There remains a need in the immunotherapy field for alternative compositions and methods that provide immunomodulatory signals to host cells for treating various diseases, such as cancer or infections. Presently disclosed embodiments address these needs and provide other related advantages.

SUMMARY

In certain aspects, the present disclosure is directed to a fusion protein, comprising an extracellular component that contains a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse.

In some examples, a length or spatial distance of a complex formed between the fusion protein and the target or a portion of such fusion protein::target complex (generally the extracellular portion of such complex) is or spans a particular distance, *e.g.*, in some examples, is a distance that is less than or less than about a certain distance. In some aspects, a distance of the fusion protein::target complex (or, typically, the extracellular portion thereof) is less than at or about 50 nm, less than at or about 40 nm, less than at or about 30 nm, or less than at or about 20 nm or equal to or less than at or about 15 nm. In some examples, it is at or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nm, such as at or about 14 or 15 nm. In some aspects, the distance is one that is similar to a distance between membranes in an immunological synapse or is a distance that is the same, about the same, or substantially the same, as a distance between the membrane proximal-most portion, *e.g.*, residue, of the extracellular domain of a TCR and the membrane proximal-most portion, *e.g.*, residue, of an MHC (*e.g.*, HLA, such as an MHCI or MHCII) molecule, with respect to a TCR-peptide/MHC complex or the distance spanned by the extracellular portions of such a complex (or spatial distance spanned by the extracellular portion known to be contained within a synapse, such as a complex containing CD8, CD4, CD28, and the respective binding partner or ligand thereof).

In some examples, spatial distances of

complexes refer to a distance between membranes of two different cells, wherein a first cell and a second cell each express on their surface a binding partner that can form a complex between the membranes when the cells are in proximity to each other. In some aspects, the distance is a distance that is the same, about the same, or substantially the same, as a distance spanned by the extracellular portions of a complex formed between a TCR and cognate interaction with an MHC molecule. In some aspects, such as where a fusion protein comprises a binding domain from a molecule ordinarily capable of entering an immunological synapse or co-localizing with an antigen receptor, the distance is similar to or the same as that spanned by a complex formed between the molecule (having the binding domain used in the fusion protein), and a natural binding partner thereof. In some aspects, such as where the fusion protein comprises a binding domain from a molecule ordinarily not capable of entering an immunological synapse or ordinarily not capable of co-localizing with an antigen receptor, the distance is different than, *e.g.*, less than or substantially less than, that spanned by a complex formed between the molecule (having the binding domain or functional portion thereof used in the fusion protein), and a natural binding partner thereof.

In some examples, a binding domain within the extracellular component of a fusion protein of this disclosure contains a target-binding portion of a molecule capable of delivering an inhibitory signal, such as of an inhibitory molecule, *e.g.*, an immunoinhibitory molecule, such as an immunoinhibitory receptor or immune checkpoint molecule. In some aspects, such a molecule is a glycoprotein, checkpoint family member. In certain examples, the fusion protein comprising a binding domain from a glycoprotein, checkpoint family member or is not a B7 or B7-binding molecule or is not a CD28-B7-superfamily member (*e.g.*, is not a CD28, CTLA4, ICOS, or other B7 family binding molecule) Exemplary glycoprotein, checkpoint family members include CD200R, SIRPα, CD279 (PD-1), CD2, CD95 (Fas), CTLA4 (CD152), CD223 (LAG3), CD272 (BTLA), A2aR, KIR, TIM3, CD300, or LPA5, or a binding variant of any such molecule. In some examples, a binding domain within the extracellular component of a fusion protein of this disclosure comprises a binding partner of any of the foregoing, or a binding variant of any such molecule. In some

aspects of such examples, the intracellular portion of a fusion protein includes a signaling domain capable of delivering a stimulatory, such as a costimulatory, signal to a lymphocyte, such as a T cell, such as a costimulatory region of CD28, 41BB, ICOS, or other costimulatory molecule. In some aspects, the intracellular portion of the fusion protein does not include an intracellular signaling domain of the inhibitory molecule, such as of a checkpoint or immunoinhibitory molecule, when the extracellular binding portion is from a checkpoint or immunoinhibitory molecule. In some aspects, a fusion protein does not include a primary signaling domain such as a CD3ζ signaling domain or other domain capable of delivering a primary signal to a T cell.

