCPHKVSAQFSSLHVRDTPKEVAQFVKDLLLHLKKLF

REGRFNKPTTTPAPRPPTAPTIASQPLSLRPEACRPAAAGGAVHTRGLDFACDIYIWPALAG

CD8hinge (48 aa) CD8tm(2)

TCGVLLSSLVITLYKRGRKKLLYIFKOPFMRPVQTTQEEDGCSCRFPREEEGGCELLGGRVKFS

4-1BB cyto

RSADAPAYQQGQNQLYNELNLGRREEYDVLDKKRRGRDPEMGGKPRRPKNPQEGLYNELQK

DKMAEAYSEIGMKGERRRGKGDGLYQGLSTATKDTYDALHMQLPPR

GMCSFRa signal peptide

IL13(EmY)

CD8hinge

CD8 transmembrane (2)

4-1BB cyto

(Gly)3

Zeta
FIGURE 20

IL13(EmY)-CD8h3-CD28tm-CD28gg-41BB-Zeta

MLLLVTLLCPELPAPAFLLIPGPVPPSTALRYIEELVNITQNKAPLCNQSMVWSINLTAGM
GMCSFRα signal peptide IL13(EmY)

YCAALESLINVSQGSCASEI KTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVVKDLLHLKLF
REGRFNAKPTTTTPAPRPPTPAPIASQPLSLRPEACRPAAAGGAVHTRLDFACDFWVVLVVVG
CD8 hinge (48 aa)
CD28tm

GVLACYSLLVTVAIFWVRSKRSGRGHDYMNMTPRPQRPRKHYQPYPYAPPRDFAAYRS
CD28gg

GGKRGRKLLYIFKQPFMRPQQTTQEEDEGCSCRFPEEEEEGGCELGGGRVFKSRSDAPAYQ
4-1BB cyto CD3ζ

QGQNQLYNELNLGRFEYDVLKDKEEGRDRPEMGKPPRRKNPQEQELNYNQKDKMAEAYSEI
GMKGERRRRGKGDGLYQQLSTATKDTYDALHMQALPR

GMCSFRα signal peptide
IL13(EmY)
CD8hinge
CD28 transmembrane
CD28gg
4-1BB cyto
(Gly)3
Zeta
FIGURE 21

IL13(EmY)-IgG4(HL-CH3)-CD4tm-41BB-Zeta

MLLVTSLLCPELPHPAFLIPPGVPVPSTALRYLIEELVNITQNKAPLCNGSMVWSINLTAGM
GMCSFRa signal peptide IL13(EmY)

YCAALESLINVSAGSAEKTQRMLSGFCPHKVSAQGFSSLHVVRDTKIEVAQFVDLLLLHLKLF

REGRFNESKYGPPCPGCCCGGGSSGSSGGGQPREPOPVYTLPPSQEEMTKNQVSLTCLVKGY
IgG4Hinge Linker IgG4-CH3

PSDIAVEWESNGQPENNYKTPPVLDSGSFFLYSRTLTDKSWQEGNVFSCSVMHEALHN

HYTQKSLSLGLDKMALIVLGGVAGLLEEFGKFGRKRLYIKQOPFMRPVQTQTEEDGCS
CD4 tm 4-1BB cyto

CRFPEEEGGCELGGRVKRSADAPAYQGOGQNNLNEMLNLGRREEYDVLKRRGRDPE
CD3ζ

MGKPQRRKNPQEGLYNELQKDAAEAYSEGKMGGERRRGGKHDGLYGQLSTATKDTYDA
LHMQALPPR

GMCSFRa signal peptide
IL13(EmY)
IgG4Hinge
Linker
IgG4-Fc-CH3
CD4 transmembrane
4-1BB cyto
(Gly)3
Zeta
FIGURE 22

IL13(EmY)-IgG4(L235E,N297Q)-CD8 tm-41BB-Zeta

MLLVTSLLLCELPHAPAFLLPGVPVPPSTALRYLIEELVNITQNKAPLCNGSMVWSDINLTAGM
GMCSFRα signal peptide  IL13(EmY)

YCAALESLINVSGLCSAIKETQRMLSGFCPHKVSAGQFSSLHVREDTHKIEVAQFVKDLMLHLKLF

REGRFNEKYGPPCPFPAPAPEFEGGPSVFLFPPKPDKTLMISRTPEVTCVVDVSDQEDPEVQF
IgG4-Fc(SmP)

