DESCRIPTION

CHIMERIC ANTIGEN RECEPTORS AND METHODS OF MAKING

BACKGROUND OF THE INVENTION

This application claims the benefit of United States Provisional Patent Application No. 61/940,339, filed February 14, 2014, the entirety of which is incorporated herein by reference.

Field of the Invention

[0001] The present invention relates generally to the field of molecular biology and medicine. More particularly, it concerns methods of generating chimeric antigen receptors (CAR).

Description of Related Art

[0002] Adoptive T cell transfer is a promising therapeutic approach that may be used for the treatment of cancer. Adoptive T cell transfer involves isolating and expanding antigen-specific T cells that can selectively kill tumor cells. Generally, T cells are removed from a subject and cultured *in vitro*. A Chimeric Antigen Receptor (CARs) may be introduced into a T cell *in vitro* to direct the T cell, once re-introduced into the subject, to selectively kill tumor cells based on expression of an antigen (*e.g.*, Wieczorek *et al.* 2013; Berry *et al.*, 2013).

[0003] One problem associated with adoptive T cell transfer is that significant variability exists between which CAR may work more effectively in certain populations of patients, *e.g.*, for treating a specific cancer. Due to the very large number of potential different CAR that could potentially be generated that might exhibit therapeutic activity against a cancer, it is presently very difficult for clinicians anticipate which CAR may display therapeutic activity against a given cancer or subtype of cancer. Due to the significant therapeutic potential of adoptive T cell transfer, there is a clear need for improved methods for identifying and generating new CARs.

SUMMARY

[0005] The present disclosure describes, in some examples, methods for the generation of CAR, and specific CAR are provided. In some examples, methods are provided for the generation of large number of CAR that may be screened for activity against a particular cancer or sub-type of cancer; in this way, CAR may be generated and identified that can exhibit improved therapeutic potential against a particular cancer or sub-type of cancer. CAR provided herein may be therapeutically administered to a subject or human patient, *e.g.*, to treat a cancer. Thus, in one aspect, the present invention refers to a nucleic acid encoding a chimeric antigen receptor comprising CAR 213 (SEQ ID: 5). In another aspect, the present invention refers to a Sleeping Beauty transposon comprising a nucleic acid encoding a chimeric antigen receptor comprising CAR 213 (SEQ ID NO: 5).

[0006] Clinical data demonstrates that a particular chimeric antigen receptor (CAR) design targeting T cells to a given tumor-associated antigen (TAA) may have varying therapeutic potential in different patients. For example, second generation CD19-specific CARs activated via chimeric CD28/CD3zeta or CD137/CD3-zeta can exhibit superior clinical responses when autologous genetically modified T cells are administered to patients with acute, rather than chronic, B-lineage leukemia. To address this problem, provided herein are methods for generating CAR species that may exhibit an improved anti-tumor effect for a given tumor.

[0007] For example, methods are provided herein that may be used to generate and screen a large number of CAR for their ability to treat a cancer from a given patient; in this way, the methods may be used to personalize a therapy for a patient and select a particular CAR that displays an improved therapeutic potential for a particular patient or subset of patients with a particular cancer. A clinical approach to gene therapy may utilize the electro-transfer of DNA plasmids from the Sleeping Beauty (SB) transposon system, *e.g.*, to reduce the cost and complexity to manufacture individual CAR designs for small subsets of patients. These methods for personalizing CAR+ T cells may utilize the generation a large number of CAR molecules that can be screened and assessed for their ability to benefit a given patient.

[0008] In some examples, there are disclosed methods for the high throughput assembly of CAR molecules using a triple site-specific recombination system (also referred to as the "EZ-CAR" Platform). In some examples, these methods can allow for the rapid combination of 3 components of a prototypical CAR from (i) the single chain variable fragment (scFv) that defines specificity, (ii) the scaffold/hinge that appends the scFv from the cell surface, and (iii) one or

more intracellular signaling domains. For example, as shown in the below examples, a CD19-specific CAR that is activated through chimeric CD28/CD3-zeta was generated using the EZ CAR platform in parallel with clinical-grade CD19RCD28mζ CAR+ T cells (CG CAR).

[0009] In some examples, a CAR provided herein or generated by methods as disclosed herein may be co-expressed in a T cell with a membrane bound IL-15. In this way, the T cell may survive or exist in a quiescent state without significant proliferation *in vitro* or *in vivo*. In contrast, as described previously T-cells expressing CAR will typically die when cytokines are withdrawn *in vitro*, and this cell death may serve as a safety feature in certain instances when the T cells are administered clinically. T cell proliferation is typically measured using a autonomous cell assay. Thus, in contrast to certain previously identified CAR, where T cells cannot persist *in vitro* without antigenic stimulation, CAR are provided herein which may induce cytotoxicity without autonomous growth *in vitro*. Depending on the particular example desired, a CAR produced by methods as disclosed herein may be expressed in a T cell either with or without co-expression in the T cell of a membrane bound IL-15.

[0010] In one example, there is disclosed a composition comprising: (a) a plurality of first vectors encoding one or more distinct antigen binding domains; (b) a plurality of second vectors encoding one or more distinct hinge domains; and (c) a plurality of third vectors encoding one or more distinct endodomains; wherein at least two of the first, second and third vectors comprise a plurality of two or more vectors encoding distinct antigen binding domains, hinge domains and/or endodomains, respectively, and further wherein the vectors comprise sites for homologous recombination to permit the generation of a fourth vector encoding a chimeric antigen receptor (CAR).

[0011] As disclosed herein, as used in reference to protein domains and polypeptides such as antigen binding domains, hinge domains, transmembrane domains, and endodomains, the term "distinct" means domains having, comprising, or consisting of different polypeptide (amino acid) sequences. For example, two "distinct" antigen binding domains may bind the same antigen (indeed, even the same epitope on that antigen); however, the antigen binding domains are "distinct" if their sequential amino acid compositions differ from each other. Likewise, two "distinct" antigen binding domains, differing in sequential amino acid composition, may also specifically bind different antigens and epitopes. Conversely, as used

herein, two molecules (polypeptides) of identical amino acid sequence are not "distinct" polypeptides.

[0012] In some examples, the plurality of first vectors encodes a plurality of distinct antigen binding domains, the plurality of second vectors encodes one hinge domain, and the plurality of third vectors encodes a plurality of distinct endodomains. In some examples, the plurality of first vectors encodes a plurality of distinct antigen binding domains, the plurality of second vectors encodes a plurality of distinct hinge domains, and the plurality of third vectors encodes a plurality of distinct endodomains. In some examples, the plurality of first vectors encodes a plurality of distinct antigen binding domains, the plurality of second vectors encodes a plurality of distinct hinge domains, and the plurality of third vectors encodes a one endodomain. In some examples, the plurality of first vectors encodes one antigen binding domain, the plurality of second vectors encodes a plurality of distinct hinge domains, and the plurality of third vectors encodes a plurality of distinct endodomains. In some examples, the antigen binding domains comprise or consist of scFv. The third vectors may encode a transmembrane domain. The second vectors may encode a transmembrane domain. In some examples, the composition further comprises a plurality of fifth vectors encoding one or more transmembrane domain; wherein the first vectors, the second vectors, the third vectors, and the fifth vectors comprise sites for homologous recombination to generate a fourth vector encoding a chimeric antigen receptor (CAR). The first vector may comprise a first sequence and a second site of homologous recombination. The second vector may comprise the second sequence of homologous recombination and a third sequence of homologous recombination. The third vector may comprise the third sequence of homologous recombination and a fourth sequence of homologous recombination. The third vector may comprise the third sequence of homologous recombination and a fourth sequence of homologous recombination. The fourth vector comprises the first sequence of homologous recombination and the fourth sequence of homologous recombination. The first vector, the second vector, and/or the third vector may encode a transposase. The transposase may be a salmonid-type Tc1-like transposase (SB). In some examples, 1, 2, 3, 4, or all of the first vector, the second vector, the third vector, the fourth vector, and/or the fifth vector is a Sleeping Beauty (SB) or *piggyBac* transposon vector. Alternately, in some examples, the first vector, the second vector, the third vector, the fourth vector, and/or the fifth vector is not a Sleeping Beauty (SB) or *piggyBac* transposon vector; for example, in some examples, a CAR may be generated without using a Sleeping Beauty (SB) or *piggyBac* vector, and then

the CAR may subsequently be inserted in a vector suitable for transfecting T cells (*e.g.*, inserted into a Sleeping Beauty (SB) vector as described, *e.g.*, in Singh *et al.*, 2015). Nonetheless, in some examples, generating a CAR already present in a vector that is suitable for transfecting T cells may simply the process or reduce the number of steps required to both generate a CAR and transfect a T cell. The distinct antigen binding domains may selectively bind different antigens. In some examples, the distinct antigen binding domains selectively bind the same antigen. The antigen binding domain may selectively bind CD19, Universal Antigen (mouse), HER-3, GD2, Gp75, CS1 protein, mesothelin, phosphatidylserine, cMyc, CD22, CD4, CD44v6, CD45, CD28, CD3, CD3e, CD123, CD138, CD52, CD56, CD74, CD30, Gp75, CD38, CD33, CD20, Her1/HER3 fusion, GD2, a carbohydrate, *Aspergillus*, ROR1, c-MET, EGFR, Dectin, Ebola, a fungus, GP, HERV-K (HERVK), NY-ESO-1, VEGF-R2, TGF-b2R, IgG4, Biotin, or O-AcGD2. The distinct antigen binding domains may consist of or comprise scFv. The hinge region may consist of or comprise the 12 AA peptide (GAGAGCAAGTACGGCCCTCCCTGCCCCCCTTGCCCT; SEQ ID NO:1), t-20 AA peptide, IgG4 Fc Δ EQ, IgG4 Fc Δ Q, (t-12AA + t-20AA), mKate, phiLov, dsRed, Venus, eGFP, CH3 HA, (CD8 α + t-20AA), Double t-20 AA, (t-20AA + CD8α), (CD8α + Leucine Zipper Basep1), (CD8α + Leucine Zipper Acid1), 2D3, CD8 α, or IgG4 Fc. At least one of the endodomains may comprise CD3ζ. At least one of the endodomains may comprise one or more ITAM domains. In some examples, at least one of the endodomains comprise (CD28 +CD3ζ), (CD28 +CD27 + CD3ζ), (CD28 + OX40 + CD3ζ), (CD28 + 4-1BB + CD3ζ), (CD28 + CD27 + OX40 + CD3ζ), (CD28 + 4-1BB + CD27 + CD3ζ), (CD28 + 4-1BB + OX40 + CD3ζ), (4-1BB + CD3ζ), (4-1BB + OX40 + CD3ζ), (4-1BB + CD27 +CD3ζ), (CD27 + CD3ζ), (CD27 + OX 40 + CD3ζ), (CD28Δ +CD3ζ), (CD28Δ +CD27 + CD3ζ), (CD28Δ + OX40 + CD3ζ), (CD28Δ + 4-1BB + CD3ζ), (CD28Δ + 4-1BB + OX40 + CD3ζ), (CD28Δ + CD27 + OX40 + CD3ζ), (CD28Δ + 4-1BB + CD27 + CD3ζ), (4-1BB + ICOS + CD3ζ), (CD28 + ICOS + CD3ζ), (ICOS + CD3ζ), CD3ζ, or CD28 only. In some examples, the CARs may be tested for activity, *e.g.*, using the iQue™ Screener (IntelliCyt, Albuquerque, NM). In some examples CARs may evaluated for one or more characteristics (*e.g.*, viability, upregulation of activation signals, upregulation of CD25, cytokine release, and/or cell killing) when expressed in cells such as T cells using a technique such as, *e.g.*, flow cytometry.

[0013] In another example, there is disclosed a composition comprising a collection of vectors encoding chimeric antigen receptors encoding a plurality of distinct antigen binding

domains, hinge domains and endodomains, the vectors of said collection being randomized with respect to said domains.

