SUMMARY

[0004] In one aspect, the present disclosure provides a polypeptide encoded by a nucleic acid comprising nucleotides 67-1485 of SEQ ID NO:5. In another aspect, the present disclosure provides a T cell expressing the polypeptide as described herein.

[0004a] In some aspects, the present disclosure provides a pharmaceutical composition comprising the T cell as described herein and a pharmaceutically acceptable carrier or diluent. In another aspects, the present disclosure provides use of the composition as described herein in the manufacture of a medicament for treating or preventing cancer in a mammal.

[0004b] In some aspects, the present disclosure provides a nucleic acid comprising nucleotides 67-1485 of SEQ ID NO: 5. In other aspects, the present disclosure provides a T cell comprising the nucleic acid as described herein.

[0004c] In some aspects, the present disclosure provides a transposon comprising the nucleic acid as described herein. In other aspects, the present disclosure provides a T cell comprising the transposon as described herein.

[0004d] In some aspects, the present disclosure provides a pharmaceutical composition comprising the T cell as described herein and a pharmaceutically acceptable carrier or diluent. In other aspects, the present disclosure provides use of the composition as described herein, in the manufacture of a medicament for treating or preventing cancer in a mammal.

[0005] The present disclosure provides, 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.

[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, provided are 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 according to the present disclosure 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 of the present disclosure or provided 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] An example of the present disclosure relates to 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] In the present disclosure, 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] Another example of the present disclosure relates to 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] Yet another example of the present disclosure relates to 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 of the present disclosure (*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 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. 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] Another example of the present disclosure relates to 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] Yet another example of the present disclosure relates to 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] Another example of the present disclosure relates to a pharmaceutical preparation comprising the transformed T cell of the present disclosure.

[0019] Yet another example of the present disclosure relates to 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] Another example of the present disclosure relates to 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, he 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 of the present disclosure 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 of the present disclosure 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 according to the present disclosure 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)

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

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 preferred examples, it may refer to a plurality of scFv regions derived from monoclonal antibodies that selectively target CD19). It is anticipated that methods of the present disclosure 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 disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred examples of the disclosure, are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

CLAIMS

WHAT IS CLAIMED IS:

1. A polypeptide encoded by a nucleic acid comprising nucleotides 67-1485 of SEQ ID NO:5.

2. The polypeptide of claim 1, wherein the nucleic acid comprises nucleotides 1-1485 of SEQ ID NO: 5.

3. The polypeptide of claim 1, wherein the nucleic acid comprises SEQ ID NO: 5.

4. The polypeptide of any one of claims 1-3, wherein the polypeptide specifically binds to CD19.

5. The polypeptide of any one of claims 1-4, wherein the polypeptide comprises a chimeric antigen receptor.

6. The polypeptide of any one of claims 1-5, wherein the polypeptide causes or promotes activation of at least one normal effector function of an immune cell comprising the polypeptide.

7. A T cell expressing the polypeptide of any one of claims 1-6.

8. The T cell of claim 7, wherein the cell is an immortalized cell.

9. The T cell of claim 7 or 8, wherein the T cell is an alpha beta T cell, a gamma delta T cell, or a NKT cell.

10. The T cell of any one of claims 7-9, further comprising a membrane-bound cytokine.

11. The T cell of claim 10, wherein the membrane-bound cytokine is membrane-bound IL-15.

12. A pharmaceutical composition comprising the T cell of any one of claims 7-11 and a pharmaceutically acceptable carrier or diluent.

13. Use of the composition of claim 12 in the manufacture of a medicament for treating or preventing cancer in a mammal.

14. The use of claim 13, wherein the cancer is a B-cell malignancy.

15. The use of claim 13, wherein the cancer is a lymphoma or a leukemia.

16. A nucleic acid comprising nucleotides 67-1485 of SEQ ID NO: 5.

17. The nucleic acid of claim 16, wherein the nucleic acid comprises nucleotides 1-1485 of SEQ ID NO: 5.

18. The nucleic acid of claim 16, wherein the nucleic acid comprises SEQ ID NO: 5.

19. The nucleic acid of any one of claims 16-18, wherein the nucleic acid encodes a polypeptide that specifically binds to CD19.

20. The nucleic acid of claim 19, wherein the polypeptide comprises a chimeric antigen receptor.

21. The nucleic acid of claim 19 or 20, wherein the polypeptide causes or promotes activation of at least one normal effector function in an immune cell comprising the polypeptide.

22. A T cell comprising the nucleic acid of any one of claims 16-21.

23. The T cell of claim 22, wherein the cell is an immortalized cell.

24. The T cell of claim 22 or 23, wherein the T cell is an alpha beta T cell, a gamma delta T cell, or an NKT cell.

25. The T cell of any one of claims 22-24, further comprising a membrane-bound cytokine.

26. The T cell of claim 25, wherein the membrane-bound cytokine is membrane-bound IL-15.

27. A transposon comprising the nucleic acid of any one of claims 16-21.

28. A T cell comprising the transposon of claim 27.

29. The T cell of claim 28, wherein the cell is an immortalized cell.

30. The T cell of claim 28 or 29, wherein the T cell is an alpha beta T cell, a gamma delta T cell, or an NKT cell.

31. The T cell of any one of claims 28-30, further comprising a membrane-bound cytokine.

32. The T cell of claim 31, wherein the membrane-bound cytokine is membrane-bound IL-15.

33. A pharmaceutical composition comprising the T cell of any one of claims 22-26 or 28-32 and a pharmaceutically acceptable carrier or diluent.

34. Use of the composition of claim 33, in the manufacture of a medicament for treating or preventing cancer in a mammal.

35. The use of claim 34, wherein the cancer is a B-cell malignancy.

36. The use of claim 34, wherein the cancer is a lymphoma or a leukemia.