SUMMARY

Novel immunotherapies for treating diseases, such as cancer, with drug combinations are provided herein. Such novel combination therapies have been discovered to exhibit unexpected improvements in reduced toxicity by, e.g., minimizing doses (synergistic potentcy) and/or outcomes by, e.g., escalating effect (synergistic efficacy). Indeed, while each drug of the inventive combinations individually produce similar or signature effects but, when administered in combination, display greatly enhanced effects. Such enhanced effect is great than that which would have been predicted or expected by the drugs' individual potentcies. As such, the combined effect is not only synergistic but also surprising and unexpected. Different methods and tools amay be used for evaluating syngistic effects of drug combinations according to the disclosure. See, for example, Tallarida, R., Quantitative Methods for Assessing Drug Synergism, *Genes & Cancer*, 2(11) 1003-1008, 2011; Meyer, C., et al., Quantifying Drug Combination Synergy Along Potency and Efficacy Axes, *Cell Systems*, 8, 97-108, Feb. 2, 2019; and Ianevski, A., SynergyFinder: a Web Application for Analyzing Drug Combination Dose-Response Matrix Data, *Bioinformatic*s, 33(15), 2413-15, 2017; each of the foregoing of which is incorporated herein by reference in its entirety. Various reference models for producing comparative interaction- or combination-indices to quantify observed effects like zero-interaction, synergism or antagonism in drug combinations are discussed in, for example, Schindler, M.Theory of Synergistic Effects: Hill-type Response Surfaces as 'Null-interaction' Models for Mixtures, *Theoretical Biology and Medical Modelling*, 14:15, 2017, which is incorporated by reference herein, in its entirety.

In one aspect, provided herein is a therapeutic composition comprising a first immune cell loaded with a protein nanogel and a second, different immune cell loaded with an immunostimulatory fusion molecule (IFM).

In one aspect, provided herein is a therapeutic composition as described herein for use as a medicament.

In one aspect, provided herein is a use of a first immune cell in the manufacture of a medicament for use in a combinatorial therapy for administration to a subject in need thereof, wherein said medicament is to be administered in combination with a second, different immune cell, wherein the first immune cell is loaded with a protein nanogel, and the second immune cell is loaded with an IFM .

In one aspect, provided herein is a use of a second immune cell in the manufacture of a medicament for use in a combinatorial therapy for administration to a subject in need thereof, wherein said medicament is to be administered in combination with a first, different immune cell, wherein the first immune cell is loaded with a protein nanogel, and the second immune cell is loaded with an IFM.

In one aspect, provided herein is a use of a first immune cell in the manufacture of a first medicament and use of a second, different immune cell in the manufacture of a second medicament, wherein the first and second medicaments are to be used in combination therapy for administration to a subject in need thereof.

More particularly, the present disclosure provides, *inter alia*, a therapeutic (e.g., cancer immunotherapy) composition comprising: a first immune cell having a surfaced loaded with a plurality of

protein nanogels and a second immune cell having a surfaced loaded with a plurality of immunostimulatory fusion molecules (IFMs, used interchangeably with "tethered fusion" or TF). In some examples, the first immune cell and the second immune cell are the same cell, i.e., the protein nanogels and the IFMs can be co-loaded on a single cell. In some examples, the first immune cell and the second immune cell are different cells, wherein the two cells (or populations of cells) can be administer together, or serially (with or without some amount of time in between). In another example, an IFM may be delivered in "free" form—i.e., in solution, unattached to a cell. Such free delivery may be administered systemically or intratumoral and may be delivered concurrently with the nanogel-loaded immune cell, or before or after the the nanogel-loaded immune cell. The administration of the nanogel-loaded cells and/or the IFM (in cell-bound and/or free form) may be repeated administration. It has been discovered that such co-administration of a nanogel and an IFM produces unexpected and surprising synergy. With the synergistic effect, greater efficacy at lower doses (for one or both compounds) and/or reduced toxicity levels can be achieved. There can be an wider dosing window with a greater span between efficacy and toxicity—i.e., there is a wider range in which to optimize dosage for efficacy before the maximal, undesired level of toxicity is reached.

Specifically, it has been surprisingly discovered that the anti-tumor activity of IL-15 nanogel (a multimer comprising chemically crosslinked IL-15/IL-15 Rα/Fc heterodimers (IL15-Fc) and a polymer)

and IL-12 tethered fusion (single-chain IL-12p70 fused to a humanized anti-CD45 Fab) surface-loaded on cells, when combined, produces an improved therapeutic profile with a synergistic effect rather than merely additive. That is, there is a statistically significant difference (increase) in the efficacy of the combination of IL-15 nanogel and IL-12 tethered fusion, compared to what would have been expected if the efficacy were purely additive.

In some examples, the protein nanogels can each include a plurality of therapeutic protein monomers reversibly cross-linked to one another via a plurality of biodegradable cross-linkers. In some examples, the protein nanogel has a size between 30 nm and 1000 nm in diameter measured by dynamic light scattering. In some examples, the cross-linker degrades, after administration into a subject in need thereof, under physiological conditions so as to release the therapeutic protein monomers from the protein nanogel. In some examples, the protein nanogel further comprises a surface modification such as polycation so as to allow the protein nanogel to associate with the first immune cell.