[0014] In yet another example, there is disclosed a method of producing a plurality of vectors each encoding a chimeric antigen receptor (CAR) comprising:(i) obtaining the composition comprising a plurality of vectors as disclosed herein (*e.g.*, as described above); and (ii) subjecting the composition to conditions sufficient to allow for the distinct antigen binding domains, hinge domains and/or endodomains comprised in or encoded by said vectors to recombine via homologous recombination to produce a plurality of fourth vectors, wherein each of said fourth vectors encodes a CAR. The method may further comprise expressing the CAR in a cell. The method may further comprise testing the CAR for activity. In some examples, one or more of the first vectors encodes a scFv region. In some examples, one or more of the third vectors encodes a transmembrane domain. In some examples, one or more of the second vectors encodes a transmembrane domain. The method may further comprise randomly incorporating by recombination a fifth vector encoding a transmembrane domain with said first vectors, second vectors, and third vectors to form said fourth vector. In some examples, said first vectors and said seconds vector are randomly attached from a plurality of vectors encoding a plurality of distinct scFv regions and a plurality of distinct hinge regions. In some examples, said first vectors and said third vectors are randomly attached from a plurality of vectors encoding a plurality of distinct scFv regions and a plurality of distinct endodomains. In some examples, said second vectors and said third vectors are randomly attached from a plurality of vectors encoding a plurality of distinct hinge regions and a plurality of distinct endodomains. In some examples, said first vectors, said second vectors, and said third vectors are randomly attached from a plurality of vectors encoding a plurality of distinct scFv regions, a plurality of distinct hinge regions, and a plurality of distinct endodomains. The method may further comprise generating said fourth vectors by random attachment of said first vectors from a first library of vectors encoding a plurality of scFv regions, random attachment of said second vectors from a second library of vectors encoding a plurality of scFv regions, and random attachment of said third vectors from a third library of vectors encoding a plurality of endodomains, to form said fourth vector encoding the CAR. The first vectors may comprise a first sequence and a second site of homologous recombination. The second vectors may comprise the second sequence of homologous recombination and a third sequence of homologous recombination. The third vectors may comprise the third sequence of homologous recombination and a fourth sequence of homologous recombination. The third vectors may comprise the third sequence of homologous recombination and a fourth sequence of homologous recombination. The fourth

vectors may comprise the first sequence of homologous recombination and the fourth sequence of homologous recombination. The first vectors, the second vectors, and/or the third vectors may encode a transposase. In some examples, a sixth vector encodes a transposase, and wherein the method comprises introducing, electroporating, or transfecting one or more of said fourth vectors and said sixth vector into a cell. The transposase may be a salmonid-type Tc1-like transposase (SB). The method may further comprise culturing or providing cells transfected with the CAR in the presence of artificial antigen presenting cells (aAPCs) that can stimulate expansion of the CAR-expressing T-cells. In some examples, each of the scFv region, the hinge region, and the endodomain are each encoded in a Sleeping Beauty (SB) or *piggyBac* transposon vector. In some examples, each of the first vector, the second vector, and/or the third vector are randomly attached by said recombination from a plurality of vectors encoding multiple distinct scFv regions, the hinge regions, and endodomains. In some examples, said first vectors, the second vectors, and the third vectors each contain a transposon; and wherein said attaching via homologous recombination comprises site specific recombination. In some examples, the first vectors and the second vectors each have a first homologous recombination site; and wherein the second vectors and the third vectors each have a second homologous recombination site. In some examples, the first vectors have a third recombination site, and wherein the fourth vectors have a fourth recombination site, wherein the third recombination site and fourth recombination site can allow for homologous recombination into a cell. The cell may be a T cell such as, *e.g.*, an alpha beta T cell, a gamma delta T cell, or NK cell, or NKT cell. In some examples, the cell is a pluripotent cell such as, *e.g.*, a stem cell or an induced pluripotent stem cell. In some examples, the cell is derived from a stem cell, an induced pluripotent stem cell, or a stem cell. The cell may be a T cell or NK cell derived from an induced pluripotent stem cell. In some examples, said distinct antigen binding domains include at least 2, 3, 4, 5, 6, 7, 8, 9, or more scFv that selectively recognize different antigens. In some examples, said distinct antigen binding domains include at least 2, 3, 4, 5, 6, 7, 8, 9, or more scFv that selectively recognize (*i.e.*, specifically bind) the same antigen. In some examples, the antigen binding domains selectively (specifically) bind CD19, Universal Antigen (mouse), HER-3, GD2, Gp75, CS1 protein, mesothelin, phosphatidylserine, cMyc, CD22, CD4, CD44v6, CD45, CD28, CD3, CD3e, CD123, CD138, CD52, CD56, CD74, CD30, Gp75, CD38, CD33, CD20, Her1/HER3 fusion, GD2, a carbohydrate, *Aspergillus*, ROR1, c-MET, EGFR, Dectin, Ebola, a fungus, GP, HERV-K, NY-ESO-1, VEGF-R2, TGF-b2R, IgG4, Biotin, or O-AcGD2. In some examples, said antigen binding domains comprise or consist of scFv. The hinge region may

encode the 12 AA peptide (GAGAGCAAGTACGGCCCTCCCTGCCCCCCTTGCCCT, SEQ ID NO:1), t-20 AA peptide, IgG4 Fc Δ EQ, IgG4 Fc Δ Q, (t-12AA + t-20AA), mKate, phiLov, dsRed, Venus, eGFP, CH3 HA, (CD8 α + t-20AA), Double t-20 AA, (t-20AA + CD8α), (CD8α + Leucine Zipper Basep1), (CD8α + Leucine Zipper Acid1), 2D3, CD8 α, or IgG4 Fc. The endodomain may encode CD3ζ. The endodomain may encode one or more ITAM domains. In some examples, the endodomain encodes (CD28 +CD3ζ), (CD28 +CD27 + CD3ζ), (CD28 + OX40 + CD3ζ), (CD28 + 4-1BB + CD3ζ), (CD28 + CD27 + OX40 + CD3ζ), (CD28 + 4-1BB + CD27 + CD3ζ), (CD28 + 4-1BB + OX40 + CD3ζ), (4-1BB + CD3ζ), (4-1BB + OX40 + CD3ζ), (4-1BB + CD27 +CD3ζ), (CD27 + CD3ζ), (CD27 + OX 40 + CD3ζ), (CD28Δ +CD3ζ), (CD28Δ +CD27 + CD3ζ), (CD28Δ + OX40 + CD3ζ), (CD28Δ + 4-1BB + CD3ζ), (CD28Δ + 4-1BB + OX40 + CD3ζ), (CD28Δ + CD27 + OX40 + CD3ζ), (CD28Δ + 4-1BB + CD27 + CD3ζ), (4-1BB + ICOS + CD3ζ), (CD28 + ICOS + CD3ζ), (ICOS + CD3ζ), CD3ζ, or CD28 only. In some examples, the CARs may be tested for activity, *e.g.*, using the iQue™ Screener (IntelliCyt, Albuquerque, NM). In some examplesCARs may evaluated for one or more characteristics (*e.g.*, viability, upregulation of activation signals, upregulation of CD25, cytokine release, and/or cell killing) when expressed in cells such as T cells using a technique such as, *e.g.*, flow cytometry. In some examples, said activity comprises ability of the CAR to selectively bind a cancer cell, selectively bind a pathogen, selectively bind a cell involved in an autoimmune disease, or promote activation of a T-cell, destruction of a T cell, differentiation of a T cell, proliferation of a T cell, de-differentation of a T cell, movement of a T cell, cytokine production by a T cell, or killing by a T cell.

[0015] In some examples, the cancer cell is an ovarian cancer, a lymphoma, a renal cell carcinoma, a B-cell malignancy, CLL, B-ALL, ALL, a leukemia, a B-cell malignancy or lymphoma, mantle cell lymphoma, an indolent B-cell lymphoma, Hodgkin lymphoma, AML, cervical cancer, breast cancer, colorectal cancer, ovarian cancer, neuroblastoma, skin cancer, melanoma, a lung cancer, osteosarcoma, glioma, an epithelial derived tumor, prostate cancer, or a pediatric cancer. The pathogen may be a virus, a fungi, or a bacteria. In some examples, said testing comprises single cell imaging, single cell genetics, assessment of single T cells or populations of T cells; measuring specific killing or serial killing, gene expression, protein expression, movement towards or away from a target, proliferation, activation-induced cell death, secretion of cytokines, or secretion of chemokines. The method may further comprise selecting a single CAR from said plurality of vectors based on a property of the single CAR. The method

may further comprise therapeutically administering the single CAR to a subject. The subject may be a mammal such as, *e.g.*, a human.

[0016] In another example, there is disclosed a polypeptide comprising or consisting of CAR 217 (SEQ ID NO: 2), CAR 194 (SEQ ID NO: 3), CAR 212 (SEQ ID NO: 4), CAR 213 (SEQ ID NO: 5), CAR 265 (SEQ ID NO: 6), CAR 214 (SEQ ID NO:56), CAR 215(SEQ ID NO:57), CAR 216 (SEQ ID NO:58), CAR 218 (SEQ ID NO:59), CAR 193 (SEQ ID NO:55), or CAR 268 (SEQ ID NO: 7).

[0017] In yet another example, there is disclosed a transformed T cell expressing the polypeptide comprising or consisting of CAR 217 (SEQ ID NO:2), CAR 194 (SEQ ID NO:3), CAR 212 (SEQ ID NO:4), CAR 213 (SEQ ID NO:5), CAR 265 (SEQ ID NO:6), CAR 214 (SEQ ID NO:56), CAR 215(SEQ ID NO:57), CAR 216 (SEQ ID NO:58), CAR 218 (SEQ ID NO:59), CAR 193 (SEQ ID NO:55), or CAR 268 (SEQ ID NO:7). The cell may be an immortalized cell. The T cell may be an alpha beta T cell, a gamma delta T cell, NK cell, NKT cell, stem cell, cells derived from stem cells, including cells of the immune system.

[0018] In yet another example, there is disclosed a pharmaceutical preparation comprising the transformed T cell as disclosed herein.

[0019] In another example, there is disclosed a nucleic acid encoding a chimeric antigen receptor comprising or consisting of CAR 217 (SEQ ID NO:2), CAR 194 (SEQ ID NO:3), CAR 212 (SEQ ID NO:4), CAR 213 (SEQ ID NO:5), CAR 265 (SEQ ID NO:6), CAR 214 (SEQ ID NO:56), CAR 215(SEQ ID NO:57), CAR 216 (SEQ ID NO:58), CAR 218 (SEQ ID NO:59), CAR 193 (SEQ ID NO:55), or CAR 268 (SEQ ID NO:7). The nucleic acid may be comprised in a T cell such as, *e.g.*, an alpha beta T cell, a gamma delta T cell, NK cell, NKT cell, stem cell, or a T cell derived from a pluripotent cell. The T cell may be comprised in a pharmaceutically acceptable carrier or excipient.

[0020] In another example, there is disclosed a composition comprising a library of different CAR encoding vectors, the vectors of said library being randomized in terms of distinct antigen binding domains, hinge domains and/or endodomains. In some examples, the library randomized in terms of distinct antigen binding domains, hinge domains, and endodomains. In some examples, the library randomized in terms of distinct antigen binding domains and endodomains. In some examples, the library randomized in terms of distinct antigen binding domains and hinge domains. In some examples, the library randomized in terms of distinct antigen hinge domains and endodomains.

