CD28 antibodies in the presence of IL-2, and finally infusing the expanded cell population back to the tumor-bearing patient (together with chemotherapy and repetitive administration of IL-2) (Dudley *et al*., *Science 298*:850, 2002). This form of adoptive T cell therapy with tumor infiltrating lymphocytes is technically cumbersome and leads to complete remission in only a minor fraction of patients with melanoma and is rarely effective in other cancers (Besser *et al*., *Clin. Cancer Res. 16*:2646, 2010).

Isolation of tumor-reactive T cell clones led to the development of another immunotherapeutic approach – the generation of recombinant T cell receptors (TCRs) specific for particular antigens, which are introduced into T cells using a vector delivery system to confer specificity for a tumor-associated peptide presented by an MHC molecule expressed on a tumor cell. A similar approach introduces a synthetic receptor, termed a chimeric antigen receptor (CAR), which contains an antigen-binding domain, which, *e.g*., in the context of anti-tumor therapy can bind to a tumor-specific or associated antigen, linked to one or more intracellular component comprising an effector domains, such as a TCR and/or costimulatory signaling domains. Unlike TILs, the basic procedure for TCR or CAR T cell immunotherapy is to genetically modify human T cells with a transgene encoding a tumor targeting moiety, *ex vivo* expansion of the recombinant T cells, and transfusing the expanded recombinant T cells back into patients. In the case of adoptive therapy with CAR T cells, the composition of the synthetic CAR structure, as well as the quality and purity of the genetically engineered T cells, will determine therapeutic efficacy against tumors *in vivo*. But, there are challenges to expanding and selecting the recombinant cell populations, as well as making sure the cells are effective and specific enough *in vivo* to avoid serious autoimmune side effects.

Currently, there remains a need in the immunotherapy field for compositions and methods for identifying, efficiently isolating/sorting, selectively expanding, *in vivo* tracking and controlling or eliminating engineered cells, such as engineered immune cells (*e.g*., T cells).

SUMMARY

In one aspect, the present disclosure refers to a single chain fusion protein, comprising a transmembrane domain disposed between an extracellular component and an intracellular component, wherein: the extracellular component comprises a plurality of tag cassettes and a connector region comprising a hinge, and wherein at least one of the plurality of tag cassettes comprises a Strep tag; and the intracellular component comprises an effector domain.

In another aspect, the present disclosure refers to a nucleic acid molecule encoding a single chain fusion protein as disclosed herein.

In another aspect, the present disclosure refers to a vector comprising a nucleic acid molecule as disclosed herein.

In another aspect, the present disclosure refers to a host cell, comprising a nucleic acid molecule encoding a single chain fusion protein as disclosed herein; optionally wherein the host cell is a T cell.

In another aspect, the present disclosure refers to an *in vitro* or *ex vivo* method for activating a cell, comprising contacting a cell comprising a single chain fusion protein as disclosed herein and/or the nucleic acid molecule as disclosed herein with a binding domain specific for the tag cassette.

In another aspect, the present disclosure refers to an *in vitro* or *ex vivo* method for promoting cell proliferation, comprising contacting a non-natural cell comprising a single chain fusion protein as disclosed herein and/or the nucleic acid molecule as disclosed herein with a binding domain specific for the tag cassette and a growth factor cytokine for a time sufficient to allow cell growth.

In another aspect, the present disclosure refers to an *in vitro* method for identifying a cell, comprising: contacting a sample comprising a cell comprising a single chain fusion protein as disclosed herein and/or the nucleic acid molecule as disclosed herein with a binding domain specific for the tag cassette, wherein the binding domain specific for the tag cassette comprises a detectable moiety, and detecting the presence of the cell in the sample.

In another aspect, the present disclosure refers to an *in vitro* method for sorting or selecting a cell or population of cells, the method comprising: contacting a sample comprising a cell comprising a single chain fusion protein as disclosed herein and/or the nucleic acid molecule as disclosed herein, with a binding domain specific for the tag cassette, and selecting or sorting away from other cells that are specifically bound by the binding domain, thereby selecting or sorting away from other cells the cell or population of cells.

In another aspect, the present disclosure refers to an *in vitro* or *ex vivo* method for enriching for or isolating a cell or population thereof, the method comprising contacting a sample comprising the cell comprising the nucleic acid molecule as disclosed herein and/or the single chain fusion protein as disclosed herein with a binding domain specific for the tag cassette and enriching for or isolating the cell away from other cells not expressing the fusion protein in the sample.

