

Engineered cells expressing trimeric complexes

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is based on an international patent applications with the application number PCT/CN2023/112027, and claims priority from said international patent applications, the entire content of which is hereby incorporated by reference into this application.

FIELD OF THE INVENTION

[0002] The instant disclosure relates generally to engineered cells expressing trimeric complexes capable of binding to FasL. It further provides fusion polypeptides which participate in the formation of the trimeric complexes.

BACKGROUND

[0003] Fas, also known as CD95, is a cell surface receptor belonging to the tumor necrosis factor (TNF) receptor family. Activation of Fas by binding to its ligand, FasL, triggers a signaling cascade that activates caspases and ultimately leads to cell death (10.1038/sj.cdd.4401305). In the context of cancer, tumor cells can exploit the Fas/FasL pathway to evade immune surveillance. Tumor cells expressing FasL can induce AICD in infiltrating T cells, thereby limiting the immune response against the tumor (10.1007/s002620050505, 10.1016/s0167-5699(98)01382-6).

[0004] In addition to FasL expression on the tumor, FasL is upregulated on CAR T cells when they encounter cancer cells which can subsequently lead to premature apoptosis limiting their persistence in patients (10.1016/s1074-7613(00)80566-x). Consequently, developing strategies to promote CAR T cell persistence while mitigating activation-induced cell death has become a key focus of research in the field of CAR T cell therapy.

BRIEF SUMMARY

[0005] The present disclosure provides novel engineered cells expressing trimeric complexes. These trimeric complexes enhance persistence and expansion of the engineered cells without the requirements of exogenous administration of antibodies or cytokines, especially in tumor microenvironment. The expressing of the trimeric complexes also ameliorates the toxicity issues with systemic administration through restricting the cytokine signal to within the engineered cells, minimizing its effect to the surrounding environment.

[0006] The disclosure provides an engineered cell expressing a trimeric complex comprising at least one fusion polypeptide, wherein the fusion polypeptide comprises, from its N to C terminus in order, a) an extracellular component comprising a Fas ectodomain; b) a transmembrane component; and c) an intracellular component comprising an IL-7R endodomain, a CD2 endodomain, or a combination thereof, wherein the intracellular component doesn't comprise a Fas endodomain. In one aspect, the trimeric complex is capable of binding to Fas ligand. In one aspect, the trimeric complex is capable of inducing the signaling of the intracellular component independent of the binding to Fas ligand.

[0007] The disclosure further provides a fusion polypeptide comprising a) an extracellular component comprising a Fas ectodomain; b) a transmembrane component; and c) an intracellular component comprising an IL-7R endodomain, a CD2 endodomain, or a combination thereof, wherein the intracellular component doesn't comprise a Fas endodomain. The disclosure also provides a trimeric complex comprising at least one fusion polypeptide comprising a) an extracellular component comprising a Fas ectodomain; b) a transmembrane component; and c) an intracellular component comprising an IL-7R endodomain, a CD2 endodomain, or a combination thereof, wherein the intracellular component doesn't comprise a Fas endodomain. In one aspect, the components in the fusion polypeptide are arranged in order from N to C terminus.

[0008] In one aspect, the extracellular component comprises or consists of an amino acid sequence that is at least 90% sequence identity to SEQ ID NO: 1. In one aspect, the extracellular component comprises a cysteine-rich domain 1, a cysteine-rich domain 2 and a cysteine-rich domain 3 of the amino acid sequence

of SEQ ID NO: 1. In one aspect, the extracellular component comprises or consists of the amino acid sequence of SEQ ID NO: 1.

[0009] In one aspect, the transmembrane component is capable of self-oligomerizing. In one aspect, the transmembrane component comprises or consists of a transmembrane domain comprising a gain-of-function insertion compared to SEQ ID NO: 10. In one aspect, the transmembrane component comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 11 to SEQ ID NO: 33. In one aspect, the transmembrane component comprises or consists of the amino acid sequence of SEQ ID NO: 11 (PILLTCPTIX₁IX₂SX₃X₄X₅X₆X₇X₈X₉X₁₀X₁₁LX₁₂X₁₃X₁₄LW), wherein X₁ is S or L; X₂ is L or S; X₃ is F or L; X₄ is F or A; X₅ is S or I; X₆ is V or L; X₇ is A or L; X₈ is L or M; X₉ is L or V; X₁₀ is V or S; X₁₁ is I or L; X₁₂ is A or L; X₁₃ is C or L; and X₁₄ is V or S. In one aspect, the transmembrane component comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 39.

[0010] In one aspect, the intracellular component comprises or consists of an IL-7R α endodomain. In one aspect, the intracellular component comprises or consists of an amino acid sequence that is at least 90% sequence identity to SEQ ID NO: 4. In one aspect, the intracellular component comprises or consists of the amino acid sequence of SEQ ID NO: 4.

[0011] In one aspect, the intracellular component comprises or consists of a CD2 endodomain. In one aspect, the intracellular component comprises or consists of a full-length CD2 endodomain or a functional fragment thereof. In one aspect, the functional fragment is truncated at the C-terminus by up to 65 amino acids compared to the full-length CD2 endodomain. In one aspect, the intracellular component comprises or consists of an amino acid sequence that is at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 5 to SEQ ID NO: 8. In one aspect, the intracellular component comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 5 to SEQ ID NO: 8.

[0012] In one aspect, the transmembrane component is capable of self-oligomerizing, the intracellular component comprises or consists of an IL-7R α endodomain that is fused by its N-terminus to the C-terminus of the transmembrane component. In one aspect, the intracellular component comprises or consists of an IL-7R α endodomain, and the transmembrane component comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 11 to SEQ ID NO: 33. In one aspect, the intracellular component comprises or consists of the amino acid sequence of SEQ ID NO: 4, and the transmembrane component comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 39.

[0013] In one aspect, the intracellular component comprises or consists of an IL-7R α endodomain and a CD2 endodomain, the transmembrane component is capable of self-oligomerizing, the IL-7R endodomain is fused by its N-terminus to the C-terminus of the transmembrane component, the IL-7R endodomain is fused by its C-terminus to the N-terminus of the CD2 endodomain. In one aspect, the intracellular component comprises or consists of a full-length CD2 endodomain or a functional fragment thereof, wherein the functional fragment is truncated at the C-terminus by up to 65 amino acids compared to the full-length CD2 endodomain; and the transmembrane component comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 11 to SEQ ID NO: 33. In one aspect, the intracellular component comprises or consists of the amino acid sequence of SEQ ID NO: 4 and an amino acid sequence selected from the group consisting of SEQ ID NO: 5 to SEQ ID NO: 8, and the transmembrane component comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 39.

[0014] In one aspect, the transmembrane component comprises or consists of a transmembrane domain selected from the group consisting of Fas, CD2, CD3s, CD35, CD3C, CD25, CD27, CD28, CD40, CD79A, CD79B, CD80, CD86, OX40, 4-1BB, SLAMF1, CTLA4, CD200R, LAG3, HVEM, BTLA, PD-L2, PD-L1, ICOS, PD-1, CD300, GITR, A2aR, DAP10, FcR α , FcR β , FcR γ , Fyn, GAL9, KIR, Lck, LAT, LRP, KG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPa, pTa, TCRA, TCR β , TIM3, TRIM, LPA5 and Zap70, the intracellular component comprises or consists of a CD2 endodomain that is fused by its N-terminus to the C-terminus of the transmembrane component. In one aspect, the CD2 endodomain is a full-length CD2 endodomain or a functional fragment thereof, wherein the functional fragment is truncated at the C-terminus by up to 65 amino acids compared to the full-length CD2 endodomain, the transmembrane component comprises or consists of Fas transmembrane domain. In one aspect, the intracellular component comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 5 to SEQ ID NO: 8, and the transmembrane component comprises or consists of the amino acid sequence of SEQ ID NO: 9.

[0015] In one aspect, the trimeric complex is expressed on the surface of the engineered cell. In one aspect, the trimeric complex is a homotrimer or a heterotrimer, wherein the heterotrimer comprises an endogenous Fas. In one aspect, the trimeric complex comprises two or three fusion polypeptides when the intracellular component comprises an IL-7R endodomain.

[0016] In one aspect, the engineered cell is an immune cell. In one aspect, the engineered cell is a lymphocyte. In one aspect, the engineered cell is a NK cell, a T cell, or a combination thereof.

[0017] In one aspect, the engineered cell comprises an antigen receptor. In one aspect, the antigen receptor is a chimeric antigen receptor or an antigen-specific TCR. In one aspect, the chimeric antigen receptor targets against CD19, CD20, CD22, CD30, BCMA, AFP, GPC3, HER2, EGFR alpha folate receptor, 5T4, $\alpha\beta$ 6 integrin, B7-H3, B7-H6, CAIX, CD16, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD138, CD171, CEA, Claudin18.2, CSPG4, DLL3, EGFR, HER2, EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, fetal AchR, FRA, GD2, GD3, MAGE-1, NY-ESO-1, IL-11R α , IL-13R α 2, Lambda, Lewis-Y, Kappa, Mesothelin, Muc1, Muc16, NCAM, NKG2D Ligands, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TAG72, TEMs, VEGFR2 or WT-1.

[0018] In one aspect, the engineered cell expresses an exogenous FasL. In one aspect, the exogenous FasL is secreted or membrane bound. In one aspect, the exogenous FasL comprises or consists of an amino acid sequence that is at least 90% sequence identity to an amino acid selected from the group consisting of SEQ ID NO: 40 to SEQ ID NO: 47. In one aspect, the exogenous FasL comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 40 to SEQ ID NO: 47.

[0019] The disclosure further provides a use of the fusion polypeptide as described herein for the enhancement of the persistence and expansion of the engineered cells. In one aspect, the fusion polypeptide is capable of participating in the formation of the trimeric complex. In one aspect, the trimeric complex is capable of inducing the signaling of the intracellular component independent of the Fas ligand binding.

[0020] The disclosure further provides an isolated nucleic acid encoding the fusion polypeptide as described herein. In one aspect, the isolated nucleic acid encodes the fusion polypeptide and an antigen receptor. In one aspect, the fusion polypeptide and the antigen receptor are linked via a viral self-cleaving polypeptide.

[0021] The disclosure further provides a vector comprising the nucleic acid as described herein. In one aspect, the vector is a viral vector. In one aspect, the viral vector is a retroviral, lentiviral, adenoviral, or adeno-associated viral vector.

[0022] The disclosure further provides a pharmaceutical formulation comprising the engineered cell, the fusion polypeptide, the isolated nucleic acid or the vector as described herein, and a pharmaceutically acceptable carrier.

[0023] The disclosure further provides a method of disease treating a subject, comprising administering the engineered cell, the fusion polypeptide, the isolated nucleic acid, the vector, or the pharmaceutical formulation as described herein. Also provided is the use of the engineered cell, the fusion polypeptide, the isolated nucleic acid, the vector, or the pharmaceutical formulation as described herein in the manufacture of a medicament for the treatment of a disease or disorder. The disclosure further provides the engineered cell, the fusion polypeptide, the isolated nucleic acid, the vector, or the pharmaceutical formulation as described herein for use as a medicament. In one aspect, the disease or disorder is a cancer or an autoimmune disease. In one aspect, the disease is selected from the group consisting of leukemia, lymphoma, lung cancer, melanoma, breast cancer, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, neuroblastoma and rhabdomyosarcoma. In one aspect, the disease is leukemia or lymphoma.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figure 1 shows the growth of T cells without further stimulation.

[0025] Figure 2A shows the EGFR⁺ T cell proliferation, T cell viability and EGFR⁺ T cell enrichment with 100 IU/mL supplemented IL2. Figure 2B shows the EGFR⁺ T cell proliferation, T cell viability and EGFR⁺ T cell enrichment absence of exogenous stimulation or cytokines. Figure 2C shows the EGFR⁺ T cell proliferation, T cell viability and EGFR⁺ T cell enrichment with 50 ng/mL trimeric FasL stimulation in the presence of supplemented 100 IU/mL IL2.

DETAILED DESCRIPTION

I. Definitions

[0026] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

[0027] The term “a” and “an” refers to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “a module” means one module or more than one module.

[0028] The terms “comprising”, “comprises” and “comprised of” as used herein are synonymous with “including”, “includes” or “containing”, “contains”, and are inclusive or open-ended and do not exclude additional, non-recited members, elements or method steps. The term “comprising” also encompasses the term “consisting of”.

[0029] “Percent (%) amino acid sequence identity” and “homology” with respect to a polypeptide are defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or MEGALIGN™ (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0030] The term “trimeric complex” or “trimer”, which can be used interchangeably, refers to a complex of three monomer polypeptides non-covalently or covalently bound. Each monomer polypeptide may be equal or different to each other. The term “homotrimer” refers to a complex comprising three monomers having identical amino acid sequences. The term “heterotrimer” refers to a complex comprising three monomers, wherein at least one monomer exhibits differences in its sequence from other monomers.