[0021] Examples of antigen binding domains, hinge regions, transmembrane domains, and endodomains that be used in methods as disclosed herein to generate a CAR are shown below in Table 1. The antigen binding domains, hinge regions, transmembrane domains, and endodomains are merely provided in Table 1 as non-limiting examples, and it is anticipated that one may select virtually any antigen binding domain (*e.g.*, targeting a cancerous cell, bacteria, fungi, virus, or virus-infected cell) as desired for the particular clinical application. In Table 1, the target of the antigen binding domain is provided (*e.g.*, "CD19" may refer to a scFv region that selectively binds CD19). In some examples, the antigen binding domain comprises or consists of a scFv that selectively binds the antigen. If desired, a portion of the scFv (*e.g.*, part of the variable region of the scFv) may be randomized if desired. In some examples, the antigen binding domain selectively binds a protein. Alternately, the antigen binding domain may selectively bind a carbohydrate expressed on a target such as, *e.g.*, a fungi, virus, bacteria, or cancerous cell. For example, in some examples, the antigen binding domain comprises or consists of Dectin-1, which can selectively bind β-glucans and carbohydrate found in fungal cell walls. In some examples, the CAR may selectively bind a virus, *e.g.*, the CAR may bind a viral protein such as a hepatitis envelope protein (*e.g.*, Krebs *et al.*, 2013). In some examples, the antigen binding domain is a cytokine. The antigen binding domain may selectively bind a protein, carbohydrate, or sugar. In some examples, a CAR is generated from a plurality of antigen binding domains that selectively bind a single target, antigen, or the antigen binding domains may have overlapping antigens. In some examples, a CAR is generated from a plurality of antigen binding domains that selectively bind different targets or antigens. The endodomain in a CAR may result in an inhibitory signal (*e.g.*, PD-1, CTLA-4, TIM-3, LAG-3, BTLA, ITIM, SHP-1, LAIR-1, TIGIT, Siglecs) or a stimulatory signal (*e.g.*,CD27, CD28, ICOS, CD134, CD137, LCK, DAP10, ITAM, ZAP-70, LAT, SLP-76, cytokines as well as cytokine receptors; as well as combinations and mutations) in a cell expressing the CAR such as, *e.g.*, a T cell or a natural killer (NK) cell. When the antigen binding region selectively recognizes an antigen, the endodomain may cause or promote the cell (*e.g.*, T cell or NK cell) comprising the CAR to activate cell killing, migrate, differentiate, de-differentiate, or result in inducing an apoptotic signal in the cell. The apoptotic signal may comprise or consist of a CTLA4 apoptotic signal and/or a PD1 (protein death 1) apoptotic signal. In some examples, more than one distinct CAR may be expressed in a cell such as, *e.g.*, a T cell or a NK cell. For example, a first CAR and a second CAR may be expressed in a cell, wherein the first CAR selectively binds an antigen on a healthy cell and induces an inhibitory signal via a first endodomain (*e.g.*, reducing the probability that the T cell or NK cell will damage the healthy cell) and the second CAR selectively binds an antigen on a target cell (*e.g.*, cancerous cell, fungi, virus-infected cell, bacteria) and induces a

stimulatory signal via a second endodomain (*e.g.*, promoting or causing cell killing of the target cell by the T cell or NK cell). A CAR generated via the methods as disclosed herein may be inserted in a target cell such as, *e.g.*, a T cell or a NK cell, as integrating DNA (*e.g.*, using electroporation and homologous recombination via a transposase/transposon vector or system) or as non-integrating DNA or RNA (*e.g.*, viral delivery of a mRNA using a viral vector such as, *e.g.*, a lentivirus or retrovirus). In some examples, the T cell encoding a CAR as disclosed herein is an immortalized cell; such immortalized cells may function may be used to evaluate or measure the therapeutic potential or toxicity of the CAR. In this way, many CARs may be screened for a desired pharmacological profile, toxicity towards diseased cells or pathogens, lack of toxicity in healthy cells, and/or therapeutic efficacy.

Table 1. DNA molecules that can be combined as Antigen binding domain-hinge-signaling domains to generate CARs.

Antigen-binding Domain (*e.g.*, an ScFv that selectively bind a target listed below)
CD19 (mouse) (*e.g.*, SEQ ID NO:8)
CD19 (human) (*e.g.*, SEQ ID NO:9)
CD19 (humanized)
Universal Antigen (mouse) (Rushworth *et al.*, 2014)
CD22 (*e.g.*, scFv from Jabbour *et al.*, 2014 or Kong *et al.*, 2014)
CD4 (*e.g.*, scFv from Humblet-Baron *et al.*, 2015)
CD44v6 (*e.g.*, scFv from Leung 2010 or Verel 2002)
CD45 (*e.g.*, scFv from Shin *et al.*, 2011)
CD28 (*e.g.*, scFv from Czerwiński et al, 2015)
CD3 (*e.g.*, SEQ ID NO:10)
CD3e (*e.g.*, scFv from monoclonal antibody SPV-T3b, Life Technologies, Carlsbad, CA),
CD123 (*e.g.*, SEQ ID NO:11)
CD138 (*e.g.*, scFv from Sun *et al.*, 2007)
CD52 (*e.g.*, scFv from Wang *et al.*, 2015)
CD56 (*e.g.*, scFv from Kaufmann *et al.*, 1997)
CD74 (*e.g.*, scFv from Kaufman *et al.*, 2013)

CD30 (*e.g.*, SEQ ID NO:12)
Gp75 (*e.g.*, scFv from Patel *et al.*, 2008)
CD38 (*e.g.*, scFv from de Weers *et al.*, 2011)
CD33 (*e.g.*, scFv from Manero *et al.*, 2013)
CD20 (*e.g.*, scFv from Le Garff-Tavernier *et al.*, 2014 or Winiarska et al, 2014)
Her1/HER3 fusion (*e.g.*, scFv from Sarup *et al.*, 2008)
HER-3 (*e.g.*, SEQ ID NO:13)
GD2 (*e.g.*, SEQ ID NO:14)
Carbohydrates (such as an Aspergillus carbohydrate), *e.g.*, scfv from Stynen *et al.*, 1991)
ROR1 (*e.g.*, SEQ ID NO:15)
c-MET (*e.g.*, scFv from Zhuang *et al.*, 2014)
cMyc (*e.g.*, SEQ ID NO:16)
EGFR (*e.g.*, scFv from Funakoshi *et al.*, 2014)
Dectin (*e.g.*, Dectin 1 ectodomain, SEQ ID NO:17)
Dectin-1 binding site
Ebola virus (*e.g.*, scFv from Audet *et al.*, 2014 or Qiu *et al.*, 2012)
Fungal antigens (*e.g.*, scFv from Guimarães *et al.*, 2011)
GP (Qiu *et al.*, 2012)
Gp75 (*e.g.*, TA99, SEQ ID NO:18)
HERV-K (HERVK) (*e.g.*, SEQ ID NO:19)
NY-ESO-1 (*e.g.*, scFv from Schultz-Thater *et al.*, 2000)
VEGF-R2 (*e.g.*, scFv from Zhang *et al.*, 2002)
TGF-b2R (*e.g.*, scFv from Leung, 2011)
IgG4 (*e.g.*, scFv from Curtin *et al.*, 2015)
Biotin (*e.g.*, scFv from Vincent *et al.*, 1993)
O-AcGD2 (*e.g.*, scFv from Goldberg e*t al.*, 2014 or Ahmed *et al.*, 2014)
CS1 protein (*e.g.*, Elotuzumab or huLuc63, SEQ ID NO:20)
Mesothelin (*e.g.*, using the SS-1 scFv, SEQ ID NO:21)

Phosphatidylserine (*e.g.*, scFv from Gerber *et al.*, 2011)

Hinge/Scaffold
12 AA (peptide) (*e.g.*, SEQ ID NO:1)
t-20 AA (peptide) (*e.g.*, SEQ ID NO:22)
CD8 α (*e.g.*, SEQ ID NO:23)
IgG4 Fc (*e.g.*, SEQ ID NO:24)
2D3 (*e.g.*, SEQ ID NO:25)
IgG4 Fc Δ EQ (IgG4Fc N40Q) (*e.g.*, SEQ ID NO:26)
IgG4 Fc Δ Q (IgG4Fc L18E N40Q) (*e.g.* SEQ ID NO:27)
t-12AA + t-20AA
mKate (*e.g.*, SEQ ID NO:28)
phiLov (*e.g.*, SEQ ID NO:29)
dsRed (*e.g.*, SEQ ID NO:30)
Venus (*e.g.*, SEQ ID NO:31)
eGFP (*e.g.*, SEQ ID NO:32)
CH3 HA (*e.g.*, SEQ ID NO:33)
mTFP-1 (*e.g.*, SEQ ID NO:34)
CD8 α + t-20AA
Double t-20 AA
t-20AA + CD8α
CD8α + Leucine Zipper Basep1 (*e.g.*, SEQ ID NO:35)
CD8α + Leucine Zipper Acid1 (*e.g.*, SEQ ID NO:36)

Transmembrane domain
CD28 (*e.g.*, SEQ ID NO:37)
CD137 (4-1BB) (*e.g.*, SEQ ID NO:38)
CD8α (*e.g.*, SEQ ID NO:39)
CD3ζ (*e.g.*, SEQ ID NO:40)

Endo-domain (signaling domain)

CD28 +CD3ζ
CD28 +CD27 + CD3ζ
CD28 + OX40 + CD3ζ
CD28 + 4-1BB + CD3ζ
CD28 + CD27 + OX40 + CD3ζ
CD28 + 4-1BB + CD27 + CD3ζ
CD28 + 4-1BB + OX40 + CD3ζ
4-1BB + CD3ζ
4-1BB + OX40 + CD3ζ
4-1BB + CD27 +CD3ζ
CD27 + CD3ζ
CD27 + OX 40 + CD3ζ
CD28Δ +CD3ζ
CD28Δ +CD27 + CD3ζ
CD28Δ + OX40 + CD3ζ
CD28Δ + 4-1BB + CD3ζ
CD28Δ + 4-1BB + OX40 + CD3ζ
CD28Δ + CD27 + OX40 + CD3ζ
CD28Δ + 4-1BB + CD27 + CD3ζ
4-1BB + ICOS + CD3ζ
CD28 + ICOS + CD3ζ
ICOS + CD3ζ
CD3ζ
CD28 only

ζ - zeta; Δ- mutant; Note = 4-1BB is also referred to as CD137; "+" refers to the fusion of the different regions.

[0022] For example, in some examples, the following antigen-binding domains, hinge/scaffolds, transmembrane domains, and endodomains may be used, as shown in Table 2. Examples of sequences included in signaling domains, *e.g.*, in Table 1 or Table 2, include CD27 (SEQ ID NO:41), CD28 (SEQ ID NO:42), CD28Δ (SEQ ID NO:43), CD134 (OX40) (SEQ ID

NO:44), CD137 (41BB) (SEQ ID NO:45), ICOS (SEQ ID NO:46)and CD3 zeta (SEQ ID NO:47). Examples of scFv Anti-EGFR domains as listed in Table 2 include Nimotuximab (SEQ ID NO:48) and Cetuximab (SEQ ID NO:49). An example of a scFv Anti-Phosphatidylserine as listed in Table 2 is Bavituximab (SEQ ID NO:50).

