Background

[0004] Various strategies are available for stimulating T cell populations *in vitro*, including for expanding antigen-specific T cells *in vitro* for use in adoptive cellular immunotherapy or cancer therapy in which infusions of such T cells have been shown to have anti-tumor reactivity in a tumor-bearing host or for use to treat viral infections. Improved strategies are needed for expanding cell populations *in vitro*, including for research, diagnostic and therapeutic purposes. Provided are reagents, methods, articles of manufacture and kits that meet such needs.

Summary

[0004a] In one aspect, the present disclosure refers to a method for culturing T cells, the method comprising:

(a) incubating a composition comprising primary T cells in the presence of a first receptor-binding agent and a second receptor-binding agent each reversibly bound to a soluble reagent comprising a plurality of binding sites capable of reversibly binding to the first receptor-binding agent and a plurality of binding sites capable of reversibly binding to the second receptor-binding agent, wherein: the first receptor-binding agent is a stimulatory agent that specifically binds to a first molecule on the surface of the T cells in a manner that induces a TCR/CD3 complex-associated signal ~~or modulates a first signal~~ in T cells in the composition; and the second receptor-binding agent is a stimulatory agent that specifically binds to a second molecule on the surface of the T cells in a manner that induces ~~or modulates~~ a co-stimulatory ~~second~~ signal in the T cells; and

(b) ~~within~~ 1-5 days after initiation of said incubation, disrupting the reversible binding between the first receptor-binding agent and the reagent and~~/or~~ the second receptor-binding agent and the reagent, thereby generating cultured T cells that are stimulated.

[0004b] In another ~~aspect~~example, the present disclosure refers to a method for culturing T cells, the method comprising:

(a) incubating a composition comprising T cells in the presence of:

i) a first receptor-binding agent that is a stimulatory agent that specifically binds to a first molecule expressed on the surface of the T cells in a manner that induces or modulates a TCR/CD3 complex-associated signal in the T cell; and

ii) a second receptor-binding agent that is a stimulatory agent that specifically binds to a second molecule on the surface of the T cells in a manner to induce or modulate a second signal in the T cells; wherein:

the first receptor-binding agent and the second receptor-binding agent are reversibly bound to a soluble reagent comprising a plurality of binding sites capable of reversibly binding to the first receptor-binding agent and a plurality of binding sites capable of reversibly binding to the second receptor-binding agent;and

(b) within 5 days after initiation of said incubation, disrupting the reversible binding between the first receptor-binding agent and the reagent and/or the second receptor-binding agent and the reagent, thereby generating cultured T cells that are stimulated.

[0005] Provided herein are methods for incubating cells and compositions thereof, such as for culturing such cells, using reversible reagents for inducing or modulating a signal in a target cell. In some examples, the cells are T cells.

Disclosed is that the method involves incubating a composition containing T cells in the presence of an agent, such as a receptor-binding agent, *e.g.*, a stimulatory agent. The receptor-binding agent (*e.g.*, stimulatory agent) may be reversibly bound to a reagent containing a plurality of binding sites capable of reversibly binding to the receptor-binding agent. Disclosed is that the receptor-binding agent is capable of specifically binding to a molecule on the surface of the cells, *e.g.*, T cells, such as in a manner that induces or modulates a signal in the cells, *e.g.*, the T cells in the composition. In some examples, the methods involve features, such as particular steps, selection of particular agents and/or selection of particular reagents, which allow the control or adjustment of the type or strength or duration of the signal received or modulated via the reagent, and/or of properties of the output composition or cell population(s) ultimately generated by the methods. In some examples, such features are possible due to advantageous properties of the agents and reagents, such as the reversibility of binding of the individual components of the agents or reagents, and thus, the reversibility of the binding of the multimerized agents and the cells. Such properties of the reagents can be exploited to achieve control in a number of ways. For example, in some examples, the methods, via reversibility of binding, include exerting temporal control of the signal, controlling the duration of the period in which the cells are in contact with the multimerized agents, and/or the duration of the signaling induced thereby.