In some examples, the therapeutic protein monomers can include one or more cytokine molecules and/or one or more costimulatory molecules, wherein:

(i) the one or more cytokine molecules are selected from IL-15, IL-2, IL-7, IL-10, IL-12, IL-18, IL-21, IL-23, IL-4, IL-1alpha, IL-1beta, IL-5, IFNgamma, TNFa, IFNalpha, IFNbeta, GM-CSF, or GCSF; and

(ii) the one or more costimulatory molecules are selected from CD137, OX40, CD28, GITR, VISTA, anti-CD40, or CD3.

In some examples, the cross-linker can be a degradable or hydrolysable linker. In some examples, the degradable linker is a redox responsive linker. Exemplary linkers as well as methods of making and using various linkers (e.g., to make nanogels) are disclosed in PCT Application No. PCT/US2018/049594, U.S. Publication No. 2017/0080104, U.S. Patent No. 9,603,944, and U.S. Publication No. 2014/0081012, each of which is incorporated herein by reference in its entirety.

In certain examples, each IFM can be engineered to contain an immunostimulatory cytokine molecule and a targeting moiety (e.g., an antibody or an antigen-binding fragment thereof) having an affinity to an antigen on the surface of the immune cell, wherein the immunostimulatory cytokine molecule is operably linked to targeting moiety. Exemplary IFMs (also referred to as "tethered fusion" or TF) are disclosed in PCT International Publication Nos. WO 2019/010219 and WO 2019/010222, each incorporated herein by reference in its entirety.

In some examples, the immunostimulatory cytokine molecule is selected from one or more of IL-15, IL-2, IL-6, IL-7, IL-12, IL-18, IL-21, IL-23, or IL-27 or variant forms thereof. The antigen can be selected from one or more of CD45, CD4, CD8, CD3, CD11a, CD11b, CD11c, CD18, CD25, CD127, CD19, CD20, CD22, HLA-DR, CD197, CD38, CD27, CD196, CXCR3, CXCR4, CXCR5, CD84, CD229, CCR1, CCR5, CCR4, CCR6, CCR8, CCR10, CD16, CD56, CD137, OX40, or GITR.

In one example, the IFM contains IL-12, e.g., single-chain IL-12p70 fused to a humanized anti-CD45 Fab. The single-chain IL-12p70 can contain IL-12B and IL-12A joined by flexible linker. In one example, "IL-12 tethered fusion" (single-chain IL-12p70 fused to a humanized anti-CD45 Fab) can be

recombinantly expressed, purified, and then tethered onto immune cells expressing CD45 such as T cells to form T cells with surface-loaded immune agonists.

In various examples, the first and second immune cell can be provided and administered separately (e.g., sequentially) to a patient in need of, e.g., cancer immunotherapy. In some examples the immune cells can be from a population of T cells that have been enriched or trained to possess specificity against one or more tumor-associated antigens (TAAs).

Another example relates to a method for providing cancer immunotherapy, comprising administering to a patient in need thereof a plurality of immune cells each loaded with a first plurality of protein nanogels and a second plurality of immunostimulatory fusion molecules (IFMs).

A further example relates to a method for providing cancer immunotherapy, comprising: administering to a patient in need thereof a first plurality of immune cells each loaded with a plurality of protein nanogels; and administering to the patient a second plurality of immune cells each loaded with a plurality of immunostimulatory fusion molecules (IFMs).

In various examples, the cancer immunotherapy is for treatment of a cancer selected from breast, prostate, lung, ovarian, cervical, skin, melanoma, colon, stomach, liver, esophageal, kidney, throat, thyroid, pancreatic, testicular, brain, and bone cancer, leukemia, chronic lymphocytic leukemia, basal cell carcinoma, biliary tract cancer, bladder cancer, brain and central nervous system (CNS) cancer, choriocarcinoma, colorectal cancer, connective tissue cancer, endometrial cancer, eye cancer, head and neck cancer, gastric cancer, intraepithelial neoplasm, larynx cancer, lymphoma; neuroblastoma; lip, tongue, mouth and pharynx cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; sarcoma; skin cancer; thyroid cancer; and uterine cancer.

Still another example relates to a method for inducing the synergistic expansion of human $CD8^+$ T cells in a human immunotherapeutic regimen, said regimen consisting of co-administering at least two immune agonists, the first immune agonist comprising a T cell loaded with an IL-12 tethered fusion, and the second immune agonist comprising a T cell loaded with an IL-15 nanogel, wherein the co-administration of such immune agonists results in a synergistic expansion of said human $CD8^+$ T cells. In some examples, the T cell loaded with the IL-12 tethered fusion, the T cell loaded with the IL-15 nanogel, or both T cells, are specific to one or more tumor-associated antigens.