Table 2: Example of libraries used to generate CAR

ScFv
Anti –CS1 protein
Anti-mesothelin (SS-1)
Anti- CD123
Anti-CD19 human
Anti-CD19 mouse
Anti-CD3
Anti-CD30
Anti-Dectin
Anti-G2D
Anti-Gp75
Anti-HERVK
Anti-CD22
Anti-ROR-1
Anti-EGFR
Anti-HER-3
Anti-Phosphatidylserine

Hinge/Scaffold
t-12 AA (peptide)
t-20 AA (peptide)
CD8 α
IgG4 Fc
IgG4Fc Δ EQ
IgG4Fc Δ Q
t-12AA + t-20AA
mKate
phiLov
dsRed
Venus
eGFP
CH3 HA
CD8 α + t-20AA
Double t-20 AA
t-20AA + CD8α
CD8α + Leucine Zipper Basep1
CD8α + Leucine Zipper Acid1

Transmembrane domain
CD28
4-1BB
CD3ζ

Signaling Domain
CD28 +CD3ζ
CD28 +CD27 + CD3ζ
CD28 + OX40 + CD3ζ
CD28 + 4-1BB + CD3ζ
CD28 + CD27 + OX40 + CD3ζ
CD28 + 4-1BB + CD27 + CD3ζ
CD28 + 4-1BB + OX40 + CD3ζ
4-1BB + CD3ζ
4-1BB + OX40 + CD3ζ
4-1BB + CD27 +CD3ζ
CD28Δ +CD3ζ
CD28Δ +CD27 + CD3ζ
CD28Δ + OX40 + CD3ζ
CD28Δ + 4-1BB + CD3ζ
CD28Δ + 4-1BB + OX40 + CD3ζ
CD28Δ + CD27 + OX40 + CD3ζ
CD28Δ + 4-1BB + CD27 + CD3ζ
4-1BB + ICOS + CD3ζ
CD28 + ICOS + CD3ζ
ICOS + CD3 ζ
CD3 ζ
CD28 only

[0023] The term "chimeric antigen receptors (CARs)" or "CAR" as used herein, includes artificial T-cell receptors, chimeric T-cell receptors, or chimeric immunoreceptors. CARs are generally engineered receptors that may graft an artificial specificity onto a particular immune effector cell. CARs may be employed to impart the specificity of a monoclonal antibody onto a T cell, thereby allowing a large number of specific T cells to be generated, for example, for use in an adoptive cell therapy. In some examples, CARs direct specificity of the cell to a tumor associated antigen. In preferred examples, CARs comprise an endodomain (comprising an intracellular activation domain), a transmembrane domain, a hinge or scaffold region, and an extracellular domain comprising a targeting domain (*e.g.*, a scFv derived from a monoclonal antibody). In some examples, the extracellular targeting domain may be a ligand of a receptor (*e.g.*, a peptide that selectively binds a protein receptor). In some examples, one can target malignant cells by redirecting the specificity of T cells by using a CAR specific for the malignant cells (*e.g.*, by using an anti-CD19 scFv to target a cancerous B-lineage cell).

[0024] Examples of scFv regions, hinge / scaffold regions, transmembrane domains, and endodomains are shown in Table 1 and examples of related sequences are also provided herein. Note in Table 1 that the scFv regions may refer to a plurality of scFv regions for a particular target (*e.g.*, "CD19" in Table 1 may refer to a single monoclonal antibody sequence, or in some examples, it may refer to a plurality of scFv regions derived from monoclonal antibodies that selectively target CD19). It is anticipated that methods as disclosed herein may be used to generate a CAR that comprises, *e.g.*, a fusion of any combination of a scFv region, hinge/scaffold, transmembrane domain, and endodomain of Table 1. For example, in some examples, the CAR may comprise a scFv region that selectively targets CD19 (*e.g.*, derived from a mouse, human, or humanized monoclonal antibody) fused to an IgG4 Fc hinge/scaffold region, a CD28 transmembrane domain, and an endodomain comprising CD28 and CD3ζ. In some examples, the CAR may comprise a scFv region that selectively targets ROR1 fused to IgG4 Fc hinge/scaffold region, a CD28 transmembrane domain, and an endodomain comprising CD28 and CD3ζ. In some examples, the CAR may comprise a scFv region that selectively targets ROR1 fused to IgG4 Fc hinge/scaffold region, a CD28 transmembrane domain, and an endodomain comprising 4-1BB and CD3ζ. In some examples, the CAR may comprise a scFv region that selectively targets CD19 (*e.g.*, derived from a mouse, human, or humanized monoclonal antibody) fused to IgG4 Fc hinge/scaffold region, a CD28 transmembrane domain, and an endodomain comprising CD28 and CD3ζ.

[0025] As used herein, the term "antigen" is a molecule capable of being bound by an antibody or T-cell receptor. An antigen may generally be used to induce a humoral immune response and/or a cellular immune response leading to the production of B and/or T lymphocytes.

[0026] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one.

[0027] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." As used herein "another" may mean at least a second or more.

[0028] Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0029] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0031] FIG. 1. Cloning vectors used to re-assemble CARs using three donor plasmids expressing (i) specific scFv, (ii) extracellular hinge and (iii) endodomains. This approach will be adapted to generate panels of CARs that differ in hinge, transmembrane, and intracellular regions. Engineering CAR molecules from components scFv, IgG4 Fc (Long hinge); or CD8a (Medium hinge) or peptide only (Small hinge) and CD3ζ ιν combinations with different signaling domains using triple recombination site system. A library of scFv and distinct scaffolds and signaling domains encoded in three donor plasmids (entry clones), are recombined in to the expression DNA vector. This approach generated multiple CAR species in the format scFv-B-scaffold-C-signaling domain(s).

[0032] FIGS. 2A-B: (**FIG. 2A**) Expression of CAR (Fc) and $CD8^+$ in T cells after 66 days post electroporation by flow cytometry. The cells were expanded in aAPC loaded with CD19 antigen (clone 4) (**FIG. 2B**) Lysis of CD19+ EL-4 was compared to background lysis of $CD19^{neg}$ EL-4 using 4-h chromium release assay by CD19CAR+ T cells Clinical Grade (CG) CD19CAR+ T cells by triple recombination sites (EZ CAR) and CAR^{neg} T cells. The CAR^{neg} T were expanded in with irradiated and anti-CD3 (OKT3) loaded K562-derived aAPC clone #4.

[0033] FIG. 3: CAR Designs. CAR 212 = SEQ ID NO:4; CAR 213 = SEQ ID NO:5; CAR 214 = SEQ ID NO:56; CAR 215 = SEQ ID NO:57; CAR 216 = SEQ ID NO:58; CAR 217 = SEQ ID NO:2; CAR 218 = SEQ ID NO:59; CAR 193 = SEQ ID NO:55.

[0034] FIG. 4: Sleeping Beauty tracking plasmids

[0035] FIG. 5: CAR Expression.

[0036] FIG. 6: CAR Expression Kinetics

[0037] FIG. 7: Phenotype.

[0038] FIGS. 8A-B: Extended Phenotype is shown in FIG. 8A and FIG. 8B.

[0039] FIG. 9: Western Blot Analysis.

[0040] FIG. 10: Expansion Kinetics.

[0041] FIG. 11: Fold Expansion: Total Cells

[0042] FIG. 12: Fold Expansion: CAR+ T cells.

[0043] FIG. 13: Cytotoxicity.

[0044] FIG. 14: 4-1BB CARs: Cytotoxicity.

[0045] FIG. 15: TM domain: Cytotoxicity.

[0046] FIG. 16: Spacer (IgG4 vs CD8): Cytotoxicity

[0047] FIG. 17: IFN-γ production.

[0048] FIG. 18: 4-1BB CARs: IFN-γ production

[0049] FIG. 19: TM domain: IFN-γ production

[0050] FIG. 20: Spacer (IgG4 vs CD8): IFN-γ production.

[0051] FIG. 21: Safety: PCR for SB11 transposase.

[0052] FIG. 22: Safety: CAR copy number (qPCR).

[0053] FIG. 23: Safety: Autonomous Growth. As shown in the figure, a lack of autonomous growth was observed.

[0054] FIG. 24: CAR design. An example of a CAR is provided on the right-hand side of the figure.

[0055] FIG. 25: CD3-zeta. Query = SEQ ID NO:51; Subject – top = SEQ ID NO:52; Subject – middle = SEQ ID NO:53; Subject – bottom = SEQ ID NO:54.

[0056] FIG. 26: CAR designs.

[0057] FIG. 27: CARs.

[0058] FIG. 28: CAR Expression.

[0059] FIG. 29: Expansion Kinetics.

[0060] FIG. 30: Expansion Kinetics.

[0061] FIG. 31: Cytotoxicity.

[0062] FIG. 32: Cytotoxicity.

[0063] FIG. 33: Memory Markers. Percent expression of CD27, CD62L, CD28 and CCR7 on CAR^+ T cells (expressing constructs shown in FIG. 26) are shown.

[0064] FIG. 34: IFN-γ production.

[0065] FIG. 35: IFN-γ production (PMA-Ion)

[0066] FIG. 36: Autonomous Growth.

[0067] FIG. 37: CAR Copy Number.

[0068] FIG. 38: CAR Copy Number.

[0069] FIG. 39: CAR Copy Number.

[0070] FIGS. 40A-E: Transfection of 293-HEK cells with plasmids carrying the CAR DNA (pSBSO EZ CAR) by lipofectamine was performed. The transfected cells were analyzed by flow cytometry after stained with anti-Fc or anti-idiotipic (antiCD19svFv) antibodies.

[0071] FIGS. 41A-B: **FIG. 41A**, Nalm-6; EL-4 CD19+ cells; patient tumor cells with MCL and CLL (targets) and were previously modified to express GFP. $5x10^3$ target cells were incubated with increasing concentration of CD19RIgG4CD28CAR T cells, CD19RCD8αCD28 CAR T cells and CAR^{neg} T cells (used as the control) for 4 hours. After 4 hours the cells were acquired by IntelliCyt's iQue and the data analyses were made in their proprietary software. **FIG. 42B**, The graphs are representing the killing percentage of CAR T cells against tumor cells. The ratio between effector and target cells ranged from 0 to 40 cells.

[0072] FIG. 42: $5x10^3$ target cells (EL-4 CD19+ Granzyme B cells reporter) were incubated with increasing concentration of Clinical-grade CD19RIgG4CD28CAR T cells, EZ CD19RCD8αCD28 CAR T cells and CAR^{neg} T cells (used as the control) for 4 and 10 hours. After incubation time the cells were acquired by IntelliCyt's iQue and the data analyses were made in their proprietary software. The graphs are representing the killing percentage of CAR T cells against tumor cells. The ratio between effector and target cells ranged from 0 to 20 cells.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0073] Provided herein are methods for generating chimeric antigen receptors (CARs). The method utilizes a plurality of vectors each encoding an antigen binding domain (*e.g.*, a scFv region), a hinge region, a transmembrane region, and/or an endodomain. For example in some embodiments a first vector encodes the antigen binding domain, a second vector encodes the hinge region, and a third region encodes an endodomain. In some embodiments, the transmembrane region is encodes either in the second vector, in the third vector, or in a fourth vector. In some preferred embodiments, the vectors can homologously recombine to form a nucleic acid encoding a CAR comprising the antigen binding domain, the hinge region, the transmembrane region, and the endodomain. In this way, many CAR may be generated and screened for a desired activity such as, *e.g.*, selective recognition and killing of a cancerous cell expressing an antigen that is selectively bound by the CAR. The CAR may then be expressed in a cell such as a T cell or a natural killer (NK) cell as an integrating nucleic acid (*e.g.*, a DNA integrated into the host genome using a transposase/transposon) or as a non-integrating nucleic acid (*e.g.*, a mRNA delivered via a viral vector such as a lentivirus or retrovirus). The T cell or NK cell expressing the CAR may then be administered in a pharmaceutical preparation or excipient to a subject such as a human patient to treat or prevent a disease (*e.g.*, a cancer, a fungal infection, a bacterial infection, or a viral infection).

I. Library generation

[0074] Libraries encoding a plurality of scFv regions, hinge/scaffold regions, transmembrane domains, and endodomains (signaling domains) may be generated by methods known to one of skill in the art. In some embodiments, multiple possibilities are available for two or three of the scFv regions, hinge/scaffold regions, and endodomains (signaling domains). In some embodiments, multiple possibilities are available for two, three, or all of the scFv regions, hinge/scaffold regions, transmembrane domains, and endodomains (signaling domains). Examples of scFv regions, hinge/scaffold regions, transmembrane domains, and endodomains (signaling domains) are provided, *e.g.*, in Table 1. In some embodiments, the library may encode a plurality of scFv that target different antigens, such as such as multiple anti-cancer or tumor-targeting antigens; in other embodiments, the library may encode a plurality of different scFv that selectively bind a single target (*e.g.*, a single anticancer antigen such as CD19, *etc.*). In this way, the methods may be used to either identify which tumor-targeting construct may work more effectively for

a given cell sample (*e.g.*, to be used in a personalized medicine) or the methods may be used to identify new CAR that function more effectively in targeting a given antigen. The scFv region generally comprises a variable light (VL) and a variable heavy (VH) chain derived from an antibody. In some embodiments, portions of the VL and VH regions may be randomized if desired. General methods for generating libraries include, *e.g.*, the generation of yeast libraries, bacterial, phage libraries, infiltrating B cells, hybridomas (including from human and rodents), or libraries from llamas, camels, equine libraries, and in silico methods (See, *e.g.*, Lennard, 2002).