[0031] The term “fusion polypeptide” refers to a hybrid (e.g., chimeric, recombinant) polypeptide which comprises protein domains from at least two different naturally occurring proteins.

[0032] The term “extracellular domain” or “ectodomain”, which can be used interchangeably, as used herein refers to the region of a membrane protein, such as a transmembrane protein, that lies outside the vesicular membrane. Ectodomains often comprise binding domains that specifically bind to ligands or cell surface receptors, such as via a binding domain that specifically binds to the ligand or cell surface receptor. The term “Fas ectodomain” refers to a binding domain that is capable of participating in the formation of a trimer that binds to Fas ligand (FasL).

[0033] The term “endodomain” or “intracellular domain,” or “cytoplasmic domain”, which can be used interchangeably, as used herein refers to the region found in some membrane proteins, such as transmembrane proteins, which extends into the interior space defined by the cell surface membrane. In some cells, the endodomain interacts with intracellular constituents and can play a role in signal transduction and thus, in some cases, can be an intracellular signaling domain.

[0034] The term “transmembrane domain” as used herein means a domain found in a membrane protein that substantially or completely spans a lipid bilayer such as those lipid bilayers found in a biological membrane such as a mammalian cell, or in an artificial construct such as a liposome. A transmembrane protein can pass through both layers of the lipid bilayer once or multiple times.

[0035] The term “functional fragment” refers to a truncated polypeptide that retains the function of the full-length polypeptide.

[0036] The term “specifically binds” or “bind”, which can be used interchangeably, as used herein means the ability of a protein, under specific binding conditions, to bind to a target protein such that its affinity or avidity is at least 5 times as great, but optionally at least 10, 20, 30, 40, 50, 100, 250 or 500 times as great, or even at least 1000 times as great as the average affinity or avidity of the same protein to a collection of random peptides or polypeptides of sufficient statistical size. A specifically binding protein need not bind exclusively to a single target molecule but may specifically bind to a non-target molecule due to similarity in structural conformation between the target and non-target (e.g., paralogs or orthologs).

[0037] The term “endogenous” refers to a protein or a nucleic acid that is naturally occurring within a cell or naturally produced by a cell. The term “exogenous” refers to a protein or a nucleic acid that does not originate from a cell and is introduced into the cell by DNA delivery methods.

[0038] The term “autologous” refers to any material derived from the same individual to whom it is later to be re-introduced into the individual. The term “allogeneic” refers to any material derived from a

different animal of the same species as the individual to whom the material is introduced. Two or more individuals are said to be allogeneic to one another when the genes at one or more loci are not identical. In some aspects, allogeneic material from individuals of the same species may be sufficiently different genetically to interact antigenically.

[0039] The term “gain-of-function” refers to any mutation, e.g. substitution, deletion or insertion, in a protein by which the mutant protein acquires a function that is not normally associated with the protein without such mutation.

[0040] The term “expression” refers to the process by which a polypeptide is produced based on the encoding sequence of a nucleic acid molecule, such as a gene. The process may include transcription, post-transcriptional control, post-transcriptional modification, translation, post-translational control, post-translational modification, or any combination thereof.

[0041] The term “encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (e.g., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene, cDNA, or RNA, encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system.

[0042] The terms “polypeptide”, “peptide” and “protein” are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A polypeptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a peptide’s sequence. The term “polypeptide” is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide of the disclosure may be of a size of about 3 or more, 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or more, 1,000 or more, or 2,000 or more amino acids. Polypeptides may have a defined three-dimensional structure, although they do not necessarily have such structure.

[0043] The term “polynucleotide” refers to an isolated nucleic acid molecule or construct, e.g., messenger RNA (mRNA), virally-derived RNA, or plasmid DNA (pDNA). A polynucleotide may comprise a conventional phosphodiester bond or a non-conventional bond (e.g., an amide bond, such as found in peptide nucleic acids (PNA). The term nucleic acid molecule refers to any one or more nucleic acid segments, e.g., DNA or RNA fragments, present in a polynucleotide.

[0044] An “isolated” nucleic acid molecule refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location. “Isolated polynucleotide (or nucleic acid) encoding a fusion polypeptide” refers to one or more polynucleotide molecules encoding the fusion polypeptide, including such polynucleotide molecule(s) in a single vector or separate vectors, and such polynucleotide molecule(s) present at one or more locations in a host cell.

[0045] The term “vector”, as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked.

[0046] The term “subject” or “individual” is intended to include living organisms in which an immune response can be elicited (e.g., mammals, human).

[0047] As used herein, “treatment” (and grammatical variations thereof such as “treat” or “treating”) refers to clinical intervention in an attempt to alter the natural course of a disease in the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

[0048] The term “pharmaceutical composition” refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. A pharmaceutical composition usually comprises one or more pharmaceutically acceptable

carrier(s). A “pharmaceutically acceptable carrier” refers to an ingredient in a pharmaceutical composition, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

[0049] An “effective amount” of an agent, e.g., a pharmaceutical composition, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

II. Trimeric Complex and Fusion Polypeptide

[0050] The present disclosure provides a novel engineered cell expressing a trimeric complex, wherein the trimeric complex comprises at least one fusion polypeptide comprising a) an extracellular component comprising a Fas ectodomain; b) a transmembrane component; and c) an intracellular component. Compared to cells that don’t express the trimeric complex or the fusion polypeptide, the engineered cell shows enhanced persistence and/or expansion, and ameliorated toxicity.

[0051] 2.1 Extracellular component of fusion polypeptide

[0052] In one aspect, provided is the fusion polypeptide as defined herein before, wherein the extracellular component comprises a Fas ectodomain. In one aspect, the Fas ectodomain is a full-length Fas ectodomain or functional fragment thereof. In one aspect, the functional fragment of the full-length Fas ectodomain is capable of participating in the formation of a trimer that binds to Fas ligand (FasL). In one aspect, the Fas ectodomain doesn’t include a signal peptide which usually locates in 1 to 25 amino acids of wild-type Fas with the signal peptide. In one aspect, the Fas ectodomain is human Fas ectodomain.

[0053] In one aspect, the Fas ectodomain is truncated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 in its N-terminus corresponding to the wild-type Fas ectodomain, e.g. SEQ ID NO: 1. In one aspect, the Fas ectodomain comprises a substitution or a deletion at positions 6, 7, 8 and/or 9. In one aspect, the Fas ectodomain comprises a deletion at position 6 or 7. In one aspect, the Fas ectodomain comprises a point substitution at position 7. In one aspect, the Fas ectodomain comprises a point substitution S7A. In one aspect, the Fas ectodomain is truncated by 1, 2, 3, 4, 5, 6 or 7 in its C-terminus. In one aspect, the Fas ectodomain comprises a human Fas ectodomain. In one aspect, the Fas ectodomain comprises a cysteine-rich domain 1, a cysteine-rich domain 2 and a cysteine-rich domain 3 of human Fas ectodomain. In one aspect, the Fas ectodomain comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO: 1. In one aspect, the Fas ectodomain comprises or consists of the amino acid sequence of SEQ ID NO: 1.

[0054] 2.2 Transmembrane component of fusion polypeptide

[0055] In one aspect, the fusion polypeptide comprises a transmembrane component that is operably linked to the extracellular component and the intracellular component. The transmembrane component comprises a transmembrane domain that is natural or unnatural. In the fusion polypeptide comprising IL-7 α endodomain, the transmembrane domain comprises one or more mutations compared to its corresponding wildtype peptide that allows the transmembrane protein to be self-active. In the fusion polypeptide comprising CD2 endodomain only, the transmembrane domain comprises naturally occurring transmembrane.

[0056] In one aspect, the transmembrane component is capable of self-oligomerizing. Such transmembrane component may fuse by its C-terminus to the N-terminus of the IL-7R endodomain. The transmembrane domain of the transmembrane component allows homodimerization of two separate fusion polypeptides each of which comprises the transmembrane domain. In one aspect, the transmembrane domain induces structural twisting of the transmembrane and endodomain of fusion polypeptides to form a self-activating helical structure. In one aspect, the transmembrane component comprises one or more gain-of-function mutations corresponding to the naturally occurring proteins of healthy subjects. The mutation(s) may be a substitution, insertion, deletion, or combination thereof. In one aspect, the one or more mutations comprise inclusion of at least one cysteine and/or at least one proline. In one aspect, the mutant polypeptides including cysteine insertion(s) induce disulfide bond formation in the transmembrane domain. In one aspect, the mutation is a gain-of-function insertion. In one aspect, the gain-of-function insertion enables homodimerization of the polypeptides. In one aspect, the gain-of-function insertion is CPT or PPCL. In one aspect, the insertion is located after the first, second, third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, twelfth, thirteenth, fourteenth, fifteenth, sixteenth, seventeenth, eighteenth, nineteenth, twentieth, twenty-first, twenty-second, twenty-third, or twenty-fourth amino acid in SEQ ID NO: 10. In one aspect, the transmembrane domain consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 11 to SEQ ID NO: 33.

[0057] In one aspect, when the intracellular component comprises an IL-7R endodomain, the transmembrane component consists of the amino acid sequence of SEQ ID NO: 11 (PILLTCPTIX₁IX₂SX₃X₄X₅X₆X₇X₈X₉X₁₀X₁₁LX₁₂X₁₃X₁₄LW), wherein X₁ is S or L; X₂ is L or S; X₃ is F or L; X₄ is F or A; X₅ is S or I; X₆ is V or L; X₇ is A or L; X₈ is L or M; X₉ is L or V; X₁₀ is V or S; X₁₁ is I or L; X₁₂ is A or L; X₁₃ is C or L; and X₁₄ is V or S. In one aspect, X₁ is S; X₂ is L; X₃ is F or L; X₄ is F or A; X₅ is S or I; X₆ is V or L; X₇ is A or L; X₈ is L or M; X₉ is L or V; X₁₀ is V or S; X₁₁ is I or L; X₁₂ is A or L; X₁₃ is C or L; and X₁₄ is V or S. In one aspect, X₁ is S; X₂ is L; X₃ is L; X₄ is A; X₅ is I; X₆ is L; X₇ is A or L; X₈ is L or M; X₉ is L or V; X₁₀ is V or S; X₁₁ is I or L; X₁₂ is A or L; X₁₃ is C or L; and X₁₄ is V or S. In one aspect, X₁ is S; X₂ is L; X₃ is F; X₄ is F; X₅ is S; X₆ is V; X₇ is A or L; X₈ is L or M; X₉ is L or V; X₁₀ is V or S; X₁₁ is I or L; X₁₂ is A or L; X₁₃ is C or L; and X₁₄ is V or S. In one aspect, X₁ is S; X₂ is L; X₃ is L; X₄ is A; X₅ is I; X₆ is L; X₇ is L; X₈ is M; X₉ is V; X₁₀ is S; X₁₁ is I or L; X₁₂ is A or L; X₁₃ is C or L; and X₁₄ is V or S. In one aspect, X₁ is S; X₂ is L; X₃ is F; X₄ is F; X₅ is S; X₆ is V; X₇ is A; X₈ is L; X₉ is L; X₁₀ is V; X₁₁ is I or L; X₁₂ is A or L; X₁₃ is C or L; and X₁₄ is V or S. In one aspect, X₁ is S; X₂ is L; X₃ is F; X₄ is F; X₅ is S; X₆ is V; X₇ is A; X₈ is L; X₉ is L; X₁₀ is V; X₁₁ is I; X₁₂ is A; X₁₃ is C; and X₁₄ is V or S. In one aspect, the transmembrane component consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 39.

[0058] In one aspect, the transmembrane component consists of a transmembrane domain selected from the group consisting of Fas, CD2, CD3s, CD35, CD3C, CD25, CD27, CD28, CD40, CD79A, CD79B, CD80, CD86, OX40, 4- IBB, SLAMF1, CTLA4, CD200R, LAG3, HVEM, BTLA, PD-L2, PD-L1, ICOS, PD-1, CD300, GITR, A2aR, DAP10, FcR α , FcR β , FcR γ , Fyn, GAL9, KIR, Lck, LAT, LRP, KG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPa, pTa, TCR α , TCR β , TIM3, TRIM, LPA5 and Zap70. Such transmembrane component may fuse by its C-terminus to the N-terminus of the CD2 endodomain. In one aspect, the transmembrane domain has been modified compared to the naturally occurring transmembrane domain, as long as it maintains the original function. In one aspect, the transmembrane component consists of a Fas transmembrane domain. In one aspect, the Fas transmembrane domain is a human Fas transmembrane domain. In one aspect, the transmembrane component consists of an amino acid that is at least 80%, 85%, 90%, 95% sequence identity to SEQ ID NO: 9. In one aspect, the transmembrane component consists of the amino acid sequence of SEQ ID NO: 9.