In some examples, the tumor-associated antigen is one expressed by a cancer selected from breast, prostate, lung, ovarian, cervical, skin, melanoma, colon, stomach, liver, esophageal, kidney, throat, thyroid, pancreatic, testicular, brain, and bone cancer, leukemia, chronic lymphocytic leukemia, basal cell carcinoma, biliary tract cancer, bladder cancer, brain and central nervous system (CNS) cancer, choriocarcinoma, colorectal cancer, connective tissue cancer, endometrial cancer, eye cancer, head and neck cancer, gastric cancer, intraepithelial neoplasm, larynx cancer, lymphoma; neuroblastoma; lip, tongue, mouth and pharynx cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; sarcoma; skin cancer; thyroid cancer; and uterine cancer.

In some examples, the IL-12 tethered fusion comprises a humanized anti-CD45 antibody or an antibody fragment selected from a Fab, $F(ab')_2$, Fd, and a Fv. In some examples, the IL-15 nanogel comprises a pluratlity of crosslinked IL-15-Fc fusion protein monomers.

Also provided herein is a method for the treatment of cancer, comprising the concurrent administration to a mammal in need thereof a synergistic, therapeutically effective amount of two immune agonists, the first immune agonist comprising a T cell loaded with an IL-12 tethered fusion, and the second immune agonist comprising a T cell loaded with an IL-15 nanogel. In some examples, said cancer is a solid tumor. In some examples, said cancer treatment further comprises an anti-proliferative cytotoxic agent either alone or in combination with radiation therapy.

In some examples, the first and second immune agonists are administered in a ratio of either immune agoists to the other immune agonists of 1:1, 1:2, 1:3, 1:4 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, 1:25, 1:30, 1:35, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, 1:70; 1:75, 1:80, 1:85, 1:90, 1:95, 1:100, 1:120, 1:130, 1:140, 1:150, 1:160, 1:170, 1:180, 1;190, 1:200, 1:500, 1:1000. 1:5000, 1:10,000, 1:100,000, 2:3, 3:4, 2:5, 3:5, 3:10, 7:10, 9:10, 2:15, 4:15, 6:15, 7:15, 8:15, 11:15, 13:15, 14:15, 3:20, 7:20, 9:20, 11:20, 13:20, 17:20, 19:20, 1:25, 2:25, 4:25, 6:25, 7:25, 8: 25, 10:25, 11:25, 12:25, 13:25, 14:25, 16:25, 17:25, 18:25, 19:25, 21:25, 22:25, 23:25, or 24:25.

In some examples, at least one of the first and second immune agonists is administered in a dosage of about 20 million cells/m^2, 40 million cells/m^2, 100 million cells/m^2, 120 million cells/m^2, 200 million cells/m^2, 360 million cells/m^2, 600 million cells/m^2, 1 billion cells/m^2, 1.5 billion cells/m^2, 10^6 cells/m^2, about 5 x 10^6 cells/m^2 , about 10^7 cells/m^2, about 5 x 10^7 cells/m^2, about 10^8 cells/m^2, about 5 x 10^8 cells/m^2, about 10^9 cells/m^2, about 5 x 10^9 cells/m^2, about 10^{10} cells/m^2, about 5 x 10^{10} cells/m^2, or about 10^{11} cells/m^2.

In one example, an immunostimulatory fusion molecule is provided, comprising:

(a) an immunostimulatory cytokine molecule; and

(b) an immune cell targeting moiety comprising an antigen-binding fragment of an antibody having an affinity to an antigen on the surface of a target immune cell,

wherein the immunostimulatory cytokine molecule is operably linked to the antigen-binding fragment.

In another example, an immunostimulatory fusion molecule is provided, comprising:

(a) an immunostimulatory cytokine molecule; and

(b) an immune cell targeting moiety comprising an antibody having an antigen-binding site specific for an antigen on the surface of a target immune cell, wherein the antibody comprises a light chain having a C-terminus and an N-terminus, and a heavy chain having a C-terminus and an N-terminus, wherein the light chain is linked to the heavy chain by a disulfide bond,

wherein the immunostimulatory cytokine molecule is operably linked to the antibody at

the C-terminus of the light chain, the N-terminus of the light chain, or the N-terminus of the heavy chain portion.

In another example, an immunostimulatory fusion molecule is provided, comprising:

(a) an IL-12 molecule; and

(b) a T cell targeting moiety comprising a Fab fragment having an antigen-binding site specific for a CD45 cell surface receptor;

wherein the Fab fragment and the IL-12 molecule are operably linked together as a fusion molecule.

In some examples, the immune cell targeting moiety targets a T cell selected from an effector T cell, a CD4+ T cell, a CD8+ T cell, and a CTL. In some examples, the antigen is a CD45 receptor expressed on the cell surface of the T cell. In some examples, the immune cell targeting moiety comprises a Fab fragment, F(ab')2, Fv, a single chain Fv of anti-CD45 antibodies BC8, 4B2, GAP8.3 or 9.4, or humanized version of any of the foregoing. In some examples, the immunostimulatory cytokine molecule comprises an IL-12, a single chain IL-12, a subunit of IL-12, or a variant form any of the foregoing. The immunostimulatory fusion molecule can further include a single-chain Fv having an affinity to an antigen on the surface of the target immune cell, wherein optionally the single-chain Fv has an affinity to the same antigen as the antigen-binding fragment. In some examples, the single-chain Fv has an affinity to a different antigen than the antigen-binding fragment. In some examples, the antigen-binding fragment is a Fab fragment, which optionally comprises a light chain and a heavy chain fragment optionally linked by a disulfide bond, and wherein the immunostimulatory cytokine molecule is operably linked to the Fab fragment at a C-terminus of the light chain, an N-terminus of the light chain, a C-terminus of the heavy chain fragment, or an N-terminus of the heavy chain fragment.