[0075] In some embodiments, the different vectors encoding the scFv, hinge/scaffold region, transmembrane domain, and endodomain are fused to form a single vector encoding a CAR. The fusion may occur via transposon-mediated homologous recombination.

[0076] For example, in some embodiments, the vectors encoding the scFv, hinge/scaffold region, transmembrane domain, and/or endodomain may be *Sleeping Beauty* (SB) or *piggyBac* DNA plasmids. *Sleeping Beauty* (SB) and *piggyBac* DNA plasmids are described, *e.g.*, in Maiti *et al.* (2013), Singh *et al.* (2008), and Huls *et al.* (2013). In some embodiments, the transposon is mediated by a salmonid-type Tc1-like transposase (SB). In some preferred embodiments, the vector encoding the CAR is transfected or incorporated into T cells from a subject, such as a human patient with cancer, via the methods as described in Singh *et al.*, 2014 or Huls *et al.* For example, DNA vectors derived from the Sleeping Beauty (SB) system can be used to avoid expense and manufacturing difficulties associated with transducing T cells with recombinant viral vectors. After electroporation, the transposon/transposase can improve the efficiency of integration of plasmids used to express CAR and other transgenes in T cells. The SB system combined with artificial antigen-presenting cells (aAPC) can selectively propagate and produce CAR(+) T cells suitable for human application. In some embodiments, synchronous electro-transfer of two DNA plasmids, a SB transposon (encoding a CAR of interest) and a SB transposase (*e.g.*, SB11) may be followed by retrieval of stable integrants by the every-7-day additions (stimulation cycle) of γ-irradiated aAPC in the presence of soluble recombinant human IL-2 and IL-21. For example, 4 cycles (28 days of continuous culture) may be undertaken to generate clinically-appealing numbers of T cells that stably express a CAR of interest. Use of a transposon / transposase system may be utilized for delivery of T cells expressing a CAR as described, *e.g.*, in Hackett *et al.*

II. Chimeric Antigen Receptors

[0077] Embodiments of the present invention involve generation and identification of nucleic acids encoding an antigen-specific chimeric antigen receptor (CAR) polypeptide. In some embodiments, the CAR is humanized to reduce immunogenicity (hCAR).

[0078] In some embodiments, the CAR may recognize an epitope comprised of the shared space between one or more antigens. Pattern recognition receptors, such as Dectin-1, may be used to derive specificity to a carbohydrate antigen. In certain embodiments, the binding region may comprise complementary determining regions of a monoclonal antibody, variable regions of a monoclonal antibody, and/or antigen binding fragments thereof. In some embodiments the binding region is an scFv. In another embodiment, a peptide (*e.g.*, a cytokine) that binds to a receptor or cellular target may be included as a possibility or substituted for a scFv region in the binding region of a CAR. Thus, in some embodiments, a CAR may be generated from a plurality of vectors encoding multiple scFv regions and/or other targeting proteins. A complementarity determining region (CDR) is a short amino acid sequence found in the variable domains of antigen receptor (*e.g.*, immunoglobulin and T-cell receptor) proteins that complements an antigen and therefore provides the receptor with its specificity for that particular antigen. Each polypeptide chain of an antigen receptor contains three CDRs (CDR1, CDR2, and CDR3). Since the antigen receptors are typically composed of two polypeptide chains, there are six CDRs for each antigen receptor that can come into contact with the antigen -- each heavy and light chain contains three CDRs. Because most sequence variation associated with immunoglobulins and T-cell receptor selectivity are generally found in the CDRs, these regions are sometimes referred to as hypervariable domains. Among these, CDR3 shows the greatest variability as it is encoded by a recombination of the VJ (VDJ in the case of heavy chain and TCR αβ chain) regions.

[0079] A CAR-encoding nucleic acid generated via the present invention may comprise one or more human genes or gene fragments to enhance cellular immunotherapy for human patients. In some embodiments, a full length CAR cDNA or coding region may be generated via the methods described herein. The antigen binding regions or domain may comprise a fragment of the V_H and V_L chains of a single-chain variable fragment (scFv) derived from a particular human monoclonal antibody, such as those described in U.S. Patent 7,109,304, incorporated herein by reference. In some embodiments, the scFv comprises an antigen binding domains of a human antigen-specific antibody. In some embodiments, the

scFv region is an antigen-specific scFv encoded by a sequence that is optimized for human codon usage for expression in human cells.

[0080] The arrangement of the antigen-binding domain of a CAR may be multimeric, such as a diabody or multimers. The multimers can be formed by cross pairing of the variable portions of the light and heavy chains into what may be referred to as a diabody. The hinge portion of the CAR may in some embodiments be shortened or excluded (*i.e.*, generating a CAR that only includes an antigen binding domain, a transmembrane region and an intracellular signaling domain). A multiplicity of hinges may be used with the present invention, *e.g.*, as shown in Table 1. In some embodiments, the hinge region may have the first cysteine maintained, or mutated by a proline or a serine substitution, or be truncated up to the first cysteine. The Fc portion may be deleted from scFv used to as an antigen-binding region to generate CARs according to the present invention. In some embodiments, an antigen-binding region may encode just one of the Fc domains, *e.g.*, either the CH2 or CH3 domain from human immunoglobulin. One may also include the hinge, CH2, and CH3 region of a human immunoglobulin that has been modified to improve dimerization and oligermerization. In some embodiments, the hinge portion of may comprise or consist of a 8-14 amino acid peptide (*e.g.*, a 12 AA peptide), a portion of CD8α, or the IgG4 Fc. In some embodiments, the antigen binding domain may be suspended from cell surface using a domain that promotes oligomerization, such as CD8 alpha. In some embodiments, the antigen binding domain may be suspended from cell surface using a domain that is recognized by monoclonal antibody (mAb) clone 2D3 (mAb clone 2D3 described, *e.g.*, in Singh *et al.*, 2008).

[0081] The endodomain or intracellular signaling domain of a CAR can generally cause or promote the activation of at least one of the normal effector functions of an immune cell comprising the CAR. For example, the endodomain may promote an effector function of a T cell such as, *e.g.*, cytolytic activity or helper activity including the secretion of cytokines. The effector function in a naive, memory, or memory-type T cell may include antigen-dependent proliferation. The terms "intracellular signaling domain" or "endodomain" refers to the portion of a CAR that can transduce the effector function signal and/or direct the cell to perform a specialized function. While usually the entire intracellular signaling domain may be included in a CAR, in some cases a truncated portion of an endodomain may be included.

Generally, endodomains include truncated endodomains, wherein the truncated endodomain retains the ability to transduce an effector function signal in a cell.

[0082] In some embodiments, an endodomain comprises the zeta chain of the T-cell receptor or any of its homologs (*e.g.*, eta, delta, gamma, or epsilon), MB1 chain, B29, Fc RIII, Fc RI, and combinations of signaling molecules, such as CD3ζ and CD28, CD27, 4-1BB, DAP-10, OX40, and combinations thereof, as well as other similar molecules and fragments. Intracellular signaling portions of other members of the families of activating proteins can be used, such as FcγRIII and FcεRI. Examples of these alternative transmembrane and intracellular domains can be found, *e.g.*, Gross *et al.* (1992), Stancovski *et al.* (1993), Moritz *et al.* (1994), Hwu *et al.* (1995), Weijtens *et al.* (1996), and Hekele *et al.* (1996), which are incorporated herein be reference in their entirety. In some embodiments, an endodomain may comprise the human CD3ζ intracellular domain.

[0083] The antigen-specific extracellular domain and the intracellular signaling-domain are preferably linked by a transmembrane domain. Transmembrane domains that may be included in a CAR include, *e.g.*, the human IgG4 Fc hinge and Fc regions, the human CD4 transmembrane domain, the human CD28 transmembrane domain, the transmembrane human CD3ζ domain, or a cysteine mutated human CD3ζ domain, or a transmembrane domains from a human transmembrane signaling protein such as, *e.g.*, the CD16 and CD8 and erythropoietin receptor. Examples of transmembrane domains are provided, *e.g.*, in Table 1.

[0084] In some embodiments, the endodomain comprises a sequence encoding a costimulatory receptors such as, *e.g.*, a modified CD28 intracellular signaling domain, or a CD28, CD27, OX-40 (CD134), DAP10, or 4-1BB (CD137) costimulatory receptor. In some embodiments, both a primary signal initiated by CD3 ζ, an additional signal provided by a human costimulatory receptor may be included in a CAR to more effectively activate a transformed T cells, which may help improve *in vivo* persistence and the therapeutic success of the adoptive immunotherapy. As noted in Table 1, the endodomain or intracellular receptor signaling domain may comprise the zeta chain of CD3 alone or in combination with an Fcγ RIII costimulatory signaling domains such as, *e.g.*, CD28, CD27, DAP10, CD137, OX40, CD2, 4-1BB. In some embodiments, the endodomain comprises part or all of one or more of TCR zeta chain, CD28, CD27, OX40/CD134, 4-1BB/CD137, FcεRIγ, ICOS/CD278,

IL-2Rbeta/CD122, IL-2Ralpha/CD132, DAP10, DAP12, and CD40. In some embodiments, 1, 2, 3, 4 or more cytoplasmic domains may be included in an endodomain. For example, in some CARs it has been observed that at least two or three signaling domains fused together can result in an additive or synergistic effect.

[0085] In some aspects, an isolated nucleic acid segment and expression cassette including DNA sequences that encode a CAR may be generated. A variety of vectors may be used. In some preferred embodiments, the vector may allow for delivery of the DNA encoding a CAR to immune such as T cells. CAR expression may be under the control of regulated eukaryotic promoter such as, *e.g.*, the MNDU3 promoter, CMV promoter, EF1alpha promoter, or Ubiquitin promoter. Also, the vector may contain a selectable marker, if for no other reason, to facilitate their manipulation *in vitro*. In some embodiments, the CAR can be expressed from mRNA *in vitro* transcribed from a DNA template.

[0086] Chimeric antigen receptor molecules are recombinant and are distinguished by their ability to both bind antigen and transduce activation signals *via* immunoreceptor activation motifs (ITAM's) present in their cytoplasmic tails. Receptor constructs utilizing an antigen-binding moiety (for example, generated from single chain antibodies (scFv)) afford the additional advantage of being "universal" in that they can bind native antigen on the target cell surface in an HLA-independent fashion. For example, a scFv constructs may be fused to sequences coding for the intracellular portion of the CD3 complex's zeta chain (ζ), the Fc receptor gamma chain, and sky tyrosine kinase (Eshhar *et al.*, 1993; Fitzer-Attas *et al.*, 1998). Re-directed T cell effector mechanisms including tumor recognition and lysis by CTL have been documented in several murine and human antigen-scFv: ζ systems (Eshhar *et al.*, 1997; Altenschmidt *et al.*, 1997; Brocker *et al.*, 1998).

[0087] The antigen binding region may, *e.g.*, be from a human or non-human scFv. One possible problem with using non-human antigen binding regions, such as murine monoclonal antibodies, is reduced human effector functionality and a reduced ability to penetrate into tumor masses. Furthermore, non-human monoclonal antibodies can be recognized by the human host as a foreign protein, and therefore, repeated injections of such foreign antibodies might lead to the induction of immune responses leading to harmful hypersensitivity reactions. For murine-based monoclonal antibodies, this effect has been referred to as a Human Anti-Mouse Antibody (HAMA) response. In some embodiments, inclusion of human antibody or scFv sequences in a CAR may result in little or no HAMA

response as compared to some murine antibodies. Similarly, the inclusion of human sequences in a CAR may be used to reduce or avoid the risk of immune-mediated recognition or elimination by endogenous T cells that reside in the recipient and might recognize processed antigen based on HLA.

[0088] In some embodiments, the CAR comprises: a) an intracellular signaling domain, b) a transmembrane domain, c) a hinge region, and d) an extracellular domain comprising an antigen binding region. In some embodiments, the intracellular signaling domain and the transmembrane domain are encoded with the endodomain by a single vector that can be fused (*e.g.*, via transposon-directed homologous recombination) with a vector encoding a hinge region and a vector encoding an antigen binding region. In other embodiments, the intracellular signaling region and the transmembrane region may be encoded by two separate vectors that are fused (*e.g.*, via transposon-directed homologous recombination).