In certain aspects, the extracellular component or the binding portion thereof contains or is a binding domain of a molecule or ectodomain capable of specifically binding to CD200, such as a binding portion of a CD200R or variant thereof. In some examples, the binding domain is or includes a binding region of a molecule or of an ectodomain that is capable of specifically binding to a CD47, such as a SIRP ectodomain or CD47-binding region thereof, such as a SIRPα ectodomain or CD47-binding region thereof. In some examples, the binding domain is capable of binding to a PD-L1 or a PD-L2 or a LAG3 molecule. Exemplary targets may be one or more proteins whose expression is increased or upregulated in certain cells or tissues associated with or of a disease or condition to be treated or ameliorated with the fusion proteins and compositions provided herein, such as a tumor cell or tumor microenvironment, or is bound by a receptor generally upregulated on immune cells such as lymphocytes infiltrating a diseased tissue, such as a tumor.

In some examples, the extracellular component further includes one or more additional regions or domains, for example, from a molecule other than that from which the binding domain is derived or other than the molecule with which the binding domain shares identity. The one or more additional extracellular domain(s) may include a spacer region, such as one from an immunoglobulin molecule, which may contain all or a portion of a hinge, or constant region domain such as CH2 or CH3 domain, or from another cell surface molecule such as a costimulatory receptor, such as CD28. The additional extracellular domain(s) may include, in some aspects, a

multimerization domain, *e.g.*, a dimerization domain or sequence that may promote homo- or heterodimerization with another molecule, such as multimerization of two or more of the fusion proteins. In some examples, such a domain includes a portion of an extracellular domain of a CD28 molecule including at least the transmembrane-proximal-most cysteine, and generally an extracellular portion between such cysteine and the membrane, or modified variant thereof. In some aspects, such a domain includes an amino acid sequence as set forth in SEQ ID NO: 32, or portion thereof, or variant thereof such as having at least 90%, 95%, or 99 % identity thereto. In some aspects, such a domain may be included in order to facilitate or promote multimerization. In some examples, a fusion protein contains an extracellular component including a CD200-binding domain, such as an extracellular portion (or portion thereof, such as a binding domain thereof) of a CD200R, such as an extracellular portion of CD200R having an amino acid sequence as set forth in SEQ ID NO: 25 or encoded by a nucleic acid molecule as set forth in SEQ ID NO: 2, or a CD200-binding portion thereof or variant thereof or binding portion thereof. In some aspects of such examples, the extracellular portion of the fusion protein further includes a portion of an extracellular region of CD28, such as up to about 9 to about 12 amino acids thereof (*e.g.*, 9 amino acids or 12 amino acids), and in some aspects including a membrane-proximal-most cysteine residue of a CD28 extracellular region. In some such examples, the length of the CD200R portion of the extracellular region is reduced in length corresponding to the number of additional residues in the CD28-derived portion, such as by about 9 to about 12 amino acids (*e.g.*, 9 amino acids or 12 amino acids), or by a sufficient number of amino acids that the distance spanned by the extracellular portion of a complex between the fusion protein and a CD200 molecule is similar to, substantially similar to, or the same as that spanned by the extracellular portion of a complex between a human CD200R, *e.g.*, a CD200R, and CD200; or that spanned by the extracellular portion of a complex between a TCR in cognate interaction with an MHC molecule (*e.g.*, MHC I or MHCII) in binding to a cognate peptide-MHC complex; or that of an immunological synapse. In some aspects, the fusion protein further includes a transmembrane domain, such as a CD28

transmembrane, such as a transmembrane domain encoded by the sequence set forth as SEQ ID NO: 4 or portion thereof, or a modified version thereof, such as a variant modified to contain additional charged regions or residues or hydrophilic residues to facilitate intermolecular interactions. In some examples, the protein further includes a CD28 intracellular signaling domain, such as a costimulatory domain of CD28, such as one that is capable of recruiting one or more adapter molecules to a CD28 in response to ligation. In some aspects, the CD28 intracellular domain includes or is a sequence encoded by the nucleotide sequence of SEQ ID NO: 5 or a portion or functional variant thereof.