In some examples, the immunostimulatory cytokine molecule is operably linked to the antigen-binding fragment by a linker. In some examples, the linker is selected from a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, and a non-helical linker, e.g., a peptide linker comprising a Gly and a Ser. In some examples, the peptide linker is a $(GGGS)_N$ (SEQ ID NO: 124) or $(GGGGS)_N$ (SEQ ID NO: 125) linker, wherein $_N$ indicates the number of repeats of the motif and is an integer selected from 1-10.

In some examples, the antigen-binding fragment has an affinity to a CD45 receptor and comprises:

(a) a light chain variable amino acid sequence corresponding to the variable domain in the antibody portion of the amino acid sequence shown in SEQ ID NO: 82, or an amino acid sequence at least 85%, 90%, 95%, or higher identity to the variable domain of SEQ ID NO: 82; and/or

(b) a heavy chain variable amino acid sequence corresponding to the variable domain of amino acid sequence shown in SEQ ID NO: 79, or an amino acid sequence at least 85%, 90%, 95%, or higher identity to the variable domain of SEQ ID NO: 79.

In some examples, the cytokine molecule comprises an IL-12 molecule having an amino acid sequence corresponding to the amino acid sequence shown in SEQ ID NO: 50, or an amino acid

sequence at least 85%, 90%, 95%, or higher identity to the cytokine portion of SEQ ID NO: 50.

In some examples, the cytokine molecule comprises a single-chain IL-12 molecule having an IL-12A subunit linked to an IL-12B subunit through a linker having an amino acid sequence corresponding to the amino acid sequence shown in SEQ ID NO: 70, or an amino acid sequence at least 85%, 90%, 95%, or higher identity to the cytokine portion of SEQ ID NO: 70.

In some examples, the linker comprises a peptide linker having an amino acid sequence corresponding to the amino acid sequence in SEQ ID NO: 36, or an amino acid sequence at least 85%, 90%, 95%, or higher identity to the cytokine portion of SEQ ID NO: 36.

In some examples, the single-chain Fv has an amino acid sequence corresponding to the Fv portion of SEQ ID NO: 80, or an amino acid sequence at least 85%, 90%, 95%, or higher identity to the Fv portion of SEQ ID NO: 80.

In some examples, the Fab fragment comprises a light chain having a variable domain (VL) and a constant domain (CL) and a heavy chain fragment having a variable domain (VH) and a constant domain (CH1), wherein the light chain and heavy chain fragment are optionally linked by a disulfide bond, and wherein the light chain and heavy chain fragment each comprise a C-terminus and an N-terminus. In some examples, the IL-12 molecule is operably linked to the C-terminus or the N-terminus of the light chain or the heavy chain fragment.

In some examples, the immunostimulatory fusion molecule further comprises a peptide linker having a first terminus fused to the IL-12 molecule and a second terminus is fused to the Fab fragment, thereby operably linking the IL-12 molecule and the Fab fragment.

Also provided herein is an isolated nucleic acid molecule encoding any one of the immunostimulatory fusion molecule disclosed herein.

Also provided herein is a vector comprising one or more nucleic acids encoding a polypeptide corresponding to the amino acid sequence of SEQ ID NO: 36, 50, 70, 79, 80, or 82, or an amino acid sequence at least 85%, 90%, 95%, or higher identity to SEQ ID NO: 36, 50, 70, 79, 80, or 82.

Also provided herein is a host cell comprising the nucleic acid molecule or the vector disclosed herein.

A further example relates to a modified immune cell comprising:

(a) an immunostimulatory fusion molecule comprising

(i) an immunostimulatory cytokine molecule; and

(ii) an immune cell targeting moiety having an affinity to a cell surface antigen; and

(b) a target immune cell expressing or otherwise displaying the cell surface antigen,

wherein the immunostimulatory fusion molecule is bound to the surface of the immune cell through interaction with the cell surface antigen.

Another example relates to a modified immune cell comprising a healthy and/or non-malignant immune cell and the immunostimulatory fusion molecule disclosed herein bound thereto.

Another example relates to a method of preparing modified immune cells, comprising:

(a) providing a population of immune cells; and

(b) incubating the immunostimulatory fusion molecule of disclosed herein with the population of immune cells so as to permit targeted binding of the immunostimulatory fusion molecule thereto, thereby producing a population of immune cells having immunostimulatory fusion molecules bound on the cell surface.

Another example relates to a composition for use in immune cell therapy, the composition comprising:

(a) a plurality of immunostimulatory fusion molecules, each fusion molecule comprising

(i) an immunostimulatory cytokine molecule; and

(ii) an immune cell targeting moiety having an affinity to a cell surface antigen of a T cell;

(b) a population of T cells expressing or otherwise displaying the cell surface antigen, wherein the plurality of immunostimulatory fusion molecules are bound to the surface of the T cells through interaction with the cell surface antigen; and

(c) a pharmaceutically acceptable carrier, excipient, or stabilizer.