[0006] In some examples, the methods involve control, *e.g.*, precise control, of the length of the time period under which such agents are bound to cells. For example, this may be

CLAIMS

WHAT IS CLAIMED:

1. A method for culturing T cells, the method comprising:

(a) incubating a composition comprising primary T cells in the presence of a first receptor-binding agent and a second receptor-binding agent each reversibly bound to a soluble reagent comprising a plurality of binding sites capable of reversibly binding to the first receptor-binding agent and a plurality of binding sites capable of reversibly binding to the second receptor-binding agent, wherein:

the first receptor-binding agent is a stimulatory agent that specifically binds to a first molecule on the surface of the T cells in a manner that induces a TCR/CD3 complex-associated signal~~or modulates a first signal~~ in T cells in the composition; and

the second receptor-binding agent is a stimulatory agent that specifically binds to a second molecule on the surface of the T cells in a manner that induces~~ or modulates~~ a co-stimulatory ~~second~~ signal in the T cells; and

(b) ~~within~~ 1-5 days after initiation of said incubation, disrupting the reversible binding between the first receptor-binding agent and the reagent and~~/or~~ the second receptor-binding agent and the reagent, thereby generating cultured T cells that are stimulated.

2. The method of claim 1, wherein ~~:~~

~~binding of the first receptor-binding agent to the first molecule initiates a TCR/CD3 complex-associated signal in the T cells; and/or~~

the first receptor-binding agent specifically binds to a member of a TCR/CD3 complex~~.;~~ ~~and/or~~

~~the first receptor-binding agent specifically binds to CD3.~~

3. The method of claim 1 or claim 2, wherein the first receptor-binding agent specifically binds to CD3~~the first molecule is a component of the TCR/CD3 complex or is CD3~~.

~~4. The method of any of claims 1-3, wherein the second signal enhances or modifies the first signal delivered through the first molecule in the T cells.~~

45. The method of any of claims 1-34, wherein said disruption comprises introducing to the T cells a composition comprising a substance capable of reversing the bond between the first receptor-binding agent and the reagent and/or the second receptor-binding agent and the reagent.

6. A method for culturing T cells, the method comprising:

(a) incubating a composition comprising T cells in the presence of:

i) a first receptor-binding agent that is a stimulatory agent that specifically binds to a first molecule expressed on the surface of the T cells in a manner that induces or modulates a TCR/CD3 complex-associated signal in the T cell; and

ii) a second receptor-binding agent that is a stimulatory agent that specifically binds to a second molecule on the surface of the T cells in a manner to induce or modulate a second signal in the T cells; wherein:

the first receptor-binding agent and the second receptor-binding agent are reversibly bound to a soluble reagent comprising a plurality of binding sites capable of reversibly binding to the first receptor-binding agent and a plurality of binding sites capable of reversibly binding to the second receptor-binding agent; and

(b) within 5 days after initiation of said incubation, disrupting the reversible binding between the first receptor-binding agent and the reagent and/or the second receptor-binding agent and the reagent, thereby generating cultured T cells that are stimulated.

7. The method of claim 6, wherein the second signal enhances or modifies a signal delivered through the first molecule in the T cells.

8. The method of any of claims 1-7, wherein said disruption is carried out greater than 30 minutes after initiation of said incubation.

59. The method of any of claims 1-48, wherein:

said disruption is carried out between 1 hour and 4 days after initiation of said incubation, between 6 hours and 3 days after initiation of said incubation, between 12 hours and 2 days after initiation of said incubation, or between 1 day and 3 days after initiation of said incubation; or

said disruption is carried out ~~between about 1 hour and about 4 days after initiation of said incubation, between about 6 hours and about 3 days after initiation of said incubation, between about 12 hours and about 2 days after initiation of said incubation, or~~ between about 1 day and about 3 days after initiation of said incubation.~~; or~~

~~said disruption is carried out greater than or equal to about 1 hour after initiation of said incubation and within 1 day, 2 days, 3 days or 4 days after initiation of said incubation.~~

~~10. The method of any of claims 1-9, wherein:~~

~~said disruption is carried out between 1 day and 3 days after initiation of said incubation; or~~

~~said disruption is carried out between about 1 day and about 3 days after initiation of said incubation.~~

6~~11~~. The method of any of claims 1-5~~10~~, wherein said disruption is carried out about 2 days after initiation of said incubation.