In some examples, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD200R, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28. In one aspect, the present disclosure refers to a fusion protein, comprising (a) an extracellular component comprised of a binding domain that specifically binds a target, (b) an intracellular component comprised of an intracellular signaling domain, and (c) a hydrophobic component connecting the extracellular and intracellular components, wherein the extracellular component comprises an extracellular portion of a CD200R and the intracellular signaling domain comprises an intracellular signaling domain of CD28.

In some examples, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD200R, (b) the hydrophobic component comprises a

transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28 and an intracellular signaling domain of a CD137 (4-1BB).

In some examples, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD200R, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD137 (4-1BB).

In some examples, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a SIRPα, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some examples, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD279 (PD-1), (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some examples, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD95 (Fas), (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some examples, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a TIM3, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some examples, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a LAG3, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some examples, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically

binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD2, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In certain aspects, the present disclosure is directed to a nucleic acid molecule encoding a fusion protein as described herein. In another aspect, the present disclosure refers to a nucleic acid molecule encoding a fusion protein as described herein.

In certain aspects, the present disclosure is directed to a vector comprising a nucleic molecule that encodes a fusion protein as described herein. In another aspect, the present disclosure refers to a vector comprising a nucleic acid molecule as described herein.

In certain other aspects, the present disclosure is directed to a host cell comprising a fusion protein, nucleic acid, or vector as described herein. In another aspect, the present disclosure refers to a host cell, comprising a fusion protein as described herein.

In certain other aspects, a method of increasing the activity of an immune cell is provided, comprising administering to a subject in need of increased immune cell activity an effective amount of a host cell as described herein.

In other aspects, the present disclosure is directed to a method of enhancing or prolonging an immune response, comprising administering to a subject in need of enhanced or prolonged immune cell activity an effective amount of a host cell as described herein.

In still other aspects, the present disclosure provides a method of stimulating an antigen-specific T cell response, comprising administering to a subject in need of increased immune cell activity an effective amount of a host cell as described herein.

In other aspects, the present disclosure is directed to a method of inhibiting an immunosuppressive signaling pathway, comprising administering to a subject in need thereof an effective amount of a host cell as described herein.

In other aspects, the present disclosure is directed to a method of treating cancer, comprising administering to a subject having cancer a therapeutically effective amount of a host cell as described herein. In another aspect, the present disclosure refers to a use of a fusion protein as described herein, a vector as described herein, or a host cell as described herein in the manufacture of a medicament for treating cancer in a subject.

In other aspects, the present disclosure is directed to a method of inhibiting immune resistance of cancer cells, comprising administering to a subject in need thereof an effective amount of a host cell as described herein.

In still other aspects, the present disclosure provides a method for treating a tumor, comprising administering to a subject having a tumor a therapeutically effective amount of a host cell as described herein, wherein the administered host cell is capable of proliferating in an immunosuppressive tumor microenvironment.

A method of treating an infection, comprising administering to a subject having the infection a therapeutically effective amount of a host cell as described herein, is also provided by the present disclosure.

These and other aspects of the present disclosure will become apparent upon reference to the following detailed description and attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show CD200R-CD28 constructs expressed at high levels on primary murine $CD8^+$ T cells. (A) Schematic representation of exemplary CD200R-CD28 constructs. Construct “I” contains CD200R extracellular ("EC") and transmembrane ("TM") domains and a CD28 intracellular ("IC") signaling domain (CD200Rtm-CD28). Construct "II" contains the extracellular domain of CD200R and the transmembrane and intracellular domains of CD28 (CD200R-CD28tm). Constructs "III-V" also incorporate a portion of the extracellular domain of CD28 to the transmembrane-proximal cysteine to promote multimerization and enhance CD28 signaling. To account for any extra extracellular amino acids (*e.g.*, from one to about 50 amino acids; such as exemplary murine constructs disclosed here contain an extra nine (9) amino acids and exemplary human constructs disclosed here contain twelve (12) amino acids), some constructs have a truncated portion of an extracellular or intracellular domain (*e.g.*, a CD200R that preserves an N linked glycosylation site). For example, construct IV has a truncated portion of CD200R that is truncated by 3 amino acids. Construct V has a truncated portion of

CD200R that is truncated 9 amino acids. Constructs "I", "II", and "V" maintain the short spatial distance between the cells (*e.g.*,

CLAIMS

1. A fusion protein, comprising (a) an extracellular component comprised of a binding domain that specifically binds a target, (b) an intracellular component comprised of an intracellular signaling domain, and (c) a hydrophobic component connecting the extracellular and intracellular components, wherein the extracellular component comprises an extracellular portion of a CD200R and the intracellular signaling domain comprises an intracellular signaling domain of CD28.