Another example relates to a pharmaceutical composition comprising the immunostimulatory fusion molecule disclosed herein and a pharmaceutically acceptable carrier, excipient, or stabilizer.

Also provided herein is a method for the treatment of cancer in a human subject, the method comprising administering to the human subject a cell therapeutic composition, the composition comprising:

(a) a plurality of immunostimulatory fusion molecules, each fusion molecule comprising

(i) an immunostimulatory cytokine molecule; and

(ii) an immune cell targeting moiety having an affinity to a cell surface antigen of a T cell; and

(b) a population of T cells that homes to a cancer cells or a tissue in which cancer cells exist, and wherein the T cells express the cell surface antigen,

wherein the plurality of immunostimulatory fusion molecules are bound to the surface of the T cells, and wherein the cytokine molecule acts *in vivo* upon the population of T cells and/or other immune cells in the human subject to stimulate an immune response against the cancer.

In some examples, the population of T cells comprise primary T cells, expanded primary T cells, T cells derived from PBMC cells, T cells derived from cord blood cells, T cells autologous to the human subject, T cells allogeneic to the human subject, genetically-engineered T cells, CAR-T cells, effector T cells, activated T cells, CD8+ T cells, CD4+ T cells, and/or CTLs. In some examples, the cell therapeutic composition is administered to the human subject in a cell therapy course selected from an adoptive cell therapy, CAR-T cell therapy, engineered TCR T cell therapy, an antigen-trained T cell therapy, or an enriched antigen-specific T cell therapy.

In some examples, the cytokine molecule is IL-12 and/or IL-15. The immune cell targeting moiety can comprise an antibody or antigen-binding fragment thereof that binds to CD45.

In some examples, the immune cell is a healthy and/or non-malignant immune cell. In various

examples, the IFM can further include a linker for operably linking the targeting moiety and the cytokine molecule. For example, the linker can be selected from: a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, or a non-helical linker, preferably a peptide linker that optionally comprises Gly and Ser, wherein preferably the peptide linker is a $(GGGS)_N$ or $(GGGGS)_N$ linker, wherein $_N$ indicates the number of repeats of the motif and is an integer selected from 1-10.

Also provided herein is a pharmaceutical composition comprising the IFM and/or protein nanogels disclosed herein and a pharmaceutically acceptable carrier, excipient, or stabilizer.

Another example relates to a modified immune cell (e.g., for a cell therapy), comprising a healthy and/or non-malignant immune cell and the IFM and/or protein nanogels disclosed herein bound or targeted thereto.

In some examples, the cell therapy can be used for treating a cancer, preferably a solid tumor cancer or a hematological cancer.

In various examples, the cell therapy can be selected from an adoptive cell therapy, CAR-T cell therapy, engineered TCR T cell therapy, a tumor infiltrating lymphocyte therapy, an antigen-trained T cell therapy, an enriched antigen-specific T cell therapy or NK cell therapy. In certain examples, the plurality of healthy and/or non-malignant immune cells are autologous to the subject.

In some examples, the immune stimulating moiety is a cytokine molecule. In certain examples, the cytokine molecule includes a cytokine, *e.g.*, includes a cytokine chosen from one or more of IL-2, IL-6, IL-7, IL-9, IL-12, IL-15, IL-18, IL-21, IL-23, or IL-27, including variant forms thereof (*e.g.*, a cytokine derivative, a complex comprising the cytokine molecule with a polypeptide, *e.g.*, a cytokine receptor complex, and other agonist forms thereof). In one example, the cytokine molecule is an IL-15 molecule.

In some examples, the immune cell targeting moiety is capable of binding to an immune cell surface target, thereby targeting the immune stimulating moiety to the immune cell, *e.g.*, an immune effector cell (e.g., a lymphocyte). Without wishing to be bound by theory, binding of the immune cell targeting moiety to the immune cell surface target is believed to increase the concentration, e.g., the concentration over time, of the immune stimulating moiety, *e.g.*, cytokine molecule, with its corresponding receptor, *e.g.*, a cytokine receptor, on the surface of the immune cell, *e.g.*, relative to the association of the free cytokine molecule with its cytokine receptor. In some examples, the immune cell surface target is abundantly present on the surface of an immune cell (*e.g.*, outnumbers the number of receptors for the cytokine molecule present on the immune cell surface). In some examples, the immune cell targeting moiety can be chosen from an antibody molecule or a ligand molecule that binds to an immune cell surface target, *e.g.*, a target chosen from CD4, CD8, CD11a, CD19, CD20 or CD45. In one example, the immune cell targeting moiety comprises an antibody molecule or a ligand molecule that binds to CD45. In examples, the targeting moiety is believed to specifically deliver and/or increase the concentration of the cytokine molecule to the surface of an immune cell, thereby resulting in one or more of increased localization, distribution and/or enhancing the cell surface availability of the cytokine

molecule. In examples, the IFM does not substantially interfere with the signaling function of the cytokine molecule. Such targeting effect results in localized and prolonged stimulation of proliferation and activation of the immune cells, thus inducing the controlled expansion and activation of an immune response.