In another aspect, the present disclosure refers to an *in vitro* method for depleting cells, comprising contacting a cell comprising a nucleic acid molecule as disclosed herein or a single chain fusion protein as disclosed herein with a binding domain specific for the tag cassette and comprising a cell-toxic reagent, wherein binding of the binding domain to the tag cassette leads to death of the cells expressing the single chain fusion protein

In yet another aspect, the present disclosure refers to the use of a host cell as disclosed herein in the manufacture of a medicament for treating a disease in a subject, wherein the disease is selected from a cancer, an infectious disease, an autoimmune disease, an inflammatory disease, or an aging-related disease.

In certain examples, the present disclosure is directed to a single chain fusion protein, comprising an extracellular component and an intracellular component connected by a hydrophobic portion, wherein the extracellular component comprises a binding domain that specifically binds a target, a tag cassette, and a connector region comprising a hinge, and wherein the intracellular component comprises an effector domain.

In some examples, the present disclosure is directed to a chimeric antigen receptor molecule, comprising a fusion protein having one or more extracellular tag cassettes (a) located at the amino-terminus of an extracellular binding domain, (b) imbedded within an extracellular binding domain, or (c) disposed between and connecting an extracellular binding domain and an intracellular component comprising an effector domain.

In further examples, the present disclosure is directed to a single chain fusion protein, comprising a hydrophobic portion disposed between and connecting an extracellular component and an intracellular component, wherein the extracellular component comprises a tag cassette and a connector region comprising a hinge, and wherein the intracellular component comprises an effector domain.

In still further examples, the present disclosure is directed to a method for activating a cell, such as a T cell (*e.g.*, a non-natural T cell), comprising contacting a cell with a binding domain specific for a tag cassette, wherein the cell comprises a nucleic acid molecule encoding a fusion protein according to this disclosure and the binding domain specific for the tag cassette is attached to a solid surface.

In yet further examples, the present disclosure is directed to a method for promoting cell proliferation, such as T cell proliferation, comprising contacting a cell (*e.g.*, a non-natural T cell) with a binding domain specific for a tag cassette and a growth factor cytokine for a time sufficient to allow cell growth, wherein the cell comprises a nucleic acid molecule encoding a fusion protein according to this disclosure and the binding domain specific for the tag cassette is attached to a solid surface.

In certain other examples, the present disclosure is directed to a method for identifying cell, such as a T cell, comprising contacting a sample comprising a cell, such as a T cell (*e.g.*, a non-natural T cell) with a binding domain specific for a tag cassette, wherein the cell comprises a nucleic acid molecule encoding a fusion protein according to this disclosure and the binding domain specific for the tag cassette comprises a detectable moiety, and detecting the presence of the cell expressing a fusion protein in the sample.

In certain further examples, the present disclosure is directed to a method for sorting a T cell, comprising contacting a sample comprising a non-natural T cell with a binding domain specific for a tag cassette, wherein the non-natural T cell comprises a nucleic acid molecule encoding a fusion protein according to this disclosure and the binding domain specific for the tag cassette comprises a detectable moiety, and sorting

the non-natural T cell expressing a fusion protein from other cells not expressing a fusion protein in the sample.

In certain examples, the present disclosure is directed to a method for enriching or isolating a T cell, comprising contacting a sample comprising a non-natural T cell with a binding domain specific for a tag cassette, wherein the non-natural T cell comprises a nucleic acid molecule encoding a fusion protein according to this disclosure and the binding domain specific for the tag cassette comprises a detectable moiety, and enriching for or isolating the non-natural T cell expressing a fusion protein away from other cells not expressing a fusion protein in the sample.

In further examples, the present disclosure is directed to a method for depleting certain T cells, comprising contacting a non-natural T cell with a binding domain specific for a tag cassette, wherein the non-natural T cell comprises a nucleic acid molecule encoding a fusion protein according to this disclosure and wherein binding of the binding domain specific for the tag cassette leads to cell death of the T cells expressing a fusion protein.