7~~12~~. The method of any of claim~~s~~ 1-6~~11~~, wherein ~~:~~

~~the second signal is a signal other than a TCR/CD3 complex-associated signal;~~

~~the second signal is capable of enhancing or potentiating a TCR/CD3 complex-associated signal; or~~

the second molecule is a costimulatory molecule, accessory molecule, cytokine receptor, chemokine receptor, immune checkpoint molecule, or ~~is~~ a member of the TNF family or the TNF receptor family.

8~~13~~. The method of any of claims 1-7~~12~~, wherein the second molecule is selected from among CD28, CD90 (Thy-1), CD95 (Apo-/Fas), CD137 (4-1BB), CD154 (CD40L), ICOS, LAT, CD27, OX40, and HVEM.

9~~14~~. The method of any of claims 1-8~~13~~, wherein the second molecule is CD28.

1~~05~~. The method of any of claims 1-9~~14~~, wherein the first receptor-binding agent comprises ~~is~~ an anti-CD3 antibody or antigen-binding fragment thereof, and the second

receptor-binding agent comprises ~~is~~ an anti-CD28 antibody or antigen-binding fragment thereof.

116. The method of any of claims 1-10~~claim 15~~, wherein the first and second receptor-binding agents ~~are~~ comprise anti-CD3 and anti-CD28 Fabs, respectively.

127. The method of any of claims 1-116, comprising, after said disruption, further incubating the composition comprising the T cells.

138. The method of claim 127, wherein:

the incubation and further incubation are carried out in the same vessel; and/or

the method does not comprise removing the first receptor-binding agent, second receptor-binding agent, and/or reagent from the ~~cell~~ composition prior to the further incubation.~~; and/or~~

14. The method of any of claim 1-13, wherein the incubation ~~and/or further incubation~~ is carried out at or about 37 ºC ± 2 ºC.~~; and/or~~

15. The method of any of claim 1-14, wherein the incubation ~~and/or further incubation~~ is carried out in the presence of a further agent that is capable of delivering a signal to T cells.

169. The method of any of claims 1-15~~claim 17 or claim 18~~, wherein the incubation ~~or further incubation~~ is carried out in the presence of a further agent capable of enhancing or inducing proliferation of T cells, CD4+ T cells, and/or CD8+ T cells.

17~~20~~. The method of claim 15 or claim 16~~19~~, wherein the further agent is a cytokine selected from among IL-2, IL-15, and IL-7.

18~~21~~. The method of any of claims 127-17~~20~~, wherein the further incubation is carried out for a time that is no more than 14 days, no more than 12 days, no more than 10 days, no more than 8 days, or no more than 6 days.

1922. The method of any of claims 127-1821, wherein the further incubation is carried out for a time that is no more than 6 days.

~~23. The method of any of claims 6-22, wherein the T cells are primary cells from a subject.~~

~~24. The method of any of claims 1-23, wherein the T cells are unfractionated T cells, are enriched or isolated CD3+ T cells, are enriched or isolated CD4+ T cells or are enriched or isolated CD8+ T cells.~~

205. The method of any of claims 1-1924, wherein the T cells are human cells.

216. The method of any of claims 1-205, wherein the first receptor-binding agent and/or the second receptor-binding agent individually comprises~~is selected from among~~ an antibody ~~fragment, a monovalent antibody fragment~~, a proteinaceous binding molecule with antibody-like binding properties, a molecule containing Ig domains, a cytokine, a chemokine, an aptamer, ~~and~~ an MHC molecule, or ~~and~~ binding fragments thereof.