Accordingly, in one example, the disclosure provides an immunostimulatory fusion molecule (IFM) comprising an immune stimulating moiety (*e.g.*, a cytokine molecule, an agonist of a costimulatory molecule, or an inhibitor of a negative immune regulator), and an immune cell targeting moiety.

In some examples, the immune stimulating moiety, *e.g.*, the cytokine molecule, is connected to, *e.g.*, covalently linked to, the immune cell targeting moiety (e.g., directly or indirectly, e.g., via a peptide linker). In some examples, the immune cell targeting moiety of the IFM binds to a surface target, *e.g.*, surface receptor, on an immune cell, *e.g.*, an immune effector cell. In examples, the IFM associates, *e.g.*, links together, the immune stimulating moiety, *e.g.*, the cytokine molecule, and the immune cell targeting moiety to the immune cell, *e.g.*, the effector immune cell. In some examples, the IFM increases the concentration of the cytokine molecule of the IFM (e.g., the concentration of the cytokine molecule of the IFM over time, e.g., a specified period of time) on the surface of the immune cell. In examples, the increased concentration of the cytokine molecule of the IFM on the surface of the immune cell results in one or more of: (i) increased localization (e.g., level) of the cytokine molecule of the IFM to the immune cell surface, *e.g.*, relative to the free cytokine molecule; (ii) enhanced cell surface availability (*e.g.*, concentration (*e.g.*, level or amount) and/or duration of exposure) of the cytokine molecule of the IFM, *e.g.*, relative to the free cytokine molecule; (iii) increased cytokine signaling in a targeted population of immune cells, *e.g.*, a population of cells expressing a preselected surface target, *e.g.*, a surface target as described herein, *e.g.*, relative to the free cytokine molecule; (iv) prolongs cytokine signaling in the targeted cell population (*e.g.*, increases the duration of cytokine signaling by at least 8 hours, e.g, 24 hours), *e.g.*, relative to the free cytokine molecule; (v) causes immunostimulation; (vi) increases immune cell activation of and/or expansion, e.g., of the targeted population of immune cells; or (vii) shows reduced side effects, *e.g.*, a lower systemic toxicity, compared to the free cytokine molecule. In some examples, the IFM changes, *e.g.*, increases, any of (i)-(vii) to a greater extent than the free cytokine molecule, *e.g.*, by at least 8 hours, e.g., 24 hours. In one example, the cytokine molecule is an IL-15 molecule as described herein, and the immune cell targeting moiety is an anti-CD45 antibody molecule, *e.g.*, an antibody or antibody fragment that binds to CD45 as described herein.

In a related example, the disclosure provides a composition, *e.g.*, an IFM, comprising a cytokine molecule coupled to, *e.g.*, fused to, an immune cell targeting moiety. In examples, the immune cell targeting moiety binds to a target or a receptor on the immune cell. In examples, the immune cell targeting moiety includes, or is, an antibody molecule, *e.g.*, an antibody or an antibody fragment, *e.g.*, an anti-CD45 antibody molecule (*e.g.*, an IgG, a Fab, scFv), that binds a CD45 receptor on a cell, *e.g.*, an immune cell (*e.g.*, an immune effector cell, such as a lymphocyte). In examples, the composition, *e.g.*, an IFM, associates, *e.g.*, links together, the cytokine molecule and the immune cell targeting moiety to the

immune cell, *e.g.*, the effector immune cell. In examples, the anti-CD45 antibody binding to the cell increases the association of the IL-15 molecule with the cell and improves one or more of IL-12 signaling, immunostimulation, over time, *e.g.*, relative to a free IL-12 molecule (an IL-12 molecule not found in the composition). In examples, the signaling and/or immunostimulation occurs over a period of time, *e.g.*, minutes, hours, days *e.g.*, by at least 8 hours, e.g., 24 hours.

In another example, the disclosure provides a particle, *e.g.*, a nanoparticle, that comprises an immune agonist as described herein, *e.g.*, nanoparticle that comprises a protein (*e.g.*, a protein nanogel as described herein). In one example, the particle comprises the same immune agonist In other examples, the particle comprises one or more different types of immune agonist.

Compositions, *e.g.*, pharmaceutical compositions, comprising the IFMs and/or the nanogels disclosed herein, are also disclosed. In examples, the pharmaceutical compositions further include a pharmaceutically acceptable carrier, excipient, or stabilizer.

The IFMs and protein nanogels described herein can be administered directly to a subject suffering from the disorder to be treated (e.g., cancer) via e.g., intravenous or subcutaneous administration. In some examples, the immune cell targeting moiety of the IFM and protein nanogel delivers the cytokine molecules to the surface of an immune cell, thereby increasing the concentration of the cytokine molecules at the surface of the immune cell. In examples, the IFM and nanogel results in one or more of: localizes the distribution and/or enhances the cell surface availability of the cytokine molecule, thereby activating and/or stimulating the immune cell.