[0059] 2.3 Intracellular component of fusion polypeptide

[0060] In one aspect, the fusion polypeptide comprises an intracellular component comprising an IL-7R (IL7 receptor) endodomain, a CD2 endodomain, or a combination thereof, wherein the intracellular component doesn't comprise a Fas endodomain. In one aspect, an intracellular component comprises or consists of an IL-7R endodomain. In one aspect, an intracellular component comprises or consists of a CD2 endodomain. In one aspect, an intracellular component comprises or consists of an IL-7R endodomain and a CD2 endodomain. In one aspect, the Fas endodomain that is not included in the intracellular component is full-length Fas endodomain or functional fragment thereof.

[0061] In one aspect, the IL-7R endodomain is an IL-7R α endodomain. In one aspect, the IL-7R α endodomain is a human IL-7R α endodomain. In one aspect, the IL-7R endodomain has been modified compared to the naturally occurring transmembrane domain, as long as it maintains the signaling activity. In one aspect, the IL-7R endodomain is a full-length IL-7R endodomain or functional fragments thereof. In one aspect, the IL-7R endodomain is between 70-195 amino acids, 70-175 amino acids, 70-150 amino acids, 70-125 amino acids, 70-100 amino acids, 80-195 amino acids, 80-175 amino acids, 80-150 amino acids, 80-125 amino acids, 80-100 amino acids, 90-195 amino acids, 90-175 amino acids, 90-150 amino acids, 90-125 amino acids, 90-100 amino acids, 100-195 amino acids, 100-175 amino acids, 100-150 amino acids, 100-125 amino acids, 125-195 amino acids, 125-175 amino acids, 125-150 amino acids, 150-195 amino acids, 150-175 amino acids or 175-195 amino acids. In one aspect, the intracellular component comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO: 4. In one aspect, the intracellular component comprises or consists of the amino acid sequence of SEQ ID NO: 4.

[0062] In one aspect, the CD2 endodomain is a human CD2 endodomain. In one aspect, the CD2 endodomain has been modified compared to the naturally occurring CD2 endodomain, as long as it maintains the signaling activity. In one aspect, the CD2 endodomain is a full-length CD2 endodomain or functional fragments thereof. In one aspect, the functional fragment is truncated at the C-terminus by up to 65 amino acids compared to the full-length CD2 endodomain. In one aspect, the functional fragment is truncated at the C-terminus by 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid compared to the full-length CD2

endodomain. In one aspect, the functional fragment is truncated at the C-terminus by 63, 44, 22 amino acids compared to the full-length CD2 endodomain. In one aspect, the intracellular component comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO: 5 to SEQ ID NO: 8, respectively. In one aspect, the intracellular component comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 5 to SEQ ID NO: 8.

[0063] 2.4 Structure of fusion polypeptide

[0064] In one aspect, the fusion polypeptide comprises or consists of, from its N to C terminus in order, an extracellular component comprising a Fas ectodomain, a transmembrane component and an IL-7R endodomain. In one aspect, the fusion polypeptide comprises or consists of, from its N to C terminus in order, an extracellular component comprising a Fas ectodomain, a transmembrane component and a CD2 endodomain. In one aspect, the fusion polypeptide comprises or consists of, from its N to C terminus in order, an extracellular component comprising a Fas ectodomain, a transmembrane component and an IL-7R endodomain and a CD2 endodomain. In one aspect, the components fuse to each other directly or through a flexible peptide. In one aspect, the components fuse to each other directly. Exemplary fusion polypeptides are showed in Table. 4.

[0065] In one aspect, the extracellular component further comprises an ectodomain of a protein other than Fas, as long as such extracellular component maintains the function of a fusion polypeptide having Fas ectodomain only. In one aspect, the function is participating in the formation of trimeric complex. In one aspect, the protein other than Fas is selected from the group consisting of PD-1 CD30, HER2, EGFR, CD19, CD34, TGFβR, IL-4R, IL-13R, IL-8R, IL-10R, LAG3, TIGIT, CTLA4, CD19, CD27, CD28, CD52, CD134, CD 137, HER2, EGFR and NGFR.

[0066] In one aspect, the intracellular component further comprises an endodomain of a protein other than IL-7R or CD2, as long as such intracellular component maintains the signaling activity of a fusion polypeptide having IL-7R and/or CD2 endodomain only. In one aspect, the protein other than IL-7R or CD2 is selected from the group consisting of IL-2R, IL-6R, IL-12R, IL-21R, IL-23R and CD122.

[0067] 2.5 Trimeric complex comprising fusion polypeptide

[0068] The disclosure provides a trimeric complex comprising the fusion polypeptide as described herein. In one aspect, the trimeric complex is capable of binding to Fas ligand. In one aspect, the trimeric complex is capable of inducing the signaling of the intracellular component independent of the binding to Fas ligand.

[0069] In one aspect, the trimeric complex is formed on the surface of the engineered cell expressing the fusion polypeptide. In one aspect, the trimeric complex is formed automatically on the surface of the engineered cell expressing the fusion polypeptide. In one aspect, the extracellular component of the fusion polypeptide comprises a cysteine-rich domain 1, a cysteine-rich domain 2 and a cysteine-rich domain 3. Cysteine residues in these cysteine-rich domains contribute to the formation of trimeric complex. In one aspect, the trimeric complex is formed via disulfide bridge(s) between the cysteine-rich domains. In one aspect, the trimeric complex is formed via disulfide bridge(s) between the cysteine-rich domain 1.

[0070] In one aspect, the trimeric complex is a homotrimer comprising three identical fusion polypeptides. In one aspect, the trimeric complex is a heterotrimer comprising at least one fusion polypeptide. In one aspect, the one fusion polypeptide comprises a CD2 endodomain. In one aspect, the trimeric complex is a heterotrimer comprising two or three identical fusion polypeptides. In one aspect, the two or three identical fusion polypeptides comprise an IL-7R endodomain and/or a CD2 endodomain, respectively. In one aspect, the two or three identical fusion polypeptides comprise an IL-7R endodomain, respectively. In one aspect, the two or three identical fusion polypeptides comprise an IL-7R endodomain and a CD2 endodomain, respectively. In one aspect, the two or three identical fusion polypeptides comprise a CD2 endodomain, respectively. In one aspect, the two or three identical fusion polypeptides comprise a transmembrane component capable of self-oligomerizing, respectively. Surprisingly, the self-oligomerizing of transmembrane component doesn't impact the formation of the trimeric complex and function thereof. In one aspect, the heterotrimer comprises one or two endogenous Fas monomers.

III. Engineered Cell and Preparation Thereof

[0071] 3.1 Cell

[0072] In one aspect, the disclosure provides engineered cells, e.g., engineered immune cells, comprising the fusion polypeptide or trimeric complex as described herein. The source of the engineered immune cells of the disclosure may be a patient to be treated (i.e., autologous cells) or from a donor who is not the patient to be treated (e.g., allogeneic cells).

[0073] In one aspect, the cell is an immunoresponsive cell. In one aspect, the cell is a cell of the lymphoid lineage. In one aspect, the engineered immune cells are engineered T cells. In one aspect, the T cells are derived from a mammalian subject. In one aspect, the T cells are derived from a primate subject, such as a human subject. Among the sub-types and subpopulations of T cells and/or of CD4⁺ and/or of CD8⁺ T cells are naive T (TN) cells, effector T cells (TEFF), memory T cells and sub-types thereof, such as stem cell memory T (TSCM), central memory T (TCM), effector memory T (TEM), or terminally differentiated effector memory T cells, tumor-infiltrating lymphocytes (TIL), immature T cells, mature T cells, helper T cells, cytotoxic T cells, mucosa-associated invariant T (MAIT) cells, naturally occurring and adaptive regulatory T (Treg) cells, helper T cells, such as TH1 cells, TH2 cells, TH3 cells, TH17 cells, TH9 cells, TH22 cells, follicular helper T cells, α/β T cells, and δ/γ T cells. Non-limiting examples of commercially available T-cell lines include lines BCL2 (AAA) Jurkat (ATCC[®] CRL-2902[™]), BCL2 (S70A) Jurkat (ATCC[®] CRL-2900[™]), BCL2 (S87A) Jurkat (ATCC[®] CRL-2901[™]), BCL2 Jurkat (ATCC[®] CRL-2899[™]), Neo Jurkat (ATCC[®] CRL-2898[™]), TALL-104 cytotoxic human T cell line (ATCC # CRL-11386). Further examples include but are not limited to mature T-cell lines, e.g., such as Deglis, EBT-8, HPB-MLp-W, HUT 78, HUT 102, Karpas 384, Ki 225, My-La, Se-Ax, SKW-3, SMZ-1 and T34; and immature T-cell lines, e.g., ALL-SIL, Be13, CCRF-CEM, CML-T1, DND-41, DU.528, EU-9, HD-Mar, HPB-ALL, H-SB2, HT-1, JK-T1, Jurkat, Karpas 45, KE-37, KOPT-K1, K-T1, L-KAW, Loucy, MAT, MOLT-1, MOLT 3, MOLT-4, MOLT 13, MOLT-16, MT-1, MT-ALL, P12/Ichikawa, Peer, PER0117, PER-255, PF-382, PFI-285, RPMI-8402, ST-4, SUP-T1 to T14, TALL-1, TALL-101, TALL-103/2, TALL-104, TALL-105, TALL-106, TALL-107, TALL-197, TK-6, TLBR-1, -2, -3, and -4, CCRF-HSB-2 (CCL-120.1), J.RT3-T3.5 (ATCC TIB-153), J45.01 (ATCC CRL-1990), J.CaM1.6 (ATCC CRL-2063), RS4;11 (ATCC CRL-1873), CCRF-CEM (ATCC CRM-CCL-119); and cutaneous T-cell lymphoma lines, e.g., HuT78 (ATCC CRM-TIB-161), MJ[G11] (ATCC CRL-8294), HuT102 (ATCC TIB-162). Non-limiting exemplary sources for such commercially available cell lines include the American Type Culture Collection (ATCC) (Manassas, VA), and the German Collection of Microorganisms and Cell Cultures.

[0074] In certain aspects, the cells are cytotoxic T cells (also known as TC, Cytotoxic T Lymphocyte, CTL, T Killer cell, a lytic T cell, CD8⁺ T cells or killer T cell). In certain aspects, the T cell is a CD4⁺T cell. In certain aspects, the T cell can be a CD4⁺T cell or a CD8⁺T cell. In certain aspects, the cell is a tumor-specific T cell.

[0075] In one aspect, the cells are natural killer (NK) cells, Natural Killer T (NKT) cells, cytokine-induced killer (CIK) cells, tumor-infiltrating lymphocytes (TILs), lymphokine-activated killer (LAK) cells, or the like. NK cells may either be isolated or obtained from a commercially available source. Non-limiting examples of commercial NK cell lines include lines NK-92 (ATCC[®] CRL-2407[™]), NK-92MI (ATCC[®] CRL-2408[™]). Further examples include but are not limited to NK lines HANK1, KHYG-1, NKL, NK-YS, NOI-90, and YT. Non-limiting exemplary sources for such commercially available cell lines include the American Type Culture Collection (ATCC) (Manassas, VA) and the German Collection of Microorganisms and Cell Cultures.

[0076] In one aspect, the cells are B cells, monocytes or granulocytes, e.g., myeloid cells, macrophages, neutrophils, dendritic cells, mast cells, eosinophils, and/or basophils.

[0077] In one aspect, the cell is a cell of the myeloid lineage. Non-limiting examples of cells of the myeloid lineage include monocytes, macrophages, basophils, neutrophils, eosinophils, mast cell, erythrocytes, megakaryocytes, thrombocytes, and stem cells from which myeloid cells may be differentiated. In certain embodiments, the stem cell is a pluripotent stem cell (e.g., embryonic stem cell or induced pluripotent stem cell).

[0078] 3.2 Antigen receptor comprised in engineered cell

[0079] In one aspect, the disclosure provides engineered cells comprising the fusion polypeptide or trimeric complex as described herein and an antigen receptor (e.g., a chimeric antigen receptor (CAR) or an antigen-specific TCR) that binds to an antigen. The antigen-recognizing receptor can bind to a tumor antigen or a pathogen antigen. In one aspect, the antigen-recognizing receptor binds to a tumor antigen. In one aspect, the tumor antigen is a tumor-specific antigen or a tumor-associated antigen. In certain embodiments, the fusion polypeptide results in an enhancement of the function of the antigen receptor.

