

SINGLE-CHAIN CHIMERIC POLYPEPTIDES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to: U.S. Patent Application Serial No. 62/725,038, filed August 30, 2018, U.S. Patent Application Serial No. 62/817,244, filed March 12, 2019; U.S. Patent Application Serial No. 62/746,832, filed October 17, 2018; U.S. Patent Application Serial No. 62/749,506, filed October 23, 2018; U.S. Patent Application Serial No. 62/817,241, filed March 12, 2019; U.S. Patent Application Serial No. 62/816,683, filed March 11, 2019; and U.S. Patent Application Serial No. 62/881,039, filed July 31, 2019; each of which is herein incorporated by reference in its entirety.

TECHNICAL FIELD

The present disclosure relates to the field of biotechnology, and more specifically, to antigen-binding molecules.

BACKGROUND

Tissue factor (TF), a 263 amino acid integral membrane glycoprotein with a molecular weight of ~46 kDa and the trigger protein of the extrinsic blood coagulation pathway, is the primary initiator of coagulation *in vivo*. Tissue factor, normally not in contact with circulating blood, initiates the coagulation cascade upon exposure to the circulating coagulation serine protease factors. Vascular damage exposes sub-endothelial cells expressing tissue factor, resulting in the formation of a calcium-dependent, high-affinity complex with pre-existing plasma factor VIIa (FVIIa). Binding of the serine protease FVIIa to tissue factor promotes rapid cleavage of FX to FXa and FIX to FIXa. The proteolytic activity of the resulting FXa and an active membrane surface then inefficiently converts a small amount of prothrombin to thrombin. The thrombin generated by FXa initiates platelet activation and activates minute amounts of the pro-cofactors factor V (FV) and factor VIII (FVIII) to become active cofactors, factor Va (FVa) and factor VIIIa (FVIIIa). FIXa complexes with FVIIIa on the platelet surface forming the intrinsic tenase complex, which results in rapid generation of FXa. FXa

complexes with FVa to form the pro-thrombinase complex on the activated platelet surface which results in rapid cleavage of prothrombin to thrombin.

In addition to the tissue factor-FVIIa complex, a recent study showed that the tissue factor-FVIIa-FXa complex can activate FVIII, which would provide additional
5 levels of FVIIIa during the initiation phase. The extrinsic pathway is paramount in initiating coagulation via the activation of limited amounts of thrombin, whereas the intrinsic pathway maintains coagulation by dramatic amplification of the initial signal.

Much of the tissue factor expressed on a cell surface is “encrypted,” which must be “decrypted” for full participation in coagulation. The mechanism of “decryption” of
10 cell-surface tissue factor is still unclear at this time, however, exposure of anionic phospholipids plays a major role in this process. Healthy cells actively sequester anionic phospholipids such as phosphatidyl serine (PS) to the inner leaflet of the plasma membrane. Following cellular damage, activation, or increased levels of cytosolic Ca^{2+} , this bilayer asymmetry is lost, resulting in increased PS exposure on the outer leaflet,
15 which increases the specific activity of cell-surface tissue factor-FVIIa complexes. PS exposure is known to decrease the apparent K_m for activation of FIX and FX by tissue factor-FVIIa complexes, but additional mechanisms could include conformational rearrangement of tissue factor or tissue factor-FVIIa and subsequent exposure of substrate binding sites.

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SUMMARY

The present invention is based on the discovery that soluble tissue factor can be used as a scaffold for chimeric polypeptides including an antigen-binding domain. Based on this discovery provided herein are single-chain chimeric polypeptides that include: (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a second target-
25 binding domain. Also provided herein are compositions that include any of the single-chain chimeric polypeptides described herein, nucleic acids that encode any of the single-chain chimeric polypeptides described herein, and cells that include any of the nucleic acids that encode any of the single-chain chimeric polypeptides described herein. Also provided herein are methods of stimulating an immune cell and methods of treating a
30 subject in need thereof that include the use of any of the single-chain chimeric

polypeptides described herein. Also provided herein are methods of producing any of the single-chain chimeric polypeptides described herein. Additional uses of any of the single-chain chimeric polypeptides are described herein.