In other examples, the IFMs described herein can be administered in combination with an immune cell therapy in order to activate and/or stimulate the immune cell therapy either *in vivo* or *in vitro*. For example, an IFM described herein may be co-administered with a cell based therapy to a subject suffering from the disorder to be treated (e.g., cancer) via e.g., intravenous or subcutaneous administration. In other examples, a cell therapy is pulsed *in vitro* with an IFM described herein prior to administration. In some examples, the cell therapy is chosen from an adoptive cell therapy, CAR-T cell therapy, engineered TCR T cell therapy, a tumor infiltrating lymphocyte therapy, an antigen-trained T cell therapy, or an enriched antigen-specific T cell therapy.

Additional features and examples of any of the IFMs, compositions, nanoparticles, methods, uses, nucleic acids, vectors, and host cells, disclosed herein include one or more of the following.

In some examples, the immune stimulating moiety, *e.g.*, the cytokine molecule, is functionally linked, *e.g.*, covalently linked (*e.g.*, by chemical coupling, genetic or protein fusion, noncovalent association or otherwise) to the immune cell targeting moiety. For example, the immune stimulating moiety can be covalently coupled indirectly, *e.g.*, via a linker to the immune cell targeting moiety. In examples, the linker is chosen from: a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, or a non-helical linker. In some examples, the linker is a peptide linker. The peptide linker can be 5-20, 8-18, 10-15, or about 8, 9, 10, 11, 12, 13, 14, 15-20, 20-25, or 25-30 amino acids long. In some examples the peptide linker can be 30 amino acids or longer; e.g., 30-35, 35-40, 40-50 50-60 amino acids long. In some examples, the peptide linker comprises Gly and Ser, *e.g.*,

a linker comprising the amino acid sequence $(Gly_3\text{-}Ser)_n$ or $(Gly_4\text{-}Ser)_n$, wherein n indicates the number of repeats of the motif, *e.g.*, n=1, 2, 3, 4 or 5 (*e.g.*, a $(Gly_3\text{-}Ser)_2$ or $(Gly_4Ser)_2$, or a $(Gly_3\text{-}Ser)_3$ or a $(Gly_4Ser)_3$ linker). In some examples, the linker comprises the amino acid sequence of SEQ ID NO: 36, 37, 38, or 39, or an amino acid sequence substantially identical thereto (*e.g.*, having 1, 2, 3, 4, or 5 amino acid substitutions). In one example, the linker comprises an amino acid sequence GGGSGGGS (SEQ ID NO: 37). In another example, the linker comprises amino acids derived from an antibody hinge region. In certain examples the linker comprises amino acids derived from the hinge regions of IgG1, IgG2, IgG3, IgG4, IgGM, or IgGA antibodies. In examples, the linker comprises amino acids derived from an IgG hinge region, e.g., an IgG1, IgG2 or IgG4 hinge region. For example, the linker comprises a variant amino acid sequence from an IgG hinge, e.g., a variant having one or more cysteines replaced, e.g., with serines.

In other examples, the linker is a non-peptide, chemical linker. For example, the immune stimulating moiety is covalently coupled to the immune cell targeting moiety by crosslinking. Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (*e.g.*, disuccinimidyl suberate). In yet other examples, the immune stimulating moiety is directly covalently coupled to the immune cell targeting moiety, without a linker. In yet other examples, the immune stimulating moiety and the immune cell targeting moiety of the IFM are not covalently linked, *e.g.*, are non-covalently associated.

In other examples, the linker can be a protein or a fragment or derivative thereof, e.g., human albumin or an Fc domain, or a fragment or derivative thereof. In some embodiments, the immune cell targeting moiety is linked to the N-terminus and the immune stimulating moiety is linked to the C-terminus.

In other embodiments, the linker non-covalently associates the immune cell targeting moiety to the immune stimulating moiety. For example, the linker comprises a dimerization domain, e.g., a coiled coil or a leucine zipper.

Other features, objects, and advantages of the disclosure will be apparent from the description and drawings, and from the claims.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

CLAIMS

1. A therapeutic composition comprising a first immune cell loaded with a protein nanogel and a second, different immune cell loaded with an immunostimulatory fusion molecule (IFM).

2. The therapeutic composition of claim 1 for use as a medicament.

3. Use of a first immune cell in the manufacture of a medicament for use in a combinatorial therapy for administration to a subject in need thereof, wherein said medicament is to be administered in combination with a second, different immune cell,

wherein the first immune cell is loaded with a protein nanogel, and the second immune cell is loaded with an IFM.

4. Use of a second immune cell in the manufacture of a medicament for use in a combinatorial therapy for administration to a subject in need thereof, wherein said medicament is to be administered in combination with a first, different immune cell,

wherein the first immune cell is loaded with a protein nanogel, and the second immune cell is loaded with an IFM.

5. Use of a first immune cell in the manufacture of a first medicament and use of a second, different immune cell in the manufacture of a second medicament, wherein the first and second medicaments are to be used in combination therapy for administration to a subject in need thereof.

6. The use of any one of claims 3-5, wherein the first immune cell and the second immune cell are to be administered concurrently to the subject in need thereof.

7. The use of any one of claims 3-5, wherein the first immune cell and the second immune cell are to be administered separately to the subject in need thereof.