2. The fusion protein according to claim 1, wherein the expression of the fusion protein in a T cell comprising a TCR or chimeric antigen receptor specific for an antigen results in at least about a 1.5-fold, 2-fold, or 3-fold increase in survival, expansion, cytotoxicity, cytokine secretion, and/or response to multiple rounds of stimulation, by the T cell, in response to binding of the antigen and/or following administration to a subject, and/or results in at least about a 1.5-fold, 2-fold, or 3-fold increase in time of survival, disease-free survival, or amelioration of one or more disease symptom, of a subject to which the cell is administered, as compared to a cell substantially the same as the T cell but not containing the fusion protein.

3. The fusion protein according to any of claims 1 to 2, wherein the extracellular component further comprises an additional extracellular portion.

4. The fusion protein according to claim 3, wherein the extracellular component or the additional extracellular portion comprises a multimerization domain and/or a spacer.

5. The fusion protein according to claim 4, wherein the multimerization domain comprises an extracellular component modified to contain a cysteine residue within about 2 to about 15 amino acids from the hydrophobic component.

6. The fusion protein according to any one of claims 1 to 5, wherein the hydrophobic component comprises a transmembrane domain of a CD2, CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD40, CD79A, CD79B, CD80, CD86, CD95 (Fas), CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD200R, CD223 (LAG3), CD270 (HVEM), CD272 (BTLA), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), CD279 (PD-1), CD300, CD357 (GITR), A2aR, DAP10, FcRα, FcRβ, FcRγ, Fyn, GAL9, KIR, Lck, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPα, pTα, TCRα, TCRβ, TIM3, TRIM, LPA5, or Zap70.

7. The fusion protein of any of claims 1 to 6, wherein the intracellular signaling domain further comprises an intracellular signaling domain of a CD3ε, CD3δ, CD3ζ, CD25, CD27, CD40, CD47, CD79A, CD79B, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD278 (ICOS), CD357 (GITR), CARD11, DAP10, DAP12, FcRα, FcRβ, FcRγ, Fyn, Lck, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, ROR2, Ryk, Slp76, pTα, TCRα, TCRβ, TRIM, Zap70, PTCH2, or any combination thereof.

8. The fusion protein according to any of claims 1 to 7, wherein the hydrophobic component comprises a transmembrane domain of a CD28 or a CD137 (4-1BB).

9. The fusion protein according to claim 8, wherein the intracellular component comprises a second intracellular signaling domain.

10. The fusion protein according to claim 8 or 9, wherein the extracellular component comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain.

11. The fusion protein according to claim 1, wherein the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NOs.:2, 8, or 11, the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NOs.:3 or 4, and the intracellular component

comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

12. The fusion protein according to claim 11, wherein the extracellular component further comprises a multimerization domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9.

13. The fusion protein according to claim 12, wherein the intracellular component further comprises a second intracellular signaling domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:13.

14. The fusion protein according to claim 1, wherein the extracellular component is associated with a negative signal and the intracellular component is associated with a positive signal and wherein the extracellular component comprises a binding domain with an amino acid sequence as set forth in SEQ ID NOs.:31 or 34, and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and the intracellular component comprises an amino acid as set forth in SEQ ID NO.:28.

15. The fusion protein according to claim 14, wherein the intracellular component further comprises a second intracellular signaling domain having an amino acid sequence as set forth in SEQ ID NO.:36.

16. A nucleic acid molecule encoding a fusion protein according to any one of claims 1 to 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 wherein the viral vector is a lentiviral or retroviral vector.

19. The vector according to claim 17 or claim 18, further encoding an antigen-specific TCR.

20. A host cell, comprising a fusion protein according to any one of claims 1 to 15.

21. Use of a fusion protein according to any one of claims 1 to 15, a vector according to any one of claims 17 to 19, or a host cell according to claim 20 in the manufacture of a medicament for treating cancer in a subject.