[0089] In some embodiments, the antigen-specific portion of a CAR, also referred to as an extracellular domain comprising an antigen binding region, selectively targets a tumor associated antigen. A tumor associated antigen may be of any kind so long as it is expressed on the cell surface of tumor cells. Examples of tumor associated antigens that may be targeted with CARs generated via the present invention include, *e.g.*, CD19, CD20, carcinoembryonic antigen, alphafetoprotein, CA-125, MUC-1, CD56, EGFR, c-Met, AKT, Her2, Her3, epithelial tumor antigen, melanoma-associated antigen, mutated p53, mutated ras, Dectin-1, and so forth. In some embodiments that antigen specific portion of the CAR is a scFv. Examples of tumor-targeting scFv are provided in Table 1. In some embodiments, a CAR may be co-expressed with a membrane-bound cytokine, *e.g.*, to improve persistence when there is a low amount of tumor-associated antigen. For example, a CAR can be co-expressed with membrane-bound IL-15.

[0090] In some embodiments, an intracellular tumor associated antigen such as, *e.g.*, HA-1, survivin, WT1, and p53 may be targeted with a CAR. This may be achieved by a CAR expressed on a universal T cell that recognizes the processed peptide described from the intracellular tumor associated antigen in the context of HLA. In addition, the universal T cell may be genetically modified to express a T-cell receptor pairing that recognizes the intracellular processed tumor associated antigen in the context of HLA.

[0091] The pathogen recognized by a CAR may be essentially any kind of pathogen, but in some embodiments the pathogen is a fungus, bacteria, or virus. Exemplary viral pathogens include those of the families of Adenoviridae, Epstein–Barr virus (EBV), Cytomegalovirus (CMV), Respiratory Syncytial Virus (RSV), JC virus, BK virus, HSV, HHV family of viruses, Picornaviridae, Herpesviridae, Hepadnaviridae, Flaviviridae, Retroviridae, Orthomyxoviridae, Paramyxoviridae, Papovaviridae, Polyomavirus, Rhabdoviridae, and Togaviridae. Exemplary pathogenic viruses cause smallpox, influenza, mumps, measles, chickenpox, ebola, and rubella. Exemplary pathogenic fungi include *Candida*, *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Pneumocystis*, and *Stachybotrys*. Exemplary pathogenic bacteria include *Streptococcus*, *Pseudomonas*, *Shigella*, *Campylobacter*, *Staphylococcus*, *Helicobacter*, *E. coli*, *Rickettsia*, *Bacillus*, *Bordetella*, *Chlamydia*, *Spirochetes*, and *Salmonella*. In some embodiments the pathogen receptor Dectin-1 may be used to generate a CAR that recognizes the carbohydrate structure on the cell wall of fungi such as *Aspergillus*. In another embodiment, CARs can be made based on an antibody recognizing viral determinants (*e.g.*, the glycoproteins from CMV and Ebola) to interrupt viral infections and pathology.

[0092] In some embodiments, naked DNA or a suitable vector encoding a CAR can be introduced into a subject's T cells (*e.g.*, T cells obtained from a human patient with cancer or other disease). Methods of stably transfecting T cells by electroporation using naked DNA are known in the art. See, *e.g.*, U.S. Pat. No. 6,410,319. Naked DNA generally refers to the DNA encoding a chimeric receptor of the present invention contained in a plasmid expression vector in proper orientation for expression. In some embodiments, the use of naked DNA may reduce the time required to produce T cells expressing a CAR generated via methods of the present invention.

[0093] Alternatively, a viral vector (*e.g.*, a retroviral vector, adenoviral vector, adeno-associated viral vector, or lentiviral vector) can be used to introduce the chimeric construct into T cells. Generally, a vector encoding a CAR that is used for transfecting a T cell from a subject should generally be non-replicating in the subject's T cells. A large number of vectors are known that are based on viruses, where the copy number of the virus maintained in the cell is low enough to maintain viability of the cell. Illustrative vectors include the pFB-neo vectors (STRATAGENE®) as well as vectors based on HIV, SV40, EBV, HSV, or BPV.

[0094] Once it is established that the transfected or transduced T cell is capable of expressing a CAR as a surface membrane protein with the desired regulation and at a desired level, it can be determined whether the chimeric receptor is functional in the host cell to provide for the desired signal induction. Subsequently, the transduced T cells may be reintroduced or administered to the subject to activate anti-tumor responses in the subject. To facilitate administration, the transduced T cells may be made into a pharmaceutical composition or made into an implant appropriate for administration *in vivo*, with appropriate carriers or diluents, which are preferably pharmaceutically acceptable. The means of making such a composition or an implant have been described in the art (see, for instance, Remington's Pharmaceutical Sciences, 16th Ed., Mack, ed. (1980)). Where appropriate, transduced T cells expressing a CAR can be formulated into a preparation in semisolid or liquid form, such as a capsule, solution, injection, inhalant, or aerosol, in the usual ways for their respective route of administration. Means known in the art can be utilized to prevent or minimize release and absorption of the composition until it reaches the target tissue or organ, or to ensure timed-release of the composition. Generally, a pharmaceutically acceptable form is preferably employed that does not ineffectuate the cells expressing the chimeric receptor. Thus, desirably the transduced T cells can be made into a pharmaceutical composition containing a balanced salt solution such as Hanks' balanced salt solution, or normal saline.

IV. Artificial Antigen Presenting Cells

[0095] In some cases, aAPCs are useful in preparing CAR-based therapeutic compositions and cell therapy products. For general guidance regarding the preparation and use of antigen-presenting systems, see, *e.g.*, U.S. Pat. Nos. 6,225,042, 6,355,479, 6,362,001 and 6,790,662; U.S. Patent Application Publication Nos. 2009/0017000 and 2009/0004142; and International Publication No. WO2007/103009).

[0096] aAPCs may be used to expand T Cells expressing a CAR. During encounter with tumor antigen, the signals delivered to T cells by antigen-presenting cells can affect T-cell programming and their subsequent therapeutic efficacy. This has stimulated efforts to develop artificial antigen-presenting cells that allow optimal control over the signals provided to T cells (Turtle *et al.*, 2010). In addition to antibody or antigen of interest, the aAPC systems may also comprise at least one exogenous assisting molecule. Any suitable number and combination of assisting molecules may be employed. The assisting molecule may be selected from assisting molecules such as co-stimulatory molecules and adhesion molecules.

Exemplary co-stimulatory molecules include CD70 and B7.1 (also called B7 or CD80), which can bind to CD28 and/or CTLA-4 molecules on the surface of T cells, thereby affecting, *e.g.*, T-cell expansion, Th1 differentiation, short-term T-cell survival, and cytokine secretion such as interleukin (IL)-2 (see Kim *et al.*, 2004). Adhesion molecules may include carbohydrate-binding glycoproteins such as selectins, transmembrane binding glycoproteins such as integrins, calcium-dependent proteins such as cadherins, and single-pass transmembrane immunoglobulin (Ig) superfamily proteins, such as intercellular adhesion molecules (ICAMs), that promote, for example, cell-to-cell or cell-to-matrix contact. Exemplary adhesion molecules include LFA-3 and ICAMs, such as ICAM-1. Techniques, methods, and reagents useful for selection, cloning, preparation, and expression of exemplary assisting molecules, including co-stimulatory molecules and adhesion molecules, are exemplified in, *e.g.*, U.S. Pat. Nos. 6,225,042, 6,355,479, and 6,362,001.

[0097] Cells selected to become aAPCs, preferably have deficiencies in intracellular antigen-processing, intracellular peptide trafficking, and/or intracellular MHC Class I or Class II molecule-peptide loading, or are poikilothermic (*i.e.*, less sensitive to temperature challenge than mammalian cell lines), or possess both deficiencies and poikilothermic properties. Preferably, cells selected to become aAPCs also lack the ability to express at least one endogenous counterpart (*e.g.*, endogenous MHC Class I or Class II molecule and/or endogenous assisting molecules as described above) to the exogenous MHC Class I or Class II molecule and assisting molecule components that are introduced into the cells. Furthermore, aAPCs preferably retain the deficiencies and poikilothermic properties that were possessed by the cells prior to their modification to generate the aAPCs. Exemplary aAPCs either constitute or are derived from a transporter associated with antigen processing (TAP)-deficient cell line, such as an insect cell line. An exemplary poikilothermic insect cells line is a Drosophila cell line, such as a Schneider 2 cell line (*e.g.*, Schneider, J.m 1972). Illustrative methods for the preparation, growth, and culture of Schneider 2 cells, are provided in U.S. Pat. Nos. 6,225,042, 6,355,479, and 6,362,001.

[0098] aAPCs may be subjected to a freeze-thaw cycle. For example, aAPCs may be frozen by contacting a suitable receptacle containing the aAPCs with an appropriate amount of liquid nitrogen, solid carbon dioxide (dry ice), or similar low-temperature material, such that freezing occurs rapidly. The frozen aAPCs are then thawed, either by removal of the aAPCs from the low-temperature material and exposure to ambient room temperature

conditions, or by a facilitated thawing process in which a lukewarm water bath or warm hand is employed to facilitate a shorter thawing time. Additionally, aAPCs may be frozen and stored for an extended period of time prior to thawing. Frozen aAPCs may also be thawed and then lyophilized before further use. Preservatives that might detrimentally impact the freeze-thaw procedures, such as dimethyl sulfoxide (DMSO), polyethylene glycols (PEGs), and other preservatives, may be advantageously absent from media containing aAPCs that undergo the freeze-thaw cycle, or are essentially removed, such as by transfer of aAPCs to media that is essentially devoid of such preservatives.

[0099] In other preferred embodiments, xenogenic nucleic acid and nucleic acid endogenous to the aAPCs may be inactivated by crosslinking, so that essentially no cell growth, replication or expression of nucleic acid occurs after the inactivation. For example, aAPCs may be inactivated at a point subsequent to the expression of exogenous MHC and assisting molecules, presentation of such molecules on the surface of the aAPCs, and loading of presented MHC molecules with selected peptide or peptides. Accordingly, such inactivated and selected peptide loaded aAPCs, while rendered essentially incapable of proliferating or replicating, may retain selected peptide presentation function. The crosslinking can also result in aAPCS that are essentially free of contaminating microorganisms, such as bacteria and viruses, without substantially decreasing the antigen-presenting cell function of the aAPCs. Thus crosslinking can be used to maintain the important APC functions of aAPCs while helping to alleviate concerns about safety of a cell therapy product developed using the aAPCs. For methods related to crosslinking and aAPCs, see for example, U.S. Patent Application Publication No. 20090017000, which is incorporated herein by reference.

IV. Examples

[00100] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

Materials and Methods

Generation of clinical-grade DNA plasmids

[00101] The SB transposon, CoOpCD19RCD28 ζ/pSBSO, expresses the human codon optimized (CoOp) 2nd generation CoOpCD19RCD28ζ CAR under EF-1/HTLV hybrid composite promoter (InvivoGen) comprised of Elongation Factor- 1a (EF-1a [Kim *et al.*, 1990] and 59 untranslated region of the Human T-Cell Leukemia Virus (HTLV) [Singh *et al.*, 2011; Davies *et al.*, 2010]. The derivation of this DNA plasmid is described in Figure S1. The SB transposase, SB11, under the cytomegalovirus (CMV) promoter is expressed in cis from the DNA plasmid pCMV-SB11 (Singh *et al.*, 2011; Singh *et al.*, 2008). Both plasmids were sequenced in their entirety and manufactured by Waisman Clinical Biomanufacturing Facility (Madison, WI) using kanamycin for selection of the bacterial strain E. Coli DH5a.