NWYVDGVEVHNAKTKPREEQFQSTYRVSVTVLHQQWDLNGKEYKCKVSNKLPGSSIEKTIS

KAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKFYPDIAVEWESNGQPENNYKTPPVL

DSDGSFFLYLRTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGKIYIWPAPLAGTCGV
CD8 tm

LLLVTITKGRKLLYIFKQFMRPVQQTQEDGCSCRFPEEEEGGCELGGGRVKSRSADAP
4-1BB cyto  CD3ζ

AYQQGQQNQLYNELNLGRREEYDVLKDQRRGDPEMGKPRKNPQEGLYNELQKDQMAEA

YSEIGMKGERRGKGDYQGLSTATKDTYDHALHMQALPPR

GMCSFRα signal peptide
IL13(EmY)
IgG4-Fc(SmP)
CD8 transmembrane
4-1BB cyto
(Gly)3
Zeta
FIGURE 23

IL13(EmY)-Linker-CD28tm-CD28gg-41BB-Zeta

MLLVTSLLLCELPHPAFLLPPIGVPPSTALRYLIEELVNITQNKAPLCNGSMVWSINLTAGM
GMCSFRA signal peptide  IL13(EmY)

YCAALESLINVSAGCSEIAEKTRQRLMSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLHLKKLF

REGRFGGGSSGGSSGGMFVVLVVGVLACYSLLVTVAFIIFVWVRSKRSRGGHDYMNMM
  Linker  CD28(M) tm  CD28gg

TPRRPGTRKHYQPYAPPRDFAAYRSGGGRKRKKLLYIFKQPFMRPQVOTTQEDGCSRFP
  4-1BB cyto

EEECCGCELGGVRKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPERMGGK
  CD3ζ

PRKNPQEGLYNELQDKMAFAYSEIGMKGRRRGKGDGLYQGGLSTATKDTYDALMHO

ALPPR

GMCSFRA signal peptide
IL13(EmY)
Linker
CD28(M) transmembrane
CD28gg
4-1BB cyto
(Gly)3
Zeta
FIGURE 24

IL13(EmY)-HL-CD28m-CD28gg-41BB-Zeta

MLLVTSLLCELPHPAFLILPGVPVPPSTALRYLIEELVNITQNKAPLCNGSMVWSINLTAGM
GMCSFRa signal peptide   IL13(EmY)

YCAALESLINVSAGSAIEKTQRMLSGFCPHKVSAGQFSSLHVHDRTKIEVAFQVFVDLLLHLKLF

REGRFNESKYGPPCPGCCPGGSSGGGGGGMFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRS
IgG4Hinge   Linker   CD28(M) tm

CD28gg

RGGHSDYMNMTPRPGPTRKYQYAPPDPDFAAYRSGGGRGKRKGLLYIFKQFMRPVQT
4-1BB cyto

TQEEDGCSCRFPEEEEGGCGLGGRVFSRSADAPAYQGQGNQLYNELNLGRREEYDVLDK
CD3z

RRGRDPMEGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMGERRRGRKGHDGLYQGLSTA

TKDTYDALHMQALPPR

GMCSFRa signal peptide
IL13(EmY)
IgG4Hinge
Linker
CD28(M) transmembrane
CD28gg
4-1BB cyto
(Gly)3
Zeta
**Figure 25**

IL13(EmY)-IgG4(HL-CH3)-CD28tm-CD28gg-41BB-Zeta

MLLVTSLLLCELPHAPFLIPGPVPPEALRYLIEELVNITQNQKAPLCNGSMVWSINLTAGM
GMCSFRα signal peptide  IL13(EmY)