These and other examples of the present disclosure will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A – 1H show illustrations of various single chain chimeric effector molecules containing one or more affinity tag cassettes (A-D, referred to herein as a Key-ChEMs), and optionally containing one or more specific binding domains (E-G, referred to herein as a T-ChARMs). The single chain ChEMs and ChARMs contain an intracellular domain. The tag cassettes may be any type of affinity tag, such as Strep tag® II (SEQ ID NO.:1), Myc tag (SEQ ID NO.:7), V5 tag (SEQ ID NO.:8), Flag® tag (SEQ ID NO.:3), His tag, or other peptides or molecules, which are recognized by a non-endogenous cognate binding partner (*e.g.*, receptor, protein, antibody). As shown, a Key-ChEM may contain (A, B) one tag cassette, (C) two tag cassettes (Key-ChEM^2), (D) three tag cassettes (Key-ChEM^3), or more. In addition, the chimeric molecules may have multiple effector domains (*e.g.*, the molecules of A and C-G have two, while the molecule shown in B has three effector domains), and the tag cassettes may be placed in various different areas of a Key-ChEM or T-ChARM molecule. In these particular examples, T-ChARMs have one tag cassette located between the specific binding domain and the effector domain (E), at the distal end (*e.g.*, amino-terminus) of the

CLAIMS

What is claimed is:

1. A single chain fusion protein, comprising a transmembrane domain disposed between an extracellular component and an intracellular component, wherein:

the extracellular component comprises a plurality of tag cassettes and a connector region comprising a hinge, and wherein at least one of the plurality of tag cassettes comprises a Strep tag; and

the intracellular component comprises an effector domain.

2. The single chain fusion protein according to claim 1, wherein each of the plurality of tag cassettes comprises a Strep tag.

3. The single chain fusion protein according to claim 1, wherein two to five of the plurality of tag cassettes comprises a Strep tag.

4. The single chain fusion protein according to any one of claims 1-3, wherein the connector region further comprises:

(a) a linker module; and/or

(b) a linker module, wherein the linker module comprises one or more Gly_xSer_y, wherein x and y are independently an integer from 0 to 10 provided that x and y are not both 0; and/or

(c) a linker module, wherein the linker module is a $(Gly_xSer)_n$, wherein x is an integer from 1 to 5 and n is an integer from 1 to 10; and/or

(d) a linker module, wherein the linker module is a CH2CH3 or a CH3.

5. The single chain fusion protein according to any one of claims 1-4, wherein:

one or more of the plurality of tag cassettes are located within the connector region; or

one or more of the plurality of tags are located within the connector region, wherein each tag is connected to one or two linker modules comprising a $(Gly_xSer_y)_n$, wherein n is an integer from 1 to 10, and x and y are independently an integer from 0 to 10 provided that x and y are not both 0; or

one or more of the plurality of tags are located within the connector region, wherein each tag is connected to one or two linker modules having the amino acid sequence of Gly-Gly-Gly-Gly-Ser (SEQ ID NO:10), $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$ (SEQ ID NO:11), $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$-Gly-Gly-Ser (SEQ ID NO:12), or any combination thereof; or

one to five of the plurality of tags are located within the connector region; or

one to five of the plurality of tags are located within the connector region, wherein each tag is connected to one or two linker modules comprising a $(Gly_xSer_y)_n$, wherein n is an integer from 1 to 10, and x and y are independently an integer from 0 to 10 provided that x and y are not both 0; or

the connector region comprises from one to five tags, wherein each tag is connected to one or two linker modules having the amino acid sequence of Gly-Gly-Gly-Gly-Ser (SEQ ID NO:10), $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$ (SEQ ID NO:11), $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$-Gly-Gly-Ser (SEQ ID NO:12), or any combination thereof.

6. The single chain fusion protein according to any one of claims 1-5, wherein the single chain fusion protein further comprises a Strep tag, His tag, Flag® tag, Xpress® tag, Avi tag, Calmodulin tag, Polyglutamate tag, HA tag, Myc tag, Nus tag, S tag, X tag, SBP tag, Softag, V5 tag, CBP, GST, MBP, GFP, Thioredoxin tag, or any combination thereof.

7. The single chain fusion protein according to any one of claims 1-6, wherein one or more of the plurality of tag cassettes are or comprise a Strep tag having amino acid sequence Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO.:1) or Trp-Arg-His-Pro-Gln-Phe-Gly-Gly (SEQ ID NO.:2).