Generation of triple site-specific recombination DNA plasmids – EZ-Build-CARs

[00102] Using the DNA sequence from the CAR described above (CoOpCD19RCD28z/pSBSO), the parts CD19 ScFv, the hinge IgG4 Fc and the domain CD28 transmembrane and cytosolic portion conjugated with CD3ζ signaling domain were flanked by lambda recombination sites, synthetized by Geneart (Life Technologies) as PCR products. These three parts were individually inserted into pDonors221 plasmids (by the enzyme BP clonase (both from Invitrogen). The three plasmids were recombined with the triple site specific recombination Sleeping Beauty plasmid by the enzyme LR PLUS clonase (Invitrogen) generating the EZ-Build CD19CD28ζ CAR in the format scFv-**B**-scaffold-**C**-signaling domain(s) (**FIG. 1**).

Cell counting

[00103] Trypan-blue exclusion was used to distinguish live from dead cells and counted using Cellometer (Nexecelom Bioscience) (Singh *et al.*, 2011).

Isolation of PBMC

[00104] Leukapheresis products from two male volunteer healthy donors were purchased from Key Biologics LLC (Memphis, TN). The peripheral blood mononuclear cells (PBMC) were isolated by our adapting the Biosafe Sepax system (Eysins, Switzerland) for

work in compliance with cGMP. Briefly, after closing all the clamps on the CS-900 kit, 100 mL Ficoll (GE Healthcare) was aseptically transferred via 60 mL syringes to a density gradient media bag (''ficoll bag'') via Luer-lock connector and the tubing was heat sealed using a hand held sealer (Sebra, Model# 2380). The kit was spike-connected to a 1,000 mL bag containing CliniMACS buffer (PBS/EDTA, Miltenyi, Cat#70026) with 20 mL 25% Human Serum Albumin (HSA) (Baxter) (2% v/v, wash buffer) for washes, a final product bag [300 mL Transfer Pack with Coupler (Baxter/ Fenwal 4R2014)] and a reagent/blood bag. Using the density gradient-based separation protocol (v126), the syringe piston was loaded into the centrifuge chamber and the cover of the Sepax aAPC (clone #4) to selectively propagate CAR+ T cells The c-irradiated aAPC were used to numerically expand the genetically modified T cells. Thawed aAPC from WCB were propagated in CM for up to 60 days in VueLife cell culture bags and harvested using Biosafe Sepax II harvest procedure. Briefly, CS-490.1 kit was connected to a 300 mL output bag (transfer pack) via Luer lock connection. The separation chamber was installed in the pit and the tubing was inserted into the optical sensor and stopcocks aligned in T-position. After connecting the pressure sensor line, the product bag and supernatant/plasma bags were hung on the holder. The modified protocol PBSCv302 was selected from the Sepax menu and the volume of input product to be processed (initial volume) was set to #840 mL. After validation and kit test, the procedure was started. Following completion, the bags were removed, clamps closed and the kit was removed. The cells from the final product bag were aseptically removed, washed twice with wash media (10% HSA in Plasmalyte) and counted. aAPC were irradiated (100 Gy) using a CIS BIO International radiator (IBL-437 C#09433) and cryopreserved for later use in cryopreservation media using controlled-rate freezer (Planer Kryo 750). The anti-CD3 (OKT3) loaded K562-derived aAPC clone #4 was used to propagate control (CARneg) autologous control T cells that had not undergone genetic modification. The aAPC, obtained from culture, were incubated overnight in serum-free X-Vivo 15 (cat # 04-744Q, Lonza) containing 0.2% acetyl cysteine (Acetadote, Cumberland Pharmaceuticals) termed Loading Medium (LM). The next day cells were washed, irradiated (100 Gy) using a Gamma Cell 1000 Elite Cs-137 radiator (MDS Nordion), resuspended in LM at a concentration of 106 cells/mL along with 1 mg/10^6 cells of functional grade purified anti-human CD3 (clone-OKT3, 16-0037-85, eBioscience) and incubated with gentle agitation on a 3-D rotator (Lab-Line) at 4^0C for 30 minutes. Following three washes with LM the cells were used in experiments or frozen in aliquots in liquid nitrogen in vapor layer for later use.

Manufacture of CAR+ T cells

[00105] Thawed PBMC were resuspended in (i) Human T-cell kit (cat# VPA-1002, Lonza; 100 µL for $2x10^7$ cells in one cuvette), with (ii) the DNA plasmid (CoOpCD19RCD28/pSBSO) coding for CD19RCD28 CAR transposon (15 µg supercoiled DNA per $2x10^7$ PBMC per cuvette), and (iii) the DNA plasmid (pCMVSB11) coding for SB11 transposase (5 µg supercoiled DNA per $2x10^7$ PBMC per cuvette). This mixture was immediately transferred to a cuvette (Lonza), electroporated (defining culture day 0) using Nucleofector II (Program U-14, Amaxa/Lonza), rested in 10% RPMI complete media for 2 to 3 hours, and after a half-media change, incubated overnight at 37°C, 5% CO_2. The following day, cells were harvested, counted, phenotyped by flow cytometry, and co-cultured with c-irradiated aAPC at a ratio of 1:2 (CAR + T cell:aAPC), which marked culture day 1 and the beginning of a 7-day stimulation cycle. IL-21 (cat # AF-200-21, PeproTech) and IL-2 (cat # NDC 65483-116-07, Novartis) were added on a Monday-Wednesday-Friday schedule onwards of day 1 and day 7 respectively. NK cells can prevent the numeric expansion of CAR + T cells, especially if their overgrowth occurs early in the tissue culturing process. Therefore, a CD56-depletion was performed if CD3negCD56 + cells $10% using CD56 beads (cat #70206, Miltenyi Biotech, 20 mL beads/107 cells) on LS columns (cat #130-042-401, Miltenyi Biotech) in CliniMACS buffer containing 25% HSA (80 mL/107 cells).

Generation of CAR^{neg} control T cells

[00106] As a control, $5x10^6$ mock transfected PBMC were co-cultured with irradiated and anti-CD3 (OKT3) loaded K562-derived aAPC clone #4 at a ratio of 1:1 in a 7-day stimulation cycle. All the cultures were supplemented with IL-21 (30 ng/mL) from culture day 1 onwards, and IL-2 (50 U/mL) starting 7 days after the start of the culture. All cytokines were subsequently added every other day

Immunophenotype of cells

[00107] Cells were stained using antibodies in 100 mL FACS Buffer (2% FBS, 0.1% Sodium Azide) for 30 minutes at 4°C. Acquisition was performed using FACSCalibur (BD Bioscience) and analyzed using FCS Express 3.00.0612

Chromium Release Assay

[00108] T cells were evaluated for their cytotoxicity in a standard 4-hour chromium release assay using ^{51}Cr-labeled target cells. T cells were plated in triplicate at $1x10^5$, $0.5x10^5$, $0.25x10^5$, $0.125x10^5$ bottom plate (Costar). After incubation, 50 µL of supernatant was harvested onto LumaPlate (Perkin Elmer), read in TopCount NXT (Perkin Elmer) and percent specific lysis was calculated per:

$$\frac{\text{Experimental } ^{51}\text{Cr released - Spontaneous } ^{51}\text{Cr released}}{\text{Maximum } ^{51}\text{Cr released - Spontaneous } ^{51}\text{Cr released}} \times 100$$

[00109] Spontaneous and maximum release was determined by measuring chromium in the conditioned supernatant from target cells incubated with CM or 0.1% Triton X-100 (Sigma), respectively and $0.0625x10^5$ cells/well with $5x10^3$ target cells in a 96-well V (Manufacturee Novo Software, Thornhill, Ontario, Canada).

Example 2

Generation of CD19$^+$ CAR

[00110] A CD19+ CAR was generated using the methods described above in Example 1 (referred to as the "EZ" method). These CD19$^+$ CAR (CD19CAR) were compared to a clinical grade ("CG") CD19CAR generated via a previous method.

[00111] The data showed that the triple site-recombination system generated a CD19CAR (EZ) similar to the clinical grade CD19CAR (CG). The footprints left by recombination-sites in the plasmids did not interfere in the expression and function of the CAR (**FIGS. 2A-B**).

Example 3

Generation of CAR containing (CD8, CD28) Transmembrane Domains and (CD28, 4-1BB) Signaling Domains

[00112] Various CARs tested have shown similar expansion, cytotoxicity, and Th1 cytotoxicity. CD19-BB-z has shown lower production of Th2 cytokines; *in vivo*, it was efficient in controlling disease in mice (Molecular Therapy 17 (8): 1453–1464, 2009).

Nonetheless, a concern exists due to the fact that cells persisted *in vitro* without antigenic stimulation.

[00113] CARs in the clinic are shown below in Table 2. In some embodiments, a CAR of the present invention does not have the specific construct as shown in the below Table 2. Alternately, in some embodiments, methods of the present invention may be used to generate another variation of a CAR having the characteristics of the CAR mentioned below in Table 2 that is nonetheless distinct from the CAR currently being used in the clinic.

Table 2: CARs in the Clinic

Clinical Trial	UPenn	Cooper (MDACC)
Gene Transfer Method	Lentivirus	Electroporation/ Sleeping Beauty
scfv derived from	FMC63	FMC63
Scaffold	CD8alpha	IgG4
Space region	69 aa	230 aa
Transmembrane	CD8alpha	CD28
CAR signaling endomain(s)	CD137 and CD3-zeta	CD28 and CD3-zeta
Culture Method	CD3/CD28 beads	K562 aAPC
Cytokine	IL-2	IL-2 and IL-21
Culture Time	14 days	28 days
Transgene Expression in product infused	4-23%	>80%

[00114] Specific CAR construct designs are illustrated in FIG. 3. As shown in FIG. 3, a schematic of various CARs using a combination of CD19scfv, CD8a hinge or IgG4 Fc stalk, CD8 transmembrane (TM) or CD28 TM or CD137 TM and signaling through CD28 or CD137 endodomain along with CD3zeta endodomain were generated.

[00115] The CAR constructs shown in FIG. 3 were then cloned into Sleeping Beauty plasmids containing SIM and FRA tags to allow tracking in competitive repopulation studies,

when amplified using a common CVseq7 primer. The Sleeping Beauty tracking plasmids are shown in FIG. 4.

[00116] The CAR constructs shown in FIG. 3 were electroporated into T cells using Amaxa Nucleofector II and co-cultured with aAPC for 28 days in the presence of cytokines (IL2, IL-21). CAR expression the day after electroporation (day 1) and after 28 days of co-culture with aAPC (day 28) is shown. Dot-plots for CD3 and CAR are shown, where CD3 and anti-CD19scfv specific Ab was used to distinguish T cells and CAR. CAR expression results are shown in FIG. 5.

[00117] The CAR constructs shown in FIG. 3 were evaluated for CAR expression over time for 28 days and is shown. After 21 days most of the cultures had >80% CAR expression. CAR expression kinetics are shown in FIG. 6.

[00118] Percent expression of CD4 and CD8 T cells in cultures nucleofected with CARs from Fig 3 is shown after 28 days of co-culture with aAPC. These phenotype results are shown in FIG. 7.

[00119] After 28 days of co-culture CAR^+ T cells (expressing the CAR described in FIG. 3) were evaluated for expression of markers pertaining to memory (CD45RA, CCR7, CD27), activation (CD69, HLA-DR), cytotoxic (Perforin, Granzyme B), exhaustion/senescence (CD57, KLRG1, PD1), and adhesion (CD39, CD150). Results for this extended phenotype are shoen in FIGS. 8A-B.

[00120] CAR^+ T cells (expressing the CAR described in FIG. 3) were evaluated for expression of CD3 ζ using western blot. Cell lysates were run under denaturing conditions, transferred and the expression of chimeric CD3 ζ was measured using a primary mouse anti-human CD3ζ mAb and HRP-conjugated goat anti-mouse IgG using SuperSignal West Femto Maximum Sensitivity substrate. Chimeric CD3ζ bands at 52, 71 and 78 kD are observed relative to size of CAR constructs. These western blot results are shown in FIG. 9.