[0080] CARs may be chimeric type I trans-membrane proteins which connect an extracellular antigen-recognizing domain (binder) to an intracellular signaling domain (endodomain). A spacer domain may be necessary to isolate the binder from the membrane and to allow it a suitable orientation. More compact spacers can suffice e.g. the stalk from CD8a and even just the IgG1 hinge alone, depending on the antigen.

A transmembrane domain anchors the protein in the cell membrane and connects the spacer to the endodomain.

[0081] 3.2.1 Antigen Binding Domain of CAR

[0082] In one aspect, the antigen-binding domain may comprise: a single-chain variable fragment (scFv) derived from a monoclonal antibody, a natural ligand of the target antigen, a peptide with sufficient affinity for the target, a single domain binder such as a camelid, an artificial binder single as a Darpin, or a single-chain derived from a T-cell receptor. In one aspect, the antigen binding domain comprises a mono-specific, bispecific or multi-specific antibody molecule.

[0083] In one aspect, the chimeric antigen receptor targets against CD19, CD20, CD22, CD30, BCMA, AFP, ALK, GPC3, HER2, EGFR, 5T4, av β 6 integrin, B7-H3, B7-H6, CA-125, CAIX, CD5, CD13, CD16, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD138, CD160, CD171, CEA, Claudin18.2, CSPG4, DLL3, EGFR, HER2, EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, Folate binding protein, fetal Carbonic anhydrase IX, AchR, FRa, G250, GD2, GD3, MAGE-1, NY-ESO-1, IL-11R α , IL-13R α 2, Lambda, Lewis-Y, Kappa, Mesothelin, Muc1, Muc16, NCAM, NKG2D Ligands, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TAG72, TEMs, VEGFR2, Vimentin or WT-1. Some tumor-associated antigens are known in the following Table. 1. The antigen-binding domain used in the present invention may be a domain which is capable of binding a TAA as indicated therein.

Table 1. Type of the TAA

Cancer type	TAA
Diffuse Large B-cell Lymphoma	CD19, CD20, CD22
Breast cancer	ErbB2, MUC1
AML	CD13, CD33
Neuroblastoma	GD2, NCAM, ALK, GD2
B-CLL	CD19, CD52, CD160
Colorectal cancer	Folate binding protein, CA-125
Chronic Lymphocytic Leukaemia	CD5, CD19
Glioma	EGFR, Vimentin
Multiple myeloma	BCMA, CD138
Renal Cell Carcinoma	Carbonic anhydrase IX, G250
Prostate cancer	PSMA

[0084] 3.2.2 Spacer Domain of CAR

[0085] The CAR may comprise a spacer sequence to connect the antigen-binding domain with the transmembrane domain. A flexible spacer allows the antigen-binding domain to orient in different directions to facilitate binding. In one aspect, the spacer sequence comprises an IgG1 Fc region, an IgG1 hinge, an IgG4 hinge or a human CDS stalk or the mouse CDS stalk. In one aspect, the spacer comprises a linker sequence which has similar length and/or domain spacing properties as an IgG1 Fc region, an IgG1 hinge or a CDS stalk.

[0086] 3.2.3 Transmembrane Domain of CAR

[0087] The transmembrane domain is the sequence of a classical CAR that spans the membrane. In one aspect, the transmembrane domain may be derived either from a natural or from a recombinant source. In one aspect, the transmembrane domain is a natural peptide derived from any membrane-bound or transmembrane protein. In one aspect, the transmembrane domain is capable of signaling to the intracellular domain(s) whenever the CAR has bound to a target. A transmembrane domain of particular use in this invention may include at least the transmembrane region(s) of, e.g., the alpha, beta or zeta chain of T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8 (e.g., CD8 alpha, CD8 beta), CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154.

[0088] 3.2.4 Intracellular Signaling Domain of CAR

[0089] The intracellular signaling domain is generally responsible for activation of at least one of the normal effector functions. In one aspect, the intracellular signaling domains include two distinct classes of cytoplasmic signaling sequences: those that initiate antigen-dependent primary activation through the TCR

(primary intracellular signaling domains) and those that act in an antigen-independent manner to provide a secondary or costimulatory signal (secondary cytoplasmic domain, e.g., a costimulatory domain).

[0090] A primary signaling domain regulates primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary intracellular signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs (ITAMs). Examples of ITAM containing primary intracellular signaling domains that are of particular use in the invention include those of TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, CD278 (also known as "ICOS"), FceRI, DAP 10, DAP12, and CD66d. In one aspect, a CAR comprises an intracellular signaling domain, e.g., a primary signaling domain of CD3-zeta. In one aspect, a primary signaling domain comprises a modified ITAM domain, e.g., a mutated ITAM domain which has altered (e.g., increased or decreased) activity as compared to the native ITAM domain. In one aspect, a primary signaling domain comprises a modified ITAM-containing primary intracellular signaling domain, e.g., an optimized and/or truncated ITAM-containing primary intracellular signaling domain. In one aspect, the signaling domain of CD3-zeta is a mutant CD3zeta or a wild-type human CD3zeta. In one aspect, a primary signaling domain comprises one, two, three, four or more ITAM motifs.

[0091] In one aspect, the intracellular signaling domain of the CAR can comprise a primary signaling domain and a costimulatory signaling domain. In one aspect, the costimulatory molecule is a cell surface molecule other than an antigen receptor or its ligands that is required for an efficient response of lymphocytes to an antigen. Examples of such molecules include MHC class I molecule, TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SEAM proteins), activating NK cell receptors, BTFA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, FFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, FIGHT, HVEM, KIRDS2, SFAMF7, NKp80 (KFRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IF2Rbeta, IF2Rgamma, IF7R alpha, ITGA4, VFA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VFA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAF, FFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, FFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKF, DNAM1 (CD226), SFAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Fy9 (CD229), CD160 (BY55), PSGF1, CD100 (SEMA4D), CD69, SFAMF6 (NTB-A, Fyl08), SEAM (SFAMF1, CD150, IPO-3), BFAME (SFAMF8), SEFPFG (CD162), FTBR, FAT, GADS, SFP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83, and the like. The intracellular signaling sequences within the cytoplasmic portion of the CAR of the invention may be linked to each other in a random or specified order. In one aspect, the intracellular signaling domain is designed to comprise two or more, e.g., 2, 3, 4, 5, or more, costimulatory signaling domains.

[0092] In one aspect, the engineered cell comprises recombinant T cell receptors (TCRs) and/or TCRs cloned from naturally occurring T cells. TCRs or antigen-binding portions thereof include those that recognize a peptide epitope or T cell epitope of a target polypeptide, such as an antigen of a tumor, viral or autoimmune protein. In one aspect, the TCR has binding specificity for a tumor associated antigen, e.g., carcinoembryonic antigen (CEA), GP100, melanoma antigen recognized by T cells 1 (MART1), melanoma antigen A3 (MAGEA3), melanoma antigen A4 (MAGEA4) or p53.

[0093] 3.3 Exogenous FasL comprised in engineered cell

[0094] In one aspect, the disclosure provides engineered cells comprising the fusion polypeptide or trimeric complex as described herein and an exogenous FasL. In one aspect, the disclosure provides engineered cells comprising the fusion polypeptide or trimeric complex as described herein, an antigen receptor and an exogenous FasL. FasL binds to Fas, which induces apoptosis of both target cells and immunoresponsive cells. Surprisingly, the fusion polypeptide or trimeric complex of this disclosure can protect the engineered cells from fratricide or suicide killing caused by the exogenous Fas. Therefore, the co-expression of the fusion polypeptide or trimeric complex and FasL can improve at least the cytotoxicity, persistence and/or expansion of engineered cells.

[0095] In one aspect, the engineered cells overexpress the FasL. In one aspect, the FasL is constitutively expressed on the surface of engineered cells (e.g., T cells or NK cells). In one aspect, the exogenous FasL is a human FasL. In one aspect, the exogenous FasL is a murine FasL. In one aspect, the exogenous FasL is secreted. In one aspect, the exogenous FasL is membrane bound. In one aspect, the FasL has been modified compared to the naturally occurring FasL, e.g. the polypeptide comprising the amino acid sequence of SEQ ID NO: 40, as long as it maintains the apoptosis activity. In one aspect, the FasL is a full-length FasL or functional fragments thereof. In one aspect, the FasL comprises or consists of an amino acid sequence of amino acids 1 to 281, 1 to 80, 81 to 102, 1 to 102, 1 to 110, 1 to 127, 81 to 102,

103 to 281, 132 to 281, 134 to 281, or 135 to 281 of SEQ ID NO: 40. In one aspect, the FasL comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 40 to SEQ ID NO: 47. In one aspect, the intracellular component comprises or consists of the amino acid sequence selected from the group consisting of SEQ ID NO: 40 to SEQ ID NO: 47.

[0096] 3.4. Methods for genetic engineering

[0097] In one aspect, the engineered cells are prepared by various methods of the transfer of polynucleotides encoding fusion polypeptide, antigen receptors, e.g., CARs, and/or FasL. Physical methods include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Biological methods include the use of DNA and RNA vectors, e.g., a viral vector, e.g., a lentiviral vector. Chemical methods include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. Exemplary methods are shown in Table 2.

Table 2. Delivery methods for the genome editing systems

Delivery Mode		Duration of Expression	Genome integration	Type of molecule delivered
Physical (e.g. electroporation)		Transient	No	Nucleic acids and Proteins
Viral vector	retrovirus	Stable	Yes	RNA
	lentivirus	Stable	Yes	RNA
	adenovirus	Transient	No	DNA
	AAV	Stable	No	DNA
	Vaccinia Virus	Transient	No	DNA
	Herpes Simplex Virus	Stable	No	DNA
Non-viral	Cationic liposomes	Transient		Nucleic acids and Proteins
	Polymeric nanoparticles	Transient		Nucleic acids and Proteins
	Transposon	Stable	Yes	DNA
Biological non-viral delivery vehicles	Attenuated bacteria	Transient	No	Nucleic acids

[0098] In one aspect, recombinant polynucleotides are transferred into cells using recombinant infectious virus particles, e.g., adenoviral vector, AAV vector, lentiviral vector, retroviral vector, such as gamma-retroviral vectors. In one aspect, the retroviral vector or lentiviral vector has a long terminal repeat sequence (LTR). In one aspect, the vectors are self-inactivating (SIN). In one aspect, the vectors are conditionally replicating (mobilizable) vectors. In one aspect, the lentiviral vectors are derived from human, feline or simian lentiviruses. In one aspect, the retroviral vectors are derived from murine retroviruses. In one aspect, the lentiviruses or retroviruses include those derived from any avian or mammalian cell source. In one aspect, the lentiviruses or retroviruses are amphotropic, meaning that they are capable of infecting host cells of several species, including humans. In one aspect, the gene to be expressed replaces the retroviral gag, pol and/or env sequences.

[0099] In one aspect, the vector comprising the polynucleotides encoding the fusion polypeptide can contain a promoter and/or enhancer or regulatory elements to regulate expression of the encoded fusion polypeptide. In one aspect, the promoter and/or enhancer or regulatory elements can be condition-dependent promoters, enhancers, and/or regulatory elements. In one aspect, the polynucleotides encoding the fusion polypeptide can be operatively linked to a constitutive promoter. In one aspect, the promoter is selected from the group consisting of Cytomegalovirus (CMV) promoter, elongation factors-1 alpha (EF1 α) promoter, ubiquitin C promoter (UbiC), phosphor-glycerokinase promoter (PGK), simian virus 40 early promoter (SV40) and chicken β -Actin promoter coupled with CMV early enhancer (CAGG).

[0100] In one aspect, the polynucleotides is operably linked to an inducible promoter. The inducible promoter can be induced by one or more conditions, such as a physical condition, microenvironment of the engineered immune effector cell, or the physiological state of the engineered immune effector cell, an inducer (i.e., an inducing agent), or a combination thereof. In one aspect, the inducing condition does not induce the expression of endogenous genes in the engineered mammalian cell, and/or in the subject that

receives the pharmaceutical composition. In one aspect, the inducing condition is selected from the group consisting of: inducer, irradiation (such as ionizing radiation, light), temperature (such as heat), redox state, tumor environment, and the activation state of the engineered mammalian cell.

[0101] In one aspect, the polynucleotides is operatively linked to a Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE), located downstream of the polynucleotides.

[0102] In one aspect, the vector can contain a single promoter that drives the expression of one or more nucleic acid molecules. In one aspect, such nucleic acid molecules can be multi-cistronic. For example, in one aspect, transcription units can be engineered as a bi-cistronic unit containing an IRES (internal ribosome entry site), which allows co-expression of gene products (e.g., encoding a fusion polypeptide and optionally, a CAR and a FasL) by a message from a single promoter. In one aspect, a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three genes separated from one another by sequences encoding a self-cleavage peptide or a protease recognition site. In one aspect, the self-cleavage peptide is selected from the group consisting of foot-and-mouth disease virus (F2A), equine rhinitis A virus (E2A), *Thosaea asigna* virus (T2A) and porcine teschovirus-1 (P2A).