8. The single chain fusion protein according to any one of claims 1-7, wherein the transmembrane domain is a CD4, CD8, CD28 or CD27 transmembrane domain.

9. The single chain fusion protein according to any one of claims 1-8, wherein:

(a) the effector domain is from CD3ε, CD3δ, CD3ζ, CD27, CD28, CD79A, CD79B, CD134, CD137, CARD11, DAP10, FcRα, FcRβ, FcRγ, Fyn, HVEM, ICOS, Lck, LAG3, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, ROR2, Ryk, SLAMF1, Slp76, pTα, TCRα, TCRβ, TRIM, Zap70, PTCH2, or any combination thereof;

(b) the effector domain is from (i) CD3ζ and (ii) one or more of 4-1BB (CD137), CD27, CD28, and OX40 (CD134); or

(c) the effector domain is from LRP, NOTCH1, NOTCH2, NOTCH3, NOTCH4, ROR2, or Ryk.

10. The single chain fusion protein according to any one of claims 1-9, wherein the fusion protein comprises from amino-terminus to carboxy-terminus:

(a) a tag cassette, a connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(b) a first connector region, a tag, a second connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(c) a first tag, a first connector region, a second tag, a second connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(d) a first tag, a first connector region, a second tag, a second connector region, a third tag, a third connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(e) two to five tags, a connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(f) a tag, a connector region comprising an IgG hinge, a transmembrane domain, and an intracellular component comprising an effector domain from 4-1BB and CD3ζ, CD27 and CD3ζ, CD28 and CD3ζ, or CD28, 4-1BB and CD3ζ; or

(g) a tag, a connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain from LRP, NOTCH1, NOTCH2, NOTCH3, NOTCH4, ROR2, or Ryk.

11. The single chain fusion protein according to any one of claims 1-10, wherein the extracellular component further comprises a binding domain that specifically binds a target.

12. The single chain fusion protein according to claim 11, wherein the binding domain comprises a scFv, scTCR, receptor ectodomain, or ligand.

13. The single chain fusion protein of claim 11 or 12, wherein the fusion protein comprises from amino-terminus to carboxy-terminus:

(a) an extracellular binding domain, a tag, a connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(b) an extracellular binding domain, a first connector region, a tag, a second connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(c) an extracellular binding domain, a first tag, a first connector region, a second tag, a second connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(d) an extracellular binding domain, a first tag, a first connector region, a second tag, a second connector region, a third tag, a third connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(e) a tag, an extracellular binding domain, a connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(f) an extracellular binding domain, two to five tags, a connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(g) an extracellular scFv or scTCR binding domain comprising a variable region linker disposed between the variable regions and containing a tag, a connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain; or

(h) an extracellular scFv or scTCR binding domain, a tag, a connector region comprising an IgG hinge, a transmembrane domain, and an intracellular component comprising an effector domain, wherein the effector domain is from (A) 4-1BB and CD3ζ, (B) CD27 and CD3ζ, (C) CD28 and CD3ζ, (D) OX40 and CD3ζ, (E) CD28, 4-1BB and CD3ζ, (F) OX40, 4-1BB and CD3ζ, or (G) CD28, OX40 and CD3ζ; or

(i) an extracellular binding domain comprising a receptor ectodomain, a tag, a connector region comprising a hinge, a transmembrane domain, and an intracellular component comprising an effector domain, wherein the effector domain is from 4-1BB, CD27, CD28, or OX40.

14. The single chain fusion protein according to any one of claims 1-10, wherein the fusion protein further comprises:

(a) a binding domain non-covalently associated with the extracellular component; and/or

(b) a binding domain non-covalently associated with the extracellular component, wherein the non-covalently associated binding domain associates with a tag; and/or

(c) a binding domain non-covalently associated with the extracellular component, wherein the non-covalently associated binding domain is a scFv, scTCR, receptor ectodomain, or ligand; and/or

(d) a bispecific binding domain non-covalently associated with the extracellular component, wherein a first binding end of the bispecific binding domain is specific for a tag and a second binding end of the bispecific binding domain is specific for a target other than the tag; and/or

(e) a bispecific binding domain non-covalently associated with the extracellular component, wherein both binding ends of the bispecific binding domain are specific for a tag; and/or

(f) a multispecific binding domain non-covalently associated with the extracellular component, wherein a first end of the multispecific binding domain binds to a tag and a second end of the multispecific binding domain is specific for one or more targets other than the tag.