YCAALESLINVSGCSAIKTQRMGLSGFCPHVSAGQFSSLHVHRDKIEVAQFVKDLLHLKLKF

REGRFESKYGPCCPICPGGGSSGGGGGQPREPOVYTLPPSQQEMTKNQVSLTCLVKGFY
IgG4Hinge  Linker  IgG4 CH3

PSDIAVEWESNQOPENNYKTTPVLDSDGFLYSLTVDKSRWQEGNVFSCSMARTLHN

HYTQKSLSSLGLKMFWVLVVGVLACYSLLVTVAIFIWVRSKRSSRGHSDYMNMTPRRP
CD28(M) tm  CD28gg

GPTKHYQPYAPPDFAAYRSGGGKKRGKLLYIFKQPMPFVQTQTEDDGCSRFPEEEEG
4-1BB cyto

GCELGGGRVKFSGSADAPAYQQGQNQLYNEELNLGRREEYDVLKORGRDPEMGKKPRRK
CD3ζ

NPQEGLYNELQDKMAEAYSEIGMKGREERRGGKHGHDGLYQGLSTAKDTYDALHMQALPP

R

GMCSFRα signal peptide
IL13(EmY)
IgG4Hinge
Linker
IgG4 CH3
CD28 transmembrane
CD28gg
4-1BB cyto
(Gly)3
Zeta
FIGURE 26

IL13(EmY)-IgG4(L235E,N297Q)-CD28tm-CD28gg-41BB-Zeta

GMCSFRa signal peptide

MLLLVTSLCELPHPALFWPGPVPSTALRYLIEELVNITQNQKAPLCNGSMVWSINLTAGM

IL13(EmY)

YCAALESINVSIGSAIEKTRQLSAGFCHVVSAGQFSSLHVRDGTKIEVAQFVKDLLLLHKLKLF

REGRFNEQSKYGPPCPACPAPCPGGSVFLFPKPDKTMISRTPEVTCVVVDVSQEDPEVQF

IgG4-Fc(L235E,N297Q)

NWYVVDGVEVHNAKTPIEEQFQSTFYRVSTVHDQWDLNGKKEYKCKVSNKGLPSSIEKTIS

KAKGQPREFPQVYTLPPSQEEEMTKQVSLTCLVGFYPFSDAVEWESNGQPENNYKTPPPVL

DSDGSSFLYSLTVDKSRWQEGNVFSCSVMHEALHNYTQKSLSLGKMFVWLVVGGV

CD28(M) tm

LACYSSLTVAFIIFWVRSKRSRGHSDYMNNTPRRPGTPRKHQRPYPAPPDRFAAYRSGGG

CD28gg

KRGRKKLLYIFKQFMRPVQTTQEDGCSCRFPEEEGGEELGGGRVKFSRSADAPAYQOG

4-1BB cyto

CD3ξ

QNLQYELNQLGRREEYDIVLKDRRGRDPEMGGGKPRKNPQEGLYNELQKDMAEAYSEIG

MKGERRGGKHGHDGLYQQLSTATKDTYDALHMQALPPR

GMCSFRa signal peptide

IL13(EmY)

IgG4-Fc(L235E,N297Q)

CD28 (M) transmembrane

CD28gg

(Gly)3

4-1BB cyto

(Gly)3

Zeta
FIGURE 27

IL13(EmY)-CD8h3-CD8tm-41BB-Zeta

MLLVTLVCCCELPHPAFFLIPGPVPPSTALRYLIEELVNTQNQKAPLCNGSMVWSINLTAGM
GMCSFRa signal peptide  IL13(EmY)

YCAALESLINVSGCASAEKTQRMRSFHCPSAGQFSSLHVRDTKIEVAQFVKDLLLLHKLKF
REGRFNAKPTTTTPARPPPTPAPTIASQPLSLRPEACRPAAAGGAVHTRGLDFACDIYIYWAPLAG
CD8hinge (48 aa)  CD8tm

TCGVLLLSSLVITGGKGRKRLLYIFKQPFMRPVQTQEDGCSRFPEEEEUGGCELGGGRVK
4-1BB cyto  CD3ζ

FSRSADAPAYQQGNQLYNELNLGRREEYDVLDKRRGGRDPEMGKPRRKNPQEGLYNELYQ
KDKEAEYSEIMGKERRRGGKHGHDGLYQLSTATKDTYDALHMQALPPR

GMCSFRa signal peptide
IL13(EmY)
CD8hinge
CD8 transmembrane
(Gly)₃
4-1BB cyto
(Gly)₃
Zeta
INTERNATIONAL SEARCH REPORT

PCT/US2015/051089

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/715 C07K14/54 C07K19/00 C12N15/867
ADD. C12N5/0783

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)

C07K C12N

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)

EPO-Internal, WPI Data, EMBL, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Date of the actual completion of the international search: 20 January 2016

Date of mailing of the international search report: 29/01/2016

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