[0103] In one aspect, polynucleotides are transferred into T cells via electroporation. In one aspect, polynucleotides are transferred into T cells via transposition. In one aspect, polynucleotides are delivered by means of transposons including a Sleeping Beauty transposon system (SB) and/or a piggyBac (PB) transposon system.

[0104] Polynucleotides encoding fusion polypeptides may be associated with additional coding regions which encode secretory or signal peptides, which direct the secretion of the fusion polypeptide. For example, if secretion of the fusion polypeptide is desired, DNA encoding a signal sequence may be placed upstream of the fusion polypeptide. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells generally have a signal peptide fused to the N-terminus of the polypeptide, which is cleaved from the translated polypeptide to produce a secreted or "mature" form of the polypeptide. In one aspect, the signal peptide comprises a sequence of a human CD2, CD3 δ , CD3 ϵ , CD3 γ , CD3 ζ , CD4, CD8 α , CD19, CD28, CD37, CD45, 4-1BB, GM-CSFR, IL-2, CD33, Human IgKVIII, Human IgG2 H, Chymotrypsinogen, trypsinogen-2, HSA, Insulin or tPA signal peptide.

[0105] In one aspect, the polynucleotides encoding the fusion polypeptide contain a nucleic acid sequence encoding one or more marker(s). In one aspect, the one or more marker(s) is a transduction marker, surrogate marker and/or a selection marker. In one aspect, the polynucleotides encoding the fusion polypeptide contain a nucleic acid sequence encoding one or more additional modules that enhance and/or dampen responses of the cells upon adoptive transfer and encounter with ligand.

[0106] In one aspect, the polynucleotides can also encode one or more surrogate marker(s). In one aspect, the surrogate markers can include truncated forms of cell surface polypeptides, such as truncated forms that are non-functional and do not transduce or are not capable of transducing a signal or a signal ordinarily transduced by the full-length form of the cell surface polypeptide, and/or do not internalize or are not capable of internalizing. In one aspect, the truncated cell surface polypeptides including truncated forms of growth factors or other receptors such as a truncated human epidermal growth factor receptor 2 (tHER2), a truncated epidermal growth factor receptor (tEGFR), a truncated prostate-specific membrane antigen (PSMA) or modified form thereof. tEGFR can be used to identify or select cells that have been engineered with the tEGFR and an encoded exogenous protein, and/or to eliminate or separate cells expressing the encoded exogenous protein.

[0107] In one aspect, the marker is a fluorescent protein, such as green fluorescent protein (GFP), enhanced green fluorescent protein (EGFP), such as super-fold GFP (sfGFP), red fluorescent protein (RFP), such as tdTomato, mCherry, mStrawberry, AsRed2, DsRed or DsRed2, cyan fluorescent protein (CFP), blue green fluorescent protein (BFP), enhanced blue fluorescent protein (EBFP), and yellow fluorescent protein (YFP), and variants thereof, including species variants, monomeric variants, and codon-optimized and/or enhanced variants of the fluorescent proteins. In one aspect, the marker is or comprises an enzyme, such as a luciferase, the lacZ gene from *E. coli*, alkaline phosphatase, secreted embryonic alkaline phosphatase (SEAP), chloramphenicol acetyl transferase (CAT). Exemplary light-emitting reporter genes include luciferase (luc), b-galactosidase, chloramphenicol acetyltransferase (CAT), b-glucuronidase (GUS) or variants thereof.

[0108] In one aspect, the marker is a selection marker. In one aspect, the selection marker is a polypeptide that confers resistance to exogenous agents or drugs. In one aspect, the selection marker is an antibiotic resistance gene. In one aspect, the selection marker is an antibiotic resistance gene confers antibiotic resistance to a mammalian cell. In one aspect, the selection marker is selected from the group consisting of a Puromycin resistance gene, a Hygromycin resistance gene, a Blasticidin resistance gene, a

Neomycin resistance gene, a Geneticin resistance gene or a Zeocin resistance gene or a modified form thereof.

[0109] 3.5 Preparation of Engineered Cells

[0110] In one aspect, the present disclosure provides a manufacture process of the engineered cells. In one aspect, any known method for preparation may be used. In a particular aspect, the method includes transducing a population of isolated cells with the polynucleotide encoding the fusion polypeptide and selecting a subpopulation of said isolated cells that have been successfully transduced with the fusion polynucleotide thereby producing genetically modified cells, as described above.

[0111] In one aspect, the method includes acquisition, isolation, transduction, expansion steps. In a particular aspect, the method includes the following steps: (i) acquisition of an immune cell population (e.g. blood cells) (ii) isolation of a particular cell population (e.g. T cells and/or NK cells) (iii) transducing a population of isolated cells with the polynucleotide encoding the fusion polypeptide; and (iv) expanding a subpopulation of said isolated cells that have been successfully transduced with said nucleic acid sequence of step (iii) thereby producing genetically modified cells. These different steps are more particularly described below.

[0112] 3.5.1 Cell acquisition

[0113] In one aspect, the subject from which the cell is obtained for introduction of the fusion polypeptide is one having the disease or condition or in need of a cell therapy or to which cell therapy will be administered. In one aspect, the cells can be derived from a healthy donor.

[0114] In one aspect, the cells may be obtained from a sample, such as a biological sample. In one aspect, the samples are selected from whole blood, peripheral blood mononuclear cells (PBMCs), leukocytes, bone marrow, thymus, tissue biopsy, tumor, leukemia, lymphoma, lymph node, gut associated lymphoid tissue, mucosa associated lymphoid tissue, spleen, other lymphoid tissues, liver, lung, stomach, intestine, colon, kidney, pancreas, breast, bone, prostate, cervix, testes, ovaries, tonsil, or other organ, and/or cells derived therefrom. In one aspect, the cells are primary cells. In one aspect, cells from the circulating blood of a subject are obtained, e.g., by apheresis or leukapheresis. The resulting samples comprise lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and/or platelets, and in some aspects contain cells other than red blood cells and platelets.

[0115] 3.5.2 Cell isolation

[0116] Various methods are readily available for isolating immune cells from a sample, for example using Life Technologies Dynabeads[®] system; STEMcell Technologies EasySep[™], RoboSep[™], RosetteSep[™], SepMate[™]; Miltenyi Biotec MACS[™] cell separation kits, cell surface marker expression and other commercially available cell separation and isolation kits (e.g., ISOCELL from Pierce, Rockford, IL). Particular subpopulations of immune cells may be isolated through the use of beads or other binding agents available in such kits specific to unique cell surface markers. For example, MACS[™] CD4⁺ and CD8⁺ MicroBeads may be used to isolate CD4⁺ and CD8⁺ T-cells.

[0117] In one aspect, isolation of the cells includes one or more non-affinity-based cell separation steps. In one aspect, cells are washed, centrifuged, and/or incubated in the presence of one or more reagents, for example, to remove unwanted components, enrich for desired components, lyse or remove cells sensitive to particular reagents. In one aspect, cells are separated based on one or more properties, such as density, adherent properties, size, sensitivity and/or resistance to particular components.

[0118] In one aspect, the blood cells collected from the subject are washed, e.g., to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In one aspect, the cells are washed with phosphate buffered saline (PBS). In one aspect, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Initial activation steps in the absence of calcium may lead to magnified activation. In one aspect, a washing step is accomplished by a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor, Baxter) according to the manufacturer's instructions. In one aspect, a washing step is accomplished by tangential flow filtration (TFF) according to the manufacturer's instructions. In one aspect, the cells are resuspended in a variety of biocompatible buffers after washing, such as, for example, Ca²⁺/Mg²⁺ free PBS. In certain aspects, components of a blood cell sample are removed and the cells directly resuspended in culture media. In one aspect, the isolation include density-based cell separation methods, such as the preparation of white blood cells from peripheral blood by lysing the red blood cells and centrifugation through a Percoll or Ficoll gradient.

[0119] In one aspect, the isolation methods include the separation of different cell types based on the expression or presence in the cell of one or more specific molecules, such as surface markers, e.g., surface

proteins, intracellular markers, or nucleic acid. In one aspect, the separation is affinity- or immunoaffinity-based separation. The separation can be based on positive selection, in which the cells having bound the reagents are retained for further use, and/or negative selection, in which the cells having not bound to the antibody or binding partner are retained. The separation needs not result in 100% enrichment or removal of a particular cell population or cells expressing a particular marker.

[0120] In one aspect, one separation step can deplete cells expressing multiple markers simultaneously, such as by incubating cells with a plurality of antibodies or binding partners, each specific for a marker targeted for negative selection. Likewise, multiple cell types can simultaneously be positively selected by incubating cells with a plurality of antibodies or binding partners expressed on the various cell types. In one aspect, multiple rounds of separation steps are carried out, where the positively or negatively selected fraction from one step is subjected to another separation step, such as a subsequent positive or negative selection.

[0121] In some aspects, specific subpopulations of T cells, such as cells positive or expressing high levels of one or more surface markers, e.g., CD3+, CD28+, CD62L+, CCR7+, CD27+, CD127+, CD4+, CD8+, CD45RA+, and/or CD45RO+ T cells, are isolated by positive or negative selection techniques. In one aspect, T cells are isolated by incubation with anti-CD3/anti-CD28 conjugated particles or beads (e.g., DYNABEADS® M-450 CD3/CD28 T Cell Expander, MACSiBeads™, etc.). In one aspect, the time period of positive selection is about 30 minutes. In a further embodiment, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In one aspect, the time period is 10 to 24 hours. In one aspect, the incubation time period is 24 hours. For isolation of a desired population of cells by positive or negative selection, the concentration of cells and particles can be varied. In certain aspects, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and beads. In one aspect, greater than 100 million cells/mL is used.

[0122] In some aspects, T cells are separated from a sample by negative selection of markers expressed on non-T cells, such as B cells, monocytes, or other white blood cells, such as CD14. In some aspects, a CD4 or CD8 selection step is used to separate CD4+ helper and CD8+ cytotoxic T cells. Such CD4+ and CD8+ populations can be further sorted into sub-populations by positive or negative selection for markers expressed or expressed to a relatively higher degree on one or more naive, memory, and/or effector T cell subpopulations.

[0123] In some aspects, CD8+ cells are further enriched for or depleted of naive, central memory, effector memory, and/or central memory stem cells, such as by positive or negative selection based on surface antigens associated with the respective subpopulation. In some aspects, enrichment for central memory T (TCM) cells is carried out to increase efficacy, such as to improve long-term survival, expansion, and/or engraftment following administration. In some aspects, combining TCM-enriched CD8+ T cells and CD4+ T cells further enhances efficacy.

[0124] In some aspects, memory T cells are present in both CD62L+ and CD62L- subsets of CD8+ peripheral blood lymphocytes. PBMC can be enriched for or depleted of CD62L-CD8+ and/or CD62L+CD8+ fractions, such as using anti-CD8 and anti-CD62L antibodies.

[0125] In some aspects, the enrichment for central memory T (TCM) cells is based on positive or high surface expression of CD45RO, CD62L, CCR7, CD28, CD3, and/or CD127. In some aspects, it is based on negative selection for cells expressing or highly expressing CD45RA and/or granzyme B. In some aspects, isolation of a CD8+ population enriched for TCM cells is carried out by depletion of cells expressing CD4, CD14, CD45RA, and positive selection or enrichment for cells expressing CD62L. In one aspect, enrichment for central memory T (TCM) cells is carried out starting with a negative fraction of cells selected based on CD4 expression, which is subjected to a negative selection based on expression of CD14 and CD45RA, and a positive selection based on CD62L.

[0126] In one aspect, the enrichment for NK cells is based on positive or high surface expression of CD56 and CD16 and on the negative expression of CD3 and/or optionally on the presence of NKp46 or NKp30 receptors.

[0127] In one aspect, the sample or composition of cells to be separated is incubated with small, magnetizable or magnetically responsive material, such as magnetically responsive particles or microparticles, such as paramagnetic beads (e.g., Dynabeads® or MACS® beads). The magnetically responsive material, e.g., particle, generally is directly or indirectly attached to a binding partner, e.g., an antibody, that specifically binds to a molecule, e.g., surface marker, present on the cell, cells, or population of cells that it is desired to separate, e.g., that it is desired to negatively or positively select. In one aspect, the sample is placed in a magnetic field, and those cells having magnetically responsive or magnetizable particles attached thereto will be attracted to the magnet and separated from the unlabeled cells. For

positive selection, cells that are attracted to the magnet are retained; for negative selection, cells that are not attracted (unlabeled cells) are retained. In some aspects, a combination of positive and negative selection is performed during the same selection step, where the positive and negative fractions are retained and further processed or subject to further separation steps.