227. The method of any of claims 1-216, wherein ~~:~~

the first and/or the second receptor-binding agent comprises an antibody fragment.~~;~~

23. The method of any of claims 1-22, wherein the first and/or the second receptor-binding agent comprises a Fab fragment.~~;~~

24. The method of any claims 1-22, wherein the first and/or the second receptor-binding agent ~~is~~ comprises a divalent antibody fragment selected from among a $F(ab')_2$-fragment and a divalent single-chain Fv (scFv) fragment.~~;~~

25. The method of any claims 1-22, wherein the first and/or the second receptor-binding agent ~~is~~ comprises a monovalent antibody fragment selected from among a Fab fragment, an Fv fragment, and an scFv fragment.~~; and/or~~

26. The method of any claims 1-21, wherein the first and/or the second receptor-binding agent comprises~~is~~ a proteinaceous binding molecule with antibody-like binding properties selected from among aptamers, muteins based on a polypeptide of the lipocalin family, glubodies, proteins based on the ankyrin scaffold, proteins based on the crystalline scaffold, adnectins and avimers.

27~~8~~. The method of any of claims 1-26~~7~~, wherein the reagent ~~is or~~ comprises streptavidin; avidin; a mutein of streptavidin that reversibly binds biotin, a biotin analog, or a biologically active fragment thereof; a mutein of avidin or streptavidin that reversibly binds a streptavidin-binding peptide; a reagent that comprises at least two chelating groups K, wherein the at least two chelating groups are capable of binding to a transition metal ion; an agent capable of binding to an oligohistidine affinity tag; an agent capable of binding to a glutathione-S-transferase; calmodulin or an analog thereof; an agent capable of binding to calmodulin binding peptide (CBP); an agent capable of binding to a FLAG-peptide; an agent capable of binding to an HA-tag; an agent capable of binding to maltose binding protein (MBP); an agent capable of binding to an HSV epitope; an agent capable of binding to a myc epitope; or an agent capable of binding to a biotinylated carrier protein.

28~~9~~. The method of any of claims 1-27~~8~~, wherein the reagent ~~is~~ comprises an oligomer or polymer of streptavidin; avidin; a mutein of streptavidin that reversibly binds biotin or a biologically active fragment thereof; a streptavidin or avidin mutein that reversibly binds a streptavidin-binding peptide; a reagent that comprises at least two chelating groups K, wherein the at least two chelating groups are capable of binding to a transition metal ion; an agent capable of binding to an oligohistidine affinity tag; an agent capable of binding to a glutathione-S-transferase; calmodulin or an analog thereof; an agent capable of binding to calmodulin binding peptide (CBP); an agent capable of binding to a FLAG-peptide; an agent capable of binding to an HA-tag; an agent capable of binding to maltose binding protein (MBP); an agent capable of binding to an HSV epitope; an agent capable of binding to a myc epitope; or an agent capable of binding to a biotinylated carrier protein.

29~~30~~. The method of any of claims 1-28~~9~~, wherein the reagent comprises an oligomer or polymer of a streptavidin mutein.

301. The method of claim 289 or claim 29230, wherein individual molecules of the oligomer or polymer are crosslinked by a polysaccharide or a bifunctional linker.

312. The method of any of claims 278-301, wherein :

the streptavidin mutein comprises the amino acid sequence Val~~Va1~~^{44}-Thr^{45}-Ala^{46}-Arg^{47} or Ile~~Ile~~^{44}-Gly^{45}-Ala^{46}-Arg^{47} at sequence positions corresponding to positions 44 to 47 with reference to positions in streptavidin in the sequence of amino acids set forth in SEQ ID NO:1.

323. The method of any of claims 278-312, wherein the streptavidin mutein comprises:

a) the sequence of amino acids set forth in any of SEQ ID NOS: 3-6, 27, and 28;

b) a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%~~, 89%~~, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS:3-6, 27, and 28 and contains the amino acid sequence corresponding to Val~~Va1~~^{44}-Thr^{45}-Ala^{46}-Arg^{47} or Ile~~Ile~~^{44}-Gly^{45}-Ala^{46}-Arg^{47} and that reversibly binds to biotin or a biologically active form thereof, a biotin analog or a biologically active fragment thereof, or a streptavidin-binding peptide; or

c) a functional fragment of a) or b) that reversibly binds to biotin or a biologically active form thereof, a biotin analog or a biologically active fragment thereof, or a streptavidin-binding peptide.