[00121] T cells electroporated with the CAR constructs (described in FIG. 3) were stimulated with K562 aAPC at day 1 and every 7 days thereafter for 28 days. At the end of each stimulation cycle, cells were counted using trypan blue exclusion method and phenotyped for CD3 and CAR expression. The graphs shown in FIG. 10 depict inferred cell counts for total, CD3, and CAR^+ T cells over time.

[00122] Expansion of cells was measured. Fold expansion for Total Cells (FIG.11) and CAR^+ (FIG.12) T cells was calculated at day 14, 21 and 28 days of co-culture by comparing counts to day 1 (post electroporation). Results are shown in FIG. 11 and FIG. 12.

[00123] Cytotoxicity was measured for the CAR-expressing T Cells (CAR^+ T cells). CAR^+ T cells (expressing CAR described in FIG. 3) were evaluated for their cytotoxicity against $CD19^+$ tumor targets (Daudiβ_2m, NALM-6 and $CD19^+$ EL-4) as compared to $CD19^{neg}$ EL-4 in a standard 4-hr chromium release assay. Results are shown in FIG. 13, FIG. 14, FIG. 15, and FIG. 16.

[00124] Intracellular IFN-γ production. CAR^+ T cells (expressing the constructs described in FIG. 3) were incubated with ($CD19^+$ and $CD19^{neg}$) stimulator cells in the presence of protein transport inhibitor for 4-6 hr, fixed, permeabilized and stained with IFN-γ specific mAb. PMA-Ionomycin was used as a positive control. Results for intracellular IFN-γ production are shown in FIG. 17, FIG. 18, FIG. 19, and FIG. 20.

[00125] PCR for SB11 transposase. DNA isolated from CAR+ T cells (Fig 3) was amplified using SB11 specific primers in a thermal cycler. GAPDH was used as the housekeeping gene, and linearized pCMV-SB11 plasmid, genomic DNA from Jurkat cells expressing SB11 were used as positive controls. CAR^{neg} cells (No DNA) were used as negative controls. These PCR results are shown in FIG. 21.

[00126] CAR copy number was measured using quantitative PCR (qPCR). The integrated number of CAR transgene in cells (of the CAR constructs shown in FIG. 3) were evaluated by amplifying genomic DNA using primers and probes specific for the IgG4 Fc stalk and inverted/direct repeats (IR/DR). RNAse P gene was used as an internal control, and the Jurkat cell line expressing a single copy of CAR was used to generate a standard curve. Results are shown in FIG. 22.

[00127] Next, CAR+ T cells were measured for the presence or absence of autonomous growth. Aberrant growth of CAR+ T cells (expressing CAR constructs shown in FIG. 3) was monitored and measured by culturing T cells in the absence of cytokines and aAPC. Cells were counted every 7 days and the percents alive/dead cells (from day 1) were calculated and plotted. As shown in FIG. 23, more than 80% of T cells were observed to be dead by day 14 showing lack of autonomous growth.

[00128] Various CARs could be expressed (>80%), expanded (~1010) and were cytotoxic (~60%, Daudi) to similar extend. Scaffolding domains (IgG4 or CD8α) were used to build CAR and didn't effect expression or potency. Transmembrane domains (CD8, CD28) did not affect potency. 4-1BB transmembrane domain (216) affected expression (anti-scFv Ab), but not cytoxicity and cytokine production. Combination of signaling domains, CD28 and 4-1BB did not have an additive effect. CAR+ T cells exhibited memory/effector phenotype. CARs containing only 4-1BB domain (212, 214, 217) had higher CCR7 expression as compared to others. Cells expressed markers for memory (CD27hi, CD45RAhi, CCR7lo), activation (CD69med, HLA-DRhi), cytolysis (granzymehi, perforinlo), and adhesion (CD39hi, CD150lo), but negligible amounts of inhibitory markers (CD57, PD1, KLRG1) were observed. All the CARs including the ones containing 4-1BB domain lacked SB11 transposase and did not auto-proliferate.

Example 4

Generation of CAR containing CD3-zeta

[00129] CAR containing CD3ζ are provided in this example. A general diagram of CAR design is shown in FIG. 24. As shown in FIG. 24, a comparison of CAR design (FIG. 24, right) with an antibody molecule (FIG, 24, left) are shown.

[00130] CD3ζ sequences are shown in FIG. 25. The sequence of CD3zeta and its isoform are shown in FIG. 25. The CAR designs included CD3 zeta (isoform 1) which forms one of the endodomain signaling moieties and has three ITAMs.

[00131] Specific CAR constructs are shown in FIG. 26 and FIG. 27. FIG. 26 shows a schematic of CD19-specific CARs having long (IgG4), medium (CD8a hinge) and small (IgG 12 aa) stalks which signaling through CD28 or CD137 endodomains. Nomenclature of CAR molecules with different stalks and signaling are shown in FIG. 27.

[00132] CAR expression was measured. Expression of CAR (as described in FIG. 26) was measured the day after electroporation (day 1) and after 28 days of co-culture on aAPC (day 28). Dot plots of CD3 and CAR (as measured by CD19scfv-specific mAb) are shown in FIG. 28.

[00133] Expansion kinetics were measured for the CAR. T cells electroporated with CAR constructs (shown in FIG. 26) were co-cultured on aAPC in a 7-day stimulation

cycle. Cells were counted and evaluated for expression of CD3 and CAR. Results are shown in FIG. 29 and FIG. 30.

[00134] Cytotoxicity of the CAR^+ T cells was measured. At the end of 28 days of co-culture CAR^+ T cells (expressing constructs shown in FIG. 26) were evaluated for cytotoxicity against tumor targets in a chromium release assay. As shown in FIG 31, percent cytotoxicity was measured at various effector-to-target ratio for CD19RCD28 (CAR 194) and CD19RCD137 (CAR 217) CARs against $CD19^+$ and $CD19^{neg}$ tumor targets. As shown in FIG 32, data were obtained for percent lysis of $CD19^+$ EL-4 by CAR^+ T cells (expressing CAR constructs shown in FIG. 26) at E:T ratio of 20:1. The percent expression of CD27, CD62L, CD28 and CCR7 on CAR^+ T cells (expressing constructs shown in FIG. 26) was measured, and results are shown in FIG. 33.

[00135] Intracellular cytokine production was measured for the CAR^+ T cells. Stimulator cells ($CD19^+$ and $CD19^{neg}$) were incubated with CAR^+ T cells (expressing CAR shown in FIG. 26) for 4 hr in the presence of protein transport inhibitor and stained with IFN-γ and IL-2 mAb. PMA-Ionomycin served as a positive control and T cells alone served as negative control. FIG 34 shows percentage of IFN- γ producing cells after stimulation. FIG 35 shows breakdown of IFN- γ and or, IL-2 producing cells after incubation with cell stimulation cocktail (PMA-Ionomycin).

[00136] The CAR^+ T cells were measured for the presence of absence of autonomous growth. CAR^+ T cells (expressing CAR described in FIG. 26) were evaluated for their lack of aberrant growth in the absence of external stimulation (cytokines and aAPC) for 18 days. At the end of 18 days, more than 80% of the cells were dead showing lack of unwanted growth. As shown in FIG. 36, a lack of autonomous growth was observed.

[00137] CAR copy number was measured in the CAR^+ T cells. The number of copies of integrated CAR molecule was evaluated using primers/probes specific for IgG4-Fc and IR/DR regions by qPCR. As shown in FIG 37, CAR copy number integrated (of CAR shown in FIG. 26) was observed using the IR/DR probe. As shown in FIG 38 and FIG. 39, a compilation of CAR copy number data are provided in a table and graphical form for CAR constructs (for both CAR constructs shown in FIG. 3 and CAR constructs shown in FIG. 26) as tested in two separate experiments (P491;C714 and GCR357861).

[00138] These data show that CARs with various spacers can be expressed and grown *in vitro* in the culture system as described herein. All CARs were observed to have similar CAR expression. The maximum cytotoxicity of CD19+ EL-4s was observed in CARs with a CD8 hinge region. Similar expression of CD62L and CD28 was observed on all CARs tested. High integration frequency as measured by CAR copy number was observed in all CAR, except for CAR containing IgG4-Fc stalk. A lack of autonomous growth and SB11 was observed by PCR. Contrary to previous reports, inclusion of a 12aa spacer in the CAR did not confer improved functionality in these studies.

Example 5

Rapid Assembly of CARs from Principal Components

[00139] The inventors generated a CD19-specific CAR that is activated through chimeric CD28/CD3-zeta using the EZ CAR platform in parallel with clinical-grade CD19RCD28mζ CAR+ T cells (CG CAR). Both, Clinical Grade CD28/CD3-ζ and EZ CAR CD19RCD28mζ CARs sequences were inserted into Sleeping Beauty transposon vectors and electroporated into T cells. After electroporation the T cells were cultivated in presence of CD19+ artificial Antigen Presenting Cells (also called Activating and Propagating Cells, or AaPCs) for antigen specific expansion of the T cells. The expression of the CARs in the T cell's surface was measured every week by flow cytometry (Fc+ expression), showing similar CAR expression in Clinical Grade CD19 CAR T cells and EZ CD19 CAR T cells. A Chromium Release Assay (CRA) was also performed to evaluate the killing function of T cells CD19 CAR+ generated by EZ CAR platform against tumor cells. After 4 hours of incubation the percentage of specific cell lysis was observed to be 52% by the EZ CAR T cells and 49% by the CG CAR T cells.

[00140] These results demonstrate that functional CAR^+ T cells were generated using these methods. The inventors then performed a rapid production of CARs using methods as described above in combination with a library of plasmids containing the following three components of a CAR molecule: (i) anti-CD19 scFv (ii) 5 hinges with different sizes (long - IgG4a and IgG4ΔEQ, medium - CD8α, short – t-20AA and t-12AA) and (iii) different combinations of 7 signaling domains (CD27, CD28, $CD28\Delta Y^{173}\rightarrow F^{173}$, CD134, CD137, CD278) with the CD3ζ domain. Transfection of HEK 293 cells with plasmid containing the CAR transgene were used to screen 27 different CARs constructs to ensure the expression of the CAR protein in the cell surface. The high throughput testing of individual CAR

molecules was undertaken using the iQue™ Screener (Intellicyt, Albuquerque, NM), a high throughput flow cytometer, where cytotoxic assays are performed using engineered target cells expressing a fluorescent granzyme B reporter or GFP. Results are shown in FIGS. 40A-E.

[00141] Additional experiments were performed to screen the CAR molecules using IntelliCyt's iQue™. iQue™ uses high throughput flow cytometry, a complementary technology that generates information by studying large populations using multiplexing capabilities and cell-by-cell analysis. The inventors adapted this technology to inform on the therapeutic potential of T cells modified with panels of CARs. T cells from the wells can be stained for viability, as well as activation signals (*e.g.*, upregulation of CD25), cytokine release, and killing. Thus the inventors adapted the iQue Screener and harnessed its ability to perform multiplexed bead-based cytokine detection and cell-based assays. The results obtained indicate that this technology may be used to test a large number of different CAR T cells generated by the EZ CAR platform. Data was generated using IntelliCyt's iQue™, where 2 populations of CAR T cells were evaluated on their abilities to kill target cells. Results are shown in FIGS. 41A-B and FIG. 42. These results demonstrate that the CAR molecules were active and the iQue™ method may be effectively used to evaluate CAR activity.

* * *

[00142] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS

WHAT IS CLAIMED IS:

1. A nucleic acid encoding a chimeric antigen receptor comprising CAR 213 (SEQ ID NO: 5).

2. The nucleic acid of claim 1, wherein the nucleic acid is comprised in a T cell.

3. The nucleic acid of claim 1, wherein the T cell is an alpha beta T cell, a gamma delta T cell, or a NKT cell.

4. A Sleeping Beauty transposon comprising a nucleic acid encoding a chimeric antigen receptor comprising CAR 213 (SEQ ID NO: 5).

5. A transformed T cell comprising a nucleic acid encoding a chimeric antigen receptor encoded by SEQ ID NO: 5.

6. The transformed T cell of claim 5 further comprising a membrane-bound cytokine.

7. The transformed T cell of claim 6, wherein the membrane-bound cytokine is membrane-bound IL-15.