15. The single chain fusion protein according to any one of claims 11-14, wherein the binding domain is specific for CD3, CEACAM6, c-Met, EGFR, EGFRvIII, ErbB2, ErbB3, ErbB4, EphA2, IGF1R, GD2, O-acetyl GD2, O-acetyl GD3, GHRHR, GHR, FLT1, KDR, FLT4, CD44v6, CD151, CA125, CEA, CTLA-4, GITR, BTLA, TGFBR2, TGFBR1, IL6R, gp130, Lewis A, Lewis Y, TNFR1, TNFR2, PD1, PD-L1, PD-L2, HVEM, MAGE-A, mesothelin, NY-ESO-1, PSMA, RANK, ROR1, TNFRSF4, CD40, CD137, TWEAK-R, HLA, tumor or pathogen associated peptide bound to HLA, hTERT peptide bound to HLA, tyrosinase peptide bound to HLA, WT-1 peptide bound to HLA, LTβR, LIFRβ, LRP5, MUC1, OSMRβ, TCRα, TCRβ, CD19, CD20, CD22, CD25, CD28, CD30, CD33, CD52, CD56, CD80, CD81, CD86, CD123, CD171, CD276, B7H4, TLR7, TLR9, PTCH1, WT-1, Robo1, α-fetoprotein (AFP), Frizzled, OX40, or CD79b.

16. A nucleic acid molecule encoding a single chain fusion protein according to any one of claims 1-15.

17. A vector comprising a nucleic acid molecule according to claim 16.

18. The vector according to claim 17, wherein the vector is a viral vector; optionally a retroviral vector or lentiviral vector.

19. A host cell, comprising a nucleic acid molecule encoding a single chain fusion protein according to any one of claims 1-15; optionally wherein the host cell is a T cell.

20. An *in vitro* or *ex vivo* method for activating a cell, comprising contacting a cell comprising a single chain fusion protein according to any one of claims 1-15 and/or the nucleic acid molecule of claim 16 with a binding domain specific for the tag cassette.

21. An *in vitro* or *ex vivo* method for promoting cell proliferation, comprising contacting a non-natural cell comprising a single chain fusion protein according to any one of claims 1-15 and/or the nucleic acid molecule of claim 16 with a binding domain specific for the tag cassette and a growth factor cytokine for a time sufficient to allow cell growth.

22. An *in vitro* method for identifying a cell, comprising:

contacting a sample comprising a cell comprising a single chain fusion protein according to any one of claims 1-15 and/or the nucleic acid molecule of claim 16 with a binding domain specific for the tag cassette, wherein the binding domain specific for the tag cassette comprises a detectable moiety, and

detecting the presence of the cell in the sample.

23. An *in vitro* method for sorting or selecting a cell or population of cells, the method comprising:

contacting a sample comprising a cell comprising a single chain fusion protein according to any one of claims 1-15 and/or the nucleic acid molecule of claim 16, with a binding domain specific for the tag cassette, and

selecting or sorting away from other cells that are specifically bound by the binding domain, thereby selecting or sorting away from other cells the cell or population of cells.

24. An *in vitro* or *ex vivo* method for enriching for or isolating a cell or population thereof, the method comprising contacting a sample comprising the cell comprising the nucleic acid molecule of claim 16 and/or the single chain fusion protein according to any one of claims 1-15 with a binding domain specific for the tag cassette and enriching for or isolating the cell away from other cells not expressing the fusion protein in the sample.

25. An *in vitro* method for depleting cells, comprising contacting a cell comprising a nucleic acid molecule of claim 16 or a single chain fusion protein according to any one of claims 1-15 with a binding domain specific for the tag cassette and comprising a cell-toxic reagent, wherein binding of the binding domain to the tag cassette leads to death of the cells expressing the single chain fusion protein.

26. Use of a host cell according to claim 19 in the manufacture of a medicament for treating a disease in a subject, wherein the disease is selected from a cancer, an infectious disease, an autoimmune disease, an inflammatory disease, or an aging-related disease.

27. The use according to claim 26, wherein the disease is a cancer selected from a solid tumor, a leukemia, and a B-cell cancer.

28. The use according to claim 26, wherein the disease is an infectious disease selected from a viral infection, bacterial infection, or protozoan infection.