33. The method of any of claims 27-32, wherein the streptavidin mutein comprises the sequence of amino acids set forth in any of SEQ ID NOS: 3-6, 27, and 28.

34. The method of any of claims 271-33, wherein:

the reagent ~~is or~~ comprises the~~a~~ streptavidin mutein; and

the first and/or the second receptor-binding agent independently comprises a streptavidin-binding peptide selected from the group consisting of Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO: 8), Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-(GlyGlyGlySer)$_3$-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO: 17), Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-(GlyGlyGlySer)$_2$-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO: 18), and Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-(GlyGlyGlySer)$_2$Gly-Gly-Ser-Ala-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO: 19).

35. The method of any of claims 271-33, wherein the reagent is or comprises thea streptavidin mutein, and the first and/or the second receptor-binding agent independently comprises a streptavidin-binding peptide comprising the sequence set forth in SEQ ID NO: 16.

36. The method of any of claims 6-35, wherein said disruption comprises introducing to the cells a composition comprising a substance capable of reversing the bond between the first receptor-binding agent and the reagent and/or the second receptor-binding agent and the reagent.

367. The method of any of claims 4-35claim 5 or claim 36, wherein the substance is a free binding partner and/or is a competition agent.

378. The method of any of claims 27-36claims 5, 36, and 37, wherein:

the reagent is or comprises thea streptavidin mutein or biologically active fragments thereof; and

the substance comprises a streptavidin-binding peptide, biotin or a biologically active fragment thereof, or a biotin analog or biologically active fragment thereof.

389. The method of claim 378, wherein the substance comprises D-biotin.

3940. The method of claim 378, wherein :

the substance is a streptavidin-binding peptide selected from the group consisting of Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO: 8), Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-$(GlyGlyGlySer)_3$-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO: 17), Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-$(GlyGlyGlySer)_2$-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO: 18), and Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-$(GlyGlyGlySer)_2$Gly-Gly-Ser-Ala-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO: 19).

401. The method of any of claims 1-3940, wherein:

prior to the incubation, the method further comprises contacting cells with a selection agent that specifically binds to a marker comprised by T cells or target cells of the composition, thereby generating or obtaining the composition comprising the T cells or target cells; or

at least a portion of the incubation is carried out further in the presence of a selection agent that specifically binds to a marker comprised by T cells or target cells of the composition, wherein the cultured T cells are enriched for T cells or target cells comprising the marker.

412. The method of claim 401, wherein:

the selection agent is reversibly bound to the reagent, said reagent further comprising a plurality of binding sites capable of specifically binding the selection agent; or

the selection agent is reversibly bound to a further reagent comprising a plurality of binding sites capable of specifically binding to the selection agent.

423. The method of any of claims 1-412, wherein the induction ~~or modulation~~ of the TCR/CD3-associated signal and the costimulatory signal effects an increase in expansion and/or activation of the cultured T cells compared to incubation of T cells in the absence of the induction ~~or modulation~~ of the TCR/CD3-associated signal and the costimulatory signal.

434. The method of any of claims 1-423, wherein the method increases expansion and/or proliferation of T cells in the composition, alters the metabolic profile of T cells in the composition, alters the subset of CD8+ T cells in the composition, and/or increases the percentage of long-lived memory T cells in the composition; each compared to the T cells in the composition prior to the incubation~~,~~ or subsequent to the incubation but in the absence of the disruption~~, or subsequent to the incubation but in the presence of an agent that specifically binds CD28 and/or induces or modulates CD28 signaling~~.

445. The method of any of claims 1-434, further comprising introducing a recombinant nucleic acid molecule into T cells of the population, which nucleic acid molecule encodes a recombinant protein, whereby T cells express the recombinant protein.

456. The method of claim 445, wherein the recombinant protein is a chimeric antigen receptor or a transgenic T cell receptor (TCR).