Accordingly, provided herein is a single-chain chimeric polypeptide comprising:

- 5 (i) a first target-binding domain;
- (ii) a soluble tissue factor domain; and
- (iii) a second target-binding domain. In some embodiments, the first target-binding domain and the soluble tissue factor domain directly abut each other. In some embodiments, the single-chain chimeric polypeptide further comprises a linker sequence
- 10 between the first target-binding domain and the soluble tissue factor domain. In some embodiments, the soluble tissue factor domain and the second target-binding domain directly abut each other. In some embodiments, the single-chain chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain. In some embodiments, the first target-binding domain and
- 15 the second target-binding domain directly abut each other. In some embodiments, the single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the second target-binding domain. In some embodiments, the second target-binding domain and the soluble tissue factor domain directly abut each other. In some embodiments, the single-chain chimeric polypeptide further comprises a
- 20 linker sequence between the second target-binding domain and the soluble tissue factor domain. In some embodiments, the first target-binding domain and the second target-binding domain bind specifically to the same antigen. In some embodiments, the first target-binding domain and the second target-binding domain bind specifically to the same epitope. In some embodiments, the first target-binding domain and the second target-binding domain comprise the same amino acid sequence. In some embodiments, the first target-binding domain and the second target-binding domain bind specifically to different antigens. In some embodiments, one or both of the first target-binding domain and the second target-binding domain is an antigen-binding domain. In some embodiments, the first target-binding domain and the second target-binding domain are each an antigen-
- 25 binding domain. In some embodiments, the antigen-binding domain comprises a scFv or a single domain antibody. In some embodiments, one or both of the first target-binding
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domain and the second target-binding domain bind to a target selected from the group consisting of: CD16a, CD28, CD3, CD33, CD20, CD19, CD22, CD123, IL-1R, IL-1, VEGF, IL-6R, IL-4, IL-10, PDL-1, TIGIT, PD-1, TIM3, CTLA4, MICA, MICB, IL-6, IL-8, TNF α , CD26a, CD36, ULBP2, CD30, CD200, IGF-1R, MUC4AC, MUC5AC,

5 Trop-2, CMET, EGFR, HER1, HER2, HER3, PSMA, CEA, B7H3, EPCAM, BCMA, P-cadherin, CEACAM5, a UL16-binding protein, HLA-DR, DLL4, TYRO3, AXL, MER, CD122, CD155, PDGF-DD, a ligand of TGF- β receptor II (TGF- β RII), a ligand of TGF- β RIII, a ligand of DNAM-1, a ligand of NKp46, a ligand of NKp44, a ligand of NKG2D, a ligand of NKp30, a ligand for a scMHC I, a ligand for a scMHC II, a ligand for a scTCR,

10 a receptor for IL-1, a receptor for IL-2, a receptor for IL-3, a receptor for IL-7, a receptor for IL-8, a receptor for IL-10, a receptor for IL-12, a receptor for IL-15, a receptor for IL-17, a receptor for IL-18, a receptor for IL-21, a receptor for PDGF-DD, a receptor for stem cell factor (SCF), a receptor for stem cell-like tyrosine kinase 3 ligand (FLT3L), a receptor for MICA, a receptor for MICB, a receptor for a ULP16-binding protein, a

15 receptor for CD155, a receptor for CD122, and a receptor for CD28. In some embodiments, one or both of the first target-binding domain and the second target-binding domain is a soluble interleukin, a soluble cytokine protein, or a soluble cell surface protein. In some embodiments, the soluble interleukin, soluble cytokine protein, or soluble cell surface protein is selected from the group consisting of: IL-1, IL-2, IL-3,

20 IL-7, IL-8, IL-10, IL-12, IL-15, IL-17, IL-18, IL-21, PDGF-DD, SCF, FLT3L, MICA, MICB, and a ULP16-binding protein. In some embodiments, one or both of the first target-binding domain and the second target-binding domain is a soluble interleukin receptor, a soluble cytokine receptor, or a soluble cell surface receptor. In some

25 embodiments, the soluble interleukin receptor, soluble cytokine receptor, or soluble cell surface receptor is a soluble TGF- β receptor II (TGF- β RII), a soluble TGF- β RIII, a soluble NKG2D, a soluble NKp30, a soluble NKp44, a