8. The use of claim 7, wherein the first immune cell and second immune cell are to be administered sequentially to the subject in need thereof.

9. The therapeutic composition of claims 1 or 2 or the use of any one of claims 3-8, wherein the protein nanogel comprises a plurality of therapeutic protein monomers reversibly cross-linked to one another via a plurality of biodegradable cross-linkers, wherein the protein nanogel has a size between 30 nm and 1000 nm in diameter measured by dynamic light scattering, wherein the cross-linker degrades, after administration into a subject in need thereof, under physiological conditions so as to release the therapeutic protein monomers from the protein nanogel.

10. The therapeutic composition or use of claim 9, wherein the therapeutic protein monomers comprise one or more cytokine molecules selected from IL-15, IL-2, IL-7, IL-10, IL-12, IL-18, IL-21, IL-23, IL-4, IL-1α, IL-1β, IL-5, IFNγ, TNFα, IFNα, IFNβ, GM-CSF, or GCSF.

11. The therapeutic composition or use of claim 10, wherein the one or more cytokine molecules comprise an IL-15 molecule.

12. The therapeutic composition or use of claim 11, wherein the one or more cytokine molecules further comprise an IL-15Rα Sushi domain fused to an Fc.

13. The therapeutic composition or use of any one of claims 9-12, wherein the biodegradable cross-linker is hydrolysable, protease-sensitive, and/or redox-responsive.

14. The therapeutic composition or use of any one of claims 9-13, wherein the protein nanogel further comprises a polycation on its surface so as to allow the protein nanogel to associate with the first immune cell.

15. The therapeutic composition or use of any one of claims 1-14, wherein the IFM comprises an immunostimulatory cytokine molecule and a targeting moiety having an affinity to

an antigen on the surface of the second immune cell, wherein the immunostimulatory cytokine molecule is operably linked to the targeting moiety.

16. The therapeutic composition or use of claim 15, wherein the targeting moiety comprises an antibody or an antigen-binding fragment thereof that has an affinity to the antigen on the surface of the second immune cell.

17. The therapeutic composition or use of claim 15 or 16, wherein the immunostimulatory cytokine molecule is selected from one or more of IL-15, IL-2, IL-6, IL-7, IL-12, IL-18, IL-21, IL-23, IL-27, and variant forms thereof.

18. The therapeutic composition or use of claim 17, wherein the immunostimulatory cytokine molecule comprises IL-12, optionally a single chain IL-12 comprising both the IL-12A and IL-12B subunits on a single chain.

19. The therapeutic composition or use of any one of claims 15-18, wherein the antigen is selected from CD45, CD4, CD8, CD3, CD11a, CD11b, CD11c, CD18, CD25, CD127, CD19, CD20, CD22, HLA-DR, CD197, CD38, CD27, CD196, CXCR3, CXCR4, CXCR5, CD84, CD229, CCR1, CCR5, CCR4, CCR6, CCR8, CCR10, CD16, CD56, CD137, OX40, and GITR.

20. The therapeutic composition or use of claim 19, wherein the antigen is CD45.

21. The therapeutic composition or use of any one of claims 15-20, wherein the IFM comprises an anti-CD45 Fab fragment fused with a single-chain IL-12.

22. The therapeutic composition or use of any one of claims 1-21, wherein the protein nanogel comprises IL-15 and the IFM comprises IL-12.

23. The therapeutic composition or use of any one of claims 1-22, wherein the first immune cell and the second immune cell are T cells.

24. The therapeutic composition or use of claim 23, wherein the T cells comprise a $CD4^+$ T cell, a $CD8^+$ T cell, an αβ T cell, a γδ T cell, a cytotoxic T cell, a CAR-T cell, and/or an engineered T-cell receptor T cell.

25. The therapeutic composition or use of claim 23 or 24, wherein the T cells are specific for one or more tumor-associated antigens.

26. The therapeutic composition or use of any one of claims 23-25, wherein the IFM and/or the protein nanogel support expansion of the T cells *in vivo*.

27. The use of any one of claims 2-26, wherein the medicament, the first medicament, and/or the second medicament is to be used in a cancer immunotherapy.

28. The use of claim 27, wherein the cancer immunotherapy is for treatment of a solid tumor.

29. The use of claim 27 or 28, wherein the cancer immunotherapy is for treatment of cancer selected from breast, prostate, lung, ovarian, cervical, skin, melanoma, colon, stomach, liver, esophageal, kidney, throat, thyroid, pancreatic, testicular, and bone cancer, leukemia, chronic lymphocytic leukemia, basal cell carcinoma, biliary tract cancer, bladder cancer, brain and central nervous system (CNS) cancer, choriocarcinoma, colorectal cancer, connective tissue cancer, endometrial cancer, eye cancer, head and neck cancer, gastric cancer, intraepithelial neoplasm, larynx cancer, lymphoma, neuroblastoma, lip, tongue, mouth and pharynx cancer, retinoblastoma, rhabdomyosarcoma, rectal cancer, sarcoma, skin cancer, thyroid cancer, and uterine cancer.

30. The use of any one of claims 27-29, wherein the cancer treatment further comprises an anti-proliferative cytotoxic agent and/or a radiation therapy.