[0128] In one aspect, the magnetically responsive particles are left attached to the cells that are to be subsequently incubated, cultured and/or engineered; in some aspects, the particles are left attached to the cells for administration to a patient. In one aspect, the magnetically responsive particles are removed from the cells. Methods for removing magnetically responsive particles from cells are known and include, e.g., the use of competing non-labeled antibodies, magnetically responsive particles or antibodies conjugated to cleavable linkers, etc. In one aspect, the magnetically responsive particles are biodegradable.

[0129] In one aspect, the affinity-based selection is via magnetic-activated cell sorting (MACS[®]) (Miltenyi Biotec, Auburn, CA). Magnetic Activated Cell Sorting (MACS[®]) systems are capable of high-purity selection of cells having magnetized particles attached thereto. In certain embodiments, MACS[®] operates in a mode wherein the non-target and target species are sequentially eluted after the application of the external magnetic field. That is, the cells attached to magnetized particles are held in place while the unattached species are eluted. Then, after this first elution step is completed, the species that were trapped in the magnetic field and were prevented from being eluted are freed in some manner such that they can be eluted and recovered. In certain embodiments, the non-target cells are labelled and depleted from the heterogeneous population of cells.

[0130] In one aspect, a cell population described herein is collected and enriched (or depleted) via flow cytometry, in which cells stained for multiple cell surface markers are carried in a fluidic stream. In one aspect, a cell population described herein is collected and enriched (or depleted) via preparative scale (FACS)-sorting. In certain embodiments, a cell population described herein is collected and enriched (or depleted) by use of microelectromechanical systems (MEMS) chips in combination with a FACS-based detection system. In both cases, cells can be labeled with multiple markers, allowing for the isolation of well-defined T cell subsets at high purity.

[0131] In one aspect, the preparation methods include steps for freezing, e.g., cryopreserving, the cells, either before or after isolation, incubation, and/or engineering. In one aspect, the freeze and subsequent thaw step removes granulocytes and, to some extent, monocytes in the cell population. In one aspect, the cells are suspended in a freezing solution, e.g., following a washing step to remove plasma and platelets. Any of a variety of known freezing solutions and parameters in some aspects may be used. One example involves using PBS containing 20% DMSO and 8% human serum albumin (HSA), or other suitable cell freezing media. This is then diluted 1:1 with media so that the final concentration of DMSO and HSA are 10% and 4%, respectively. The cells are then frozen to -80 °C at a rate of 1 °C per minute and stored in the vapor phase of a liquid nitrogen storage tank. In one aspect, cryopreserved cells are thawed and washed as described herein and allowed to rest for one hour at room temperature prior to activation.

[0132] 3.5.3 Cell expansion

[0133] In one aspect, the provided methods include cultivation, incubation, culture, and/or genetic engineering steps. In one aspect, the cells are incubated and/or cultured prior to or in connection with genetic engineering. The incubation steps can include culture, cultivation, stimulation, activation, and/or propagation. In one aspect, the cells are incubated in the presence of stimulating conditions or a stimulatory agent. The conditions can include one or more of particular media, temperature, oxygen content, carbon dioxide content, time, agents, e.g., nutrients, amino acids, antibiotics, ions, and/or stimulatory factors, such as cytokines, chemokines, antigens, binding partners, fusion polypeptides, recombinant soluble receptors, and any other agents designed to activate the cells.

[0134] In one aspect, the stimulating conditions or agents include one or more agent, e.g., ligand, which is capable of stimulating or activating an intracellular signaling domain of a TCR complex. In some aspects, the agent turns on or initiates TCR/CD3 intracellular signaling cascade in a T cell. Such agents can include antibodies, such as those specific for a TCR component and/or co-stimulatory receptor, e.g., anti-CD3, anti-CD28, for example, bound to solid support such as a bead (e.g., Dynabeads[®]), and/or one or more cytokines. In one aspect, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 80, 85, 90, 95, 100 125 or 150 million cells/mL is used.

[0135] In one aspect, the mixture may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In another embodiment, the mixture may be cultured for 21 days. In one embodiment, the beads and the T cells are cultured together for about eight days. In another embodiment, the beads and cells are cultured together for 2-3 days. Several cycles of stimulation may also be desired such that culture time of T cells can be 60 days or more. Conditions appropriate for T cell

culture include an appropriate media that may contain factors necessary for proliferation and viability, including interleukin-2 (IL-2), insulin, IFN- γ , IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGF β , and TNF- α or any other additives. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, AIM-V, DMEM, MEM, α -MEM, F-12, X-Vivo 15, and X-Vivo 20, optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, e.g., penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (e.g., 37 °C) and atmosphere (e.g., air plus 5% CO₂).

[0136] In one aspect, NK cell populations can be expanded *in vitro* using interleukin-2 (IL-2) IL-15, IL-15/IL-15RA complex, IL-18 and IL-12. In one aspect, the NK cells are *ex vivo* expanded for at least about 5 days, for example, not less than about 10 days, not less than about 15 days, or not less than about 20 days before administration to the patient.

IV. Pharmaceutical Composition

[0137] Pharmaceutical compositions of the present disclosure may comprise engineered cells in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the present disclosure are in one aspect formulated for intravenous administration.

[0138] In one aspect, the pharmaceutical composition is substantially free of, e.g., there are no detectable levels of a contaminant, e.g., selected from the group consisting of endotoxin, mycoplasma, replication competent lentivirus (RCL), p24, VSV-G nucleic acid, HIV gag, residual anti-CD3/anti-CD28 coated beads, mouse antibodies, pooled human serum, bovine serum albumin, bovine serum, culture media components, vector packaging cell or plasmid components, a bacterium and a fungus. In one aspect, the bacterium is at least one selected from the group consisting of *Alcaligenes faecalis*, *Candida albicans*, *Escherichia coli*, *Haemophilus influenza*, *Neisseria meningitides*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* group A.

[0139] When “an immunologically effective amount”, “an anti-tumor effective amount”, “a tumor-inhibiting effective amount”, or “therapeutic amount” is indicated, the precise amount of the compositions of the present disclosure to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the T cells described herein may be administered at a dosage of 10⁴ to 10⁹ cells/kg body weight, in some instances 10⁵ to 10⁶ cells/kg body weight, including all integer values within those ranges. T cell compositions may also be administered one time or multiple times at these dosages. The cells can be administered by using infusion techniques that are commonly known in immunotherapy.

V. Method of Treatment

[0140] Engineered cells or pharmaceutical compositions of the present disclosure may be administered in a manner appropriate to the disease to be treated (or prevented). The diseases may comprise solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma and brain metastases);

the non-solid tumors, such as leukemias, including acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

[0141] The disease may also comprises autoimmune disease selected from the group consisting of inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (e.g. atopic dermatitis); systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); respiratory distress syndrome (including adult respiratory distress syndrome; ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T-cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus (e.g. Type I diabetes mellitus or insulin dependent diabetes mellitus); multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjogren's syndrome; juvenile onset diabetes; and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia; myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; anti-phospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullous; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Bechet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia. In one embodiment the medicament is for treatment of multiple sclerosis. In one preferred embodiment the medicament is for the treatment of secondary progressive multiple sclerosis.

[0142] The quantity and frequency of administration will be determined by the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials. The administration of the subject compositions may be carried out in any convenient manner, including by aerosol injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient transarterially, subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. In one aspect, the T cell compositions of the present invention are administered to a patient by intradermal or subcutaneous injection. In one aspect, the compositions are directly injected into an organ of interest (e.g., an organ affected by a neoplasm). Alternatively, the compositions are provided indirectly to the organ of interest, for example, by administration into the circulatory system (e.g., the tumor vasculature). Expansion and differentiation agents can be provided prior to, during or after administration of the cells or compositions to increase production of T cells or NK cells in vitro or in vivo.

[0143] In one aspect, lymphodepletion is performed on a subject, e.g., prior to administering one or more cells described herein. In one aspect, the lymphodepletion comprises administering one or more of melphalan, cytoxan, cyclophosphamide, and fludarabine.

[0144] In one aspect, the engineered cells are administered as part of a combination treatment, such as simultaneously with or sequentially with, in any order, another therapeutic intervention, such as an antibody or engineered cell or receptor or agent, such as a cytotoxic or therapeutic agent. The cells or antibodies in some embodiments are co-administered with one or more additional therapeutic agents or in connection with another therapeutic intervention, either simultaneously or sequentially in any order. In one aspect, the cells are co-administered with another therapy sufficiently close in time such that the cell populations enhance the effect of one or more additional therapeutic agents, or vice versa. In one aspect, the cells or antibodies are administered prior to the one or more additional therapeutic agents. In one aspect, the cells or antibodies are administered after to the one or more additional therapeutic agents, such as anti-cancer agents. In the context of the present disclosure, it is contemplated that cell therapy could be used similarly in conjunction with chemotherapeutic, radiotherapeutic, or immunotherapeutic intervention, as well as pro-apoptotic or cell cycle regulating agents such as immune checkpoint inhibitor.

[0145] Alternatively, the present therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and present disclosure are applied

separately to the individual, one would generally ensure that a significant period of time did not expire between the times of each delivery, such that the agent and therapy would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several week (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations. It is expected that the treatment cycles would be repeated if necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the cell therapy.

VI. Exemplary Sequences

Table 3. Sequences of the present disclosure

SEQ ID No:	Name	Sequence
1	Fas ecto	QVTDINSKGLELRKTVTTVETQNLEGLHHDGQFCHKPCPPGERKARDC TVNGDEPDCVPCQEGKEYTDKAHFSSKCRRCRLCDEGHGLEVEINCT RTQNTKCRCKPNFFCNSTVCEHCDPCTKCEHGIKECTLTSNTKCKEEG SRSN
2	CD34 ecto	SLDNNGTATPELPTQGTFNSVSTNVSYQETTTSTLSTLHPVSQHGNC EATTNITETTVKFTSTSVITSVYGNTNSSVQSQTSVISTVFTTPANVSTPE TTLKPSLSPGNVSDLTSTSTSLATSPTKPYTSSSPILSDIKAEIKCSGIREV KLTQGICLEQNKTSSEAEFKKDRGEGLARVLCGEEQADADAGAQCVC LLLAQSEVRPQCLLLVLANRTEISSKLQLMKKHQSDLKKLGILDFTEQ DVASHQSYSQKT
3	dFas endo	KRKEVQKTCRKHRRKENQGSHESTLNPETVALKDITSDSENSNFRNEI QSLV
4	IL-7R α endo	KKRIKPIVWPSLPDHHKKTLEHLCKKPRKNLNVSNPESFLDCQIHRVDD IQARDEVEGFLQDTPPQQLSESEKQRLGGDVQSPNCPSEDVVITPESFG RDSSLTCLAGNVSAACDAPILSSSRSLDCRESGKNGPHVYQDLLLLSLGTT NSTLPPPSLQSGILTLNPVAQGGPILTSLSNQEEAYVTMSSFYQNNQ
5	CD2 t1	KRKKQRSRRNDEELETRAHRVATEERGRKPHQIPASTPQNPATSQHPP PPPGHRSQAPSHRPPPPGHRVQHQPQKRPPAPSGTQVHQKGPPLPRP RVQPKPPHGAENSLSPSSN
6	CD2 t2	KRKKQRSRRNDEELETRAHRVATEERGRKPHQIPASTPQNPATSQHPP PPPGHRSQAPSHRPPPPGHRVQHQPQKRPPAPSGTQVHQKGPPLP
7	CD2 t3	KRKKQRSRRNDEELETRAHRVATEERGRKPHQIPASTPQNPATSQHPP PPPGH
8	CD2 t4	KRKKQRSRRNDEELETRAHRVATEERGRKPHQIPASTPQNPATSQHPP PPPGHRSQAPSHRPPPPGHRVQHQP
9	Fas TM	LGWLCLLLPIPLIVWV
10	IL-7R α TM	PILLTISILSFFSVALLVILACVLW
11	IL-7R α TM1	PILLTCPTIX ₁ IX ₂ SX ₃ X ₄ X ₅ X ₆ X ₇ X ₈ X ₉ X ₁₀ X ₁₁ LX ₁₂ X ₁₃ X ₁₄ LW
12	IL-7R α TM2	PILNPCLTISILSFFSVALLVILACVLW
13	IL-7R α TM3	PTCLTISILSFFSVALLVILACVLW
14	IL-7R α TM4	PSANCGAISILSFFSVALLVILACVLW
15	IL-7R α TM5	PILLVSCPTISILSFFSVALLVILACVLW
16	IL-7R α TM6	PILLIISIQWLSFFSVALLVILACVLW
17	IL-7R α TM7	NSPCLTISILSFFSVALLVILACVLW
18	IL-7R α TM8	PCLEGLTISILSFFSVALLVILACVLW
19	IL-7R α TM9	PILLTISILSFFWNLLVILACVLW
20	IL-7R α TM10	RFCPHISILSFFSVALLVILACVLW

21	IL-7R α TM11	IKCILSFFSVALLVILACVLW
22	IL-7R α TM12	PIFHPFNCGPISILSFFSVALLVILACVLW
23	IL-7R α TM13	PILLMCPTISILSFFSVALLVILACVLW
24	IL-7R α TM14	PILLTISILSFFSGPSLALLVILACVLW
25	IL-7R α TM15	PILRLGCVTISILSFFSVALLVILACVLW
26	IL-7R α TM16	PIPQGGCILSFFSVALLVILACVLW
27	IL-7R α TM17	LQSCILSFFSVALLVILACVLW
28	IL-7R α TM18	PIFPHQHTISILSFFSVALLVILACVLW
29	IL-7R α TM19	PILLTISKCHLSFFSVALLVILACVLW
30	IL-7R α TM20	PILLTCHLISILSFFSVALLVILACVLW
31	IL-7R α TM21	PIFSCGPLTISILSFFSVALLVILACVLW
32	IL-7R α TM22	PILLPPCLTISILSFFSVALLVILACVLW
33	IL-7R α TM23	PILLTPVCSVTISILSFFSVALLVILACVLW
34	IL-7R α TM1-1	PILLTCPTISILSFFSVALLVILACVLW
35	IL-7R α TM1-2	PILLTCPTISILSFFSVALLVLLLSLW
36	IL-7R α TM1-3	PILLTCPTISILSFFSVLMVSLLLSLW
37	IL-7R α TM1-4	PILLTCPTISILSLAILLMVSLLLSLW
38	IL-7R α TM1-5	PILLTCPTILISSLAILLMVSLLLSLW
39	IL-7R α TM1-6	PILLTCPTISILSFFSVALLVILACSLW
40	wt-hFasL	MQQPFNYPPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPP PPPLPPPPPPPLPLPLPLPLKRGNHSTGLCLLMFFMVLVALVGLGL GMFQLFHLQKELAELESTSQMHTASSLEKQIGHPSPPPEKKELRKVA HLTGKSNSRSMPLWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSK VYFRGQSCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQM WARSSYLGA VFNLTADHLYVNVSELSLVNFEESTFFGLYKL
41	mt1-hFas	MQQPFNYPPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPP PPPLPPPPPPPLPLPLPLPLKRGNHSTGLCLLMFFMVLVALVGLGL GMFQLFHLQKEPSPPPEKKELRKVAHLTGKSNSRSMPLWEDTYGIVL LSGVKYKKGGLVINETGLYFVYSKVYFRGQSCNNLPLSHKVYMRNSK YPQDLVMMEGKMMSYCTTGQMWARSSYLGA VFNLTADHLYVNVSE ELSLVNFEESTFFGLYKL
42	mt2-hFas	MQQPFNYPPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPP PPPLPPPPPPPLPLPLPLPLKRGNHSTGLCLLMFFMVLVALVGLGL GMFQLFHLQKELAELESTSQMHTASSLGHPSPPEKKELRKVAHLTG KSNSRSMPLWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYFR GQSCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWARSS YLGA VFNLTADHLYVNVSELSLVNFEESTFFGLYKL
43	mt3-hFas	MQQPFNYPPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRGNHST GLCLLMFFMVLVALVGLGLGMFQLFHLQKELAELESTSQMHTASS LEKQIGHPSPPPEKKELRKVAHLTGKSNSRSMPLWEDTYGIVLLSGV KYKKGGLVINETGLYFVYSKVYFRGQSCNNLPLSHKVYMRNSKYPQD LVMMEGKMMSYCTTGQMWARSSYLGA VFNLTADHLYVNVSELSL VNFEESTFFGLYKL
44	mt4-hFas	MQQPFNYPPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPP PPPLPPPPPPPLPLPLPLPLEEGNHSTGLCLLMFFMVLVALVGLGL GMFQLFHLQKELAELESTSQMHTASSLEKQIGHPSPPPEKKELRKVA HLTGKSNSRSMPLWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSK VYFRGQSCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQM

		WARSSYLGA VFNLT SADHLY VNVSELSLVNFEE SQTFGLYKL
45	mt5-hFas	MQQPFNY PYPQIY WVDSSASSPW APPGTVLPCPTS VPRRPGQRRPPPPP PPPLPPPPPPPLPPLPLPLEEEGNHSTGLCCLVMFFMVLVALVGLGL GMFQLFHLQKELAE LRESTSQMHTASSLEGHPSPPEKKELRKVAHLT GKSNSRSMPL EWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYF RGQSCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWARS SYLGA VFNLT SADHLY VNVSELSLVNFEE SQTFGLYKL
46	mt6-hFas	MQQPFNY PYPQIY WVDSSASSPW APPGTVLPCPTS VPRRPGQRRPPPPP PPPLPPPPPPPLPPLPLPLEEEGNHSTGLCCLVMFFMVLVALVGLGL GMFQLFHLQKELAE LRESTSQMHTASSLEGHPSPPEKKELRKVAHLT GKSNSRSMPL EWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYF GQSCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWARS YLGA VFNLT SADHLY VNVSELSLVNFEE SQTFGLYKL
47	wt-muFas	MQQPMNYP CQIFWVDSSATSSW APPGSVFPCPSCGPRGPDQRRPPPPP PPPVSP LPPSQPLPLPPLTPLKKKD HNTNLWLPVVF MVLVALVGMG LGM YQLFHLQKELAE LREFTNQSLKVSSF EKQIANPSTPSEKKEPR SVA HLTG NPHSR SIPLEWEDTYGTALISGVKYKKGGLVINETGLYFVYSKV YFRGQSCNNQPLNHKVYMRNSKYPEDLVLMEEKRLNYCTTGQIWAH SSYLGA VFNLT SADHLY VNISQLSLINFEE SKTFGLYKL
48	SP-1	MLGIWTL LPLVLT S VARLSSKSVNA
49	SP-2	METDTLL L WVLL L WVPGSTG
49	tEGFR	METDTLL L WVLL L WVPGSTGRKVCNGIGIGEFKDSLSINATNIKHFKN CTSI S GDLHILPVA FRGDSFTHTPPLDPQELDILKTVKEITGFLLIQAWPE NRTDLHAFENLEIRGR TKQH GQFSLAVVSLNITSLGLRSLKEISDGDVII SGNKNLCYANTINWKKLFGTSGQKTKIISNRGENSCKATGQVCHALCS PEGCWGP EPRDCVSCRNVSRGRECVDKCNLLEGE PREFVENSECIQCH PECLPQAMNITCTGRGPDNCIQCAHYIDGPHCVKTC PAGVMGENNTL VWKYADAGHVCHLCPNCTY GCTGPGLEGCPTNGPKIPSIATGMVGA LLLLLVVALGIGLFM
50	T2A	EGRGSLL TCGDVEENPGP
51	E2A	QCTNYALL KLAGDVESNPGP

VIII. Example

[0146] The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

[0147] Example 1: Generation of fusion polypeptides

[0148] Plasmid constructs were designed to encode a marker of transduction (tEGFR) on its 3' of the ORF followed by a sequence encoding a self-cleaving 2A peptide derived from the equine rhinitis A virus. Following this was the sequence encoding the signal peptide (SP-1) and extracellular domain of human Fas followed by a transmembrane sequence (TM1-1~TM1-6, Fas TM) and then the endodomain of human IL7Ra and/or human CD2 (F-1, F-3~F-7; F-2). In some instances, the sequences encoding truncated sections of the CD2 endodomain proceeds after the IL7Ra endodomain sequence (F-8~F-11). As a functional negative control, a plasmid only encoding the tEGFR transduction marker was developed (Ctrl). As a positive control for protection against FasL mediated apoptosis, a plasmid was designed encoding tEGFR followed by the 2A sequences from equine rhinitis A virus followed by the signal peptide (SP-1), extracellular domain, transmembrane and truncated endodomain of human Fas (construct comprising C-1). As a positive control for cytokine receptor signaling, a plasmid was designed encoding tEGFR followed by the 2A sequences from equine rhinitis A virus followed by the signal peptide (SP-2) and extracellular domain from human CD34 followed by transmembrane sequence TM1-1 and the endodomain from human IL7Ra (construct comprising C-2). The construct comprising C-3 was designed encoding tEGFR followed by the 2A sequences from equine rhinitis A virus followed by a signal peptide (SP-2) and extracellular domain from human TGFβR followed by transmembrane sequence TM1-1 and the endodomain from human IL7Ra.

Table 4. Structures of fusion polypeptides (from N- to C-terminus)

No	Ectodomain	Transmembrane domain	First Endodomain	Second Endodomain
F-1	Fas	IL-7R α TM1-1	IL-7R α	(none)
F-2	Fas	Fas TM	CD2 t1	(none)
F-3	Fas	IL-7R α TM1-2	IL-7R α	(none)
F-4	Fas	IL-7R α TM1-3	IL-7R α	(none)
F-5	Fas	IL-7R α TM1-4	IL-7R α	(none)
F-6	Fas	IL-7R α TM1-5	IL-7R α	(none)
F-7	Fas	IL-7R α TM1-6	IL-7R α	(none)
F-8	Fas	IL-7R α TM1-1	IL-7R α	CD2 t1
F-9	Fas	IL-7R α TM1-1	IL-7R α	CD2 t2
F-10	Fas	IL-7R α TM1-1	IL-7R α	CD2 t3
F-11	Fas	IL-7R α TM1-1	IL-7R α	CD2 t4
C-1	Fas	Fas TM	dFas	(none)
C-2	CD34	IL-7R α TM1-1	IL-7R α	(none)
C-3	TGF β R2	IL-7R α TM1-1	IL-7R α	

[0149] Example 2: Apoptosis resistance of engineered T cell

[0150] Over activation/stimulation of T cells by the surrounding tumor can lead to Fas/FasL mediated AICD and can subsequently result in a decreased number of viable T cells. T cells were transduced with lentivirus vectors expressing the fusion polypeptides as described in Example 1. Transduced T cells (5×10^5 T cells) were added to the wells and were cultured for 7 days without further stimulation in an incubator.

[0151] At the point of seeding cells into the 24-well plate (day 0), cells were spun by centrifugation, stained for EGFR (Biolegend, #352908). High Speed, High Throughput Cell Counter (Nexcelom) was used to determine the live T cell numbers.

[0152] At day 1, 3, 7, 8, 10, 15 during incubation, T cells were mixed gently, 60 μ L cell suspension was removed for surviving T cell count by High Speed, High Throughput Cell Counter (Nexcelom). At day 3, 7, 10 after detection of live T cell number, if T cell density over 1×10^6 cells/mL, then will be adjusted to 5×10^5 cells/mL for culture. Live T cell numbers during incubation were made relative to the day 0 analysis to measure proliferation fold difference. Figure. 1 shows the growth of transduced T cells (7 healthy donors) after initial seeding (5×10^5 T cells) and left without further stimulation. Here we observe a significant improvement in the expansion of T cells transduced with the F-1 over the control constructs C-1 ($p=0.0135$, F-1 vs. C-1) and Ctrl ($p=0.0063$, F-1 vs. Ctrl). These data show the enhanced growth capacity of F-1 in unstimulated conditions.

[0153] Example 3: Persistence of engineered T cells

[0154] Improved persistence of CAR T cells correlates with clinical efficacy in many settings (10.1186/s40364-022-00434-9). In order to expand and maintain durable remission responses, T cells are required to persist in environments with low levels of stimulation and cytokines. To test the ability of the cells to survive these harsh conditions we subjected the transduced T cells to starvation assays. Briefly, primary T cells from 4 healthy donors were transduced with either Ctrl, C-2 or F-1. All transduced T cells were spun by centrifugation, stained for EGFR to evaluate the transduction efficiency. And then, the transduced T cells were normalized to be 50% transduced EGFR⁺ through the addition of non-transduced T cells and then seeded at the same density (1×10^6 T cells) for 7 days in an incubator. At day 3, 7 during incubation, cell suspension was harvest for counting living T cell and EGFR⁺ T cell enrichment.

[0155] Figure 2B shows that in the absence of cytokine or stimulation, T cells transduced with Ctrl are unable to proliferate and cellular viability decreases dramatically over 7 days to approximately 40% ($p=0.0002$, C-2 vs. Ctrl; $p=0.0003$, F-1 vs. Ctrl). Both C-2 and F-1 have a significant survival advantage over the Ctrl ($p<0.0001$, C-2 vs. Ctrl; $p=0.0002$, F-1 vs. Ctrl), showing linear growth over the 7 day period ($p=0.0008$, C-2 vs. Ctrl; $p=0.0014$, F-1 vs. Ctrl) and sustained viability comparable to the same conditions

supplemented with external IL2 (100 IU/mL, Cytiva, #29062790) (Figure 2A and 2B). In addition, C-2 and F-1 were able to enrich in the transduced T cell population over this period compared to the Ctrl (Figure 2B) or compared to the condition with supplemented IL2 (100 IU/mL) (Figure 2A). This demonstrates that T cells transduced with F-1 are able to deliver a cytokine survival signal and promote growth and viability in the absence of exogenous stimulation or cytokines. To further interrogate the constructs' ability to withstand Fas/FasL mediated apoptosis, the transduced T cells were exposed to trimeric FasL stimulation (50 ng/mL, Adipogene, AG-40B-0130-3010) in the presence of supplemented IL2 (100 IU/mL) and the cell growth, viability and enrichment was assessed over a 7 day period. Here we demonstrate that T cells transduced with either Ctrl or C-2 are unable to grow, have reduced viability and enrichment of the transduced population. In contrast, T cells transduced with F-1 continue to proliferate and grow to similar levels to the condition without FasL ($p=0.0035$, F-1 vs. Ctrl; $p=0.0037$, F-1 vs. C-2). We also observed a significant improvement in viability ($p=0.0007$, F-1 vs. Ctrl; $p=0.0146$, F-1 vs. C-2) and enrichment ($p=0.0003$, F-1 vs. Ctrl; $p=0.0003$, F-1 vs. C-2) over this 7 day period (Figure 2C).

[0156] Example 4: Persistence of modules post FasL stimulation

[0157] To further interrogate the fusion proteins' (sharing same extracellular domain of human Fas and different endodomain of either human IL7Ra or CD2) ability to withstand Fas/FasL mediated apoptosis, the transduced T cells (7 healthy donors) were exposed to 50 ng/mL trimeric FasL stimulation in the presence of supplemented IL-2 (100 IU/mL) for 7 days in an incubator. At day 1, 3, 7 during incubation, T cells were mixed gently and removed for surviving T cell count by High Speed, High Throughput Cell Counter (Nexcelom). Live T cell densities during incubation were made relative to the initial seeding (5×10^5 T cells/mL) of day 0 to measure proliferation fold difference. As shown in Figure 3, the T cells transduced with various fusion proteins (sharing same extracellular domain of human Fas) demonstrate enhanced persistence to FasL induced apoptosis, in which the cells expressing F1 displayed a slightly higher proliferation level than those with C1 and F2.

[0158] Example 5: Expansion of modules post non-IL-2 starvation

[0159] To further interrogate the fusion proteins' (sharing different extracellular domain from either human Fas or TGF β R and same transmembrane domain and endodomain) expansion ability in cytokine starvation, we subjected the transduced T cells to non-IL-2 conditions. Briefly, primary T cells from 4 healthy donors were transduced with either Ctrl, C-3 or F-1. All transduced T cells were spun by centrifugation, stained for EGFR to evaluate the transduction efficiency. And then, the EGFR⁺ T cells were normalized to be 50% through the addition of non-transduced T cells and then seeded at the same density (1×10^6 T cells) for 7 days in an incubator. At day 3, 7 during incubation, cell suspension was harvested for living T cell counting by Cell Counter and EGFR⁺ T cell detection by flow cytometry. EGFR⁺ T cell numbers during incubation were made relative to the day 0 analysis to measure proliferation fold difference.

[0160] Figure 4 shows that T cells transduced with C-3 are unable to proliferate in the absence of cytokine over the 7-day period (C-3 vs. F-1, $p=0.0277$ at day3, $p=0.0060$ at day7). Above results demonstrate that the function of IL-7Ra TM-endo would be prevented by the definite extracellular domain.

CLAIMS

1. An engineered cell expressing a trimeric complex comprising at least one fusion polypeptide, wherein the fusion polypeptide comprises, from its N to C terminus in order,
 - a) an extracellular component comprising a Fas ectodomain;
 - b) a transmembrane component; and
 - c) an intracellular component comprising an IL-7R endodomain, a CD2 endodomain, or a combination thereof, wherein the intracellular component doesn't comprise a Fas endodomain; wherein the trimeric complex is capable of binding to Fas ligand.
2. The engineered cell of claim 1, wherein the extracellular component comprises or consists of an amino acid sequence that is at least 90% sequence identity to SEQ ID NO: 1;

preferably, the extracellular component comprises a cysteine-rich domain 1, a cysteine-rich domain 2 and a cysteine-rich domain 3 of the amino acid sequence of SEQ ID NO: 1;

more preferably, the extracellular component comprises or consists of the amino acid sequence of SEQ ID NO: 1;
3. The engineered cell of claim 1 or 2, wherein the transmembrane component is capable of self-oligomerizing;

preferably, the transmembrane component consists of a transmembrane domain comprising a gain-of-function insertion compared to SEQ ID NO: 10;

more preferably, the transmembrane component consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 11 to SEQ ID NO: 33.
4. The engineered cell of claim 3, wherein the transmembrane component consists of the amino acid sequence of SEQ ID NO: 11 (PILLTCPTIX₁IX₂SX₃X₄X₅X₆X₇X₈X₉X₁₀X₁₁LX₁₂X₁₃X₁₄LW), wherein X₁ is S or L; X₂ is L or S; X₃ is F or L; X₄ is F or A; X₅ is S or I; X₆ is V or L; X₇ is A or L; X₈ is L or M; X₉ is L or V; X₁₀ is V or S; X₁₁ is I or L; X₁₂ is A or L; X₁₃ is C or L; and X₁₄ is V or S;

preferably, the transmembrane component consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 39.
5. The engineered cell of any one of claims 1 to 4, wherein the intracellular component comprises or consists of an IL-7R α endodomain;

preferably, the intracellular component comprises or consists of an amino acid sequence that is at least 90% sequence identity to SEQ ID NO: 4;

more preferably, the intracellular component comprises or consists of the amino acid sequence of SEQ ID NO: 4.
6. The engineered cell of any one of claims 1 to 5, wherein the intracellular component comprises or consists of a CD2 endodomain;

preferably, the intracellular component comprises or consists of a full-length CD2 endodomain or a functional fragment thereof, wherein the functional fragment is truncated at the C-terminus by up to 65 amino acids compared to the full-length CD2 endodomain;

more preferably, the intracellular component comprises or consists of an amino acid sequence that is at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 5 to SEQ ID NO: 8, or comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 5 to SEQ ID NO: 8.
7. The engineered cell of any one of claims 1 to 6, wherein the transmembrane component is capable of self-oligomerizing, the intracellular component comprises or consists of an IL-7R α endodomain that is fused by its N-terminus to the C-terminus of the transmembrane component;

preferably, the transmembrane component consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 11 to SEQ ID NO: 33;

more preferably, the intracellular component comprises or consists of the amino acid sequence of SEQ ID NO: 4, and the transmembrane component consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 39.

8. The engineered cell of any one of claims 1 to 7, wherein the intracellular component comprises or consists of an IL-7R α endodomain and a CD2 endodomain, the transmembrane component is capable of self-oligomerizing, the IL-7R endodomain is fused by its N-terminus to the C-terminus of the transmembrane component, the IL-7R endodomain is fused by its C-terminus to the N-terminus of the CD2 endodomain;

preferably, the intracellular component consists of a full-length CD2 endodomain or a functional fragment thereof, wherein the functional fragment is truncated at the C-terminus by up to 65 amino acids compared to the full-length CD2 endodomain; and the transmembrane component consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 11 to SEQ ID NO: 33;

more preferably, the intracellular component comprises or consists of the amino acid sequence of SEQ ID NO: 4 and an amino acid sequence selected from the group consisting of SEQ ID NO: 5 to SEQ ID NO: 8; and the transmembrane component consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 34 to SEQ ID NO: 39.

9. The engineered cell of any one of claims 1, 2 and 6, wherein the transmembrane component consists of a transmembrane domain selected from the group consisting of Fas, CD2, CD3s, CD35, CD3C, CD25, CD27, CD28, CD40, CD79A, CD79B, CD80, CD86, OX40, 4- IBB, SLAMF1, CTLA4, CD200R, LAG3, HVEM, BTLA, PD-L2, PD-L1, ICOS, PD-1, CD300, GITR, A2aR, DAP10, FcR α , FcR β , FcR γ , Fyn, GAL9, KIR, Lck, LAT, LRP, KG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPa, pTa, TCR α , TCR β , TIM3, TRIM, LPA5 and Zap70, the intracellular component comprises or consists of a CD2 endodomain that is fused by its N-terminus to the C-terminus of the transmembrane component;

preferably, the CD2 endodomain is a full-length CD2 endodomain or a functional fragment thereof, wherein the functional fragment is truncated at the C-terminus by up to 65 amino acids compared to the full-length CD2 endodomain, the transmembrane component consists of Fas transmembrane domain;

more preferably, the intracellular component consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 5 to SEQ ID NO: 8, and the transmembrane component consists of the amino acid sequence of SEQ ID NO: 9.

10. The engineered cell of any one of claims 1 to 9, wherein the trimeric complex is expressed on the surface of the engineered cell;

preferably, the trimeric complex is a homotrimer or a heterotrimer, wherein the heterotrimer comprises an endogenous Fas;

more preferably, the trimeric complex comprises two or three fusion polypeptides when the intracellular component comprises an IL-7R endodomain.

11. The engineered cell of any one of claims 1 to 10, wherein the engineered cell is an immune cell, preferably, the engineered cell is a lymphocyte; more preferably, the engineered cell is a NK cell, a T cell, or a combination thereof.

12. The engineered cell of any one of claims 1 to 11, wherein the engineered cell expresses an antigen receptor; preferably, the antigen receptor is a chimeric antigen receptor or an antigen-specific TCR; more preferably, the chimeric antigen receptor targets against CD19, CD20, CD22, CD30, BCMA, AFP, ALK, GPC3, HER2, EGFR alpha folate receptor, 5T4, $\alpha\beta$ 6 integrin, B7-H3, B7-H6, CAIX, CD16, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD138, CD171, CEA, Claudin18.2, CSPG4, DLL3, EGFR, HER2, EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, fetal AchR, FRa, GD2, GD3, MAGE-1, NY-ESO-1, IL-11R α , IL-13R α 2, Lambda, Lewis-Y, Kappa, Mesothelin, Muc1, Muc16, NCAM, NKG2D Ligands, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TAG72, TEMs, VEGFR2 or WT-1.

13. The engineered cell of any one of claims 1 to 12, wherein the engineered cell expresses an exogenous FasL; preferably, the exogenous FasL is secreted or membrane bound; more preferably, the exogenous FasL comprises or consists of an amino acid sequence that is at least 90% sequence identity to an amino acid selected from the group consisting of SEQ ID NO: 40 to SEQ ID NO: 47, or comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 40 to SEQ ID NO: 47.

14. A fusion polypeptide comprised in the engineered cell of any one of claims 1 to 13.

15. A use of the fusion polypeptide of claim 14 for the enhancement of the persistence and expansion of the engineered cells; preferably, the fusion polypeptide is capable of participating in the formation of the trimeric complex; more preferably, the trimeric complex is capable of inducing the signaling of the intracellular component independent of the Fas ligand binding.
16. An isolated nucleic acid encoding the fusion polypeptide of claim 14, preferably, the isolated nucleic acid encodes the fusion polypeptide and an antigen receptor, more preferably, the fusion polypeptide and the antigen receptor are linked via a viral self-cleaving polypeptide.
17. A vector comprising the nucleic acid of claim 16, preferably, the vector is a viral vector, more preferably, the viral vector is a retroviral, lentiviral, adenoviral, or adeno-associated viral vector.
18. A pharmaceutical formulation comprising the engineered cell of any one of claims 1-13, the fusion polypeptide of claim 14, the isolated nucleic acid of claims 16 or the vector of claims 17, and a pharmaceutically acceptable carrier.
19. A method of disease treating a subject, comprising administering the engineered cell of any one of claims 1-13, the fusion polypeptide of claim 14, the isolated nucleic acid of claims 16, the vector of claims 17, or the pharmaceutical formulation of claim 18 to the subject; preferably, the disease is selected from the group consisting of leukemia, lymphoma, lung cancer, melanoma, breast cancer, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, neuroblastoma and rhabdomyosarcoma; more preferably, the disease is leukemia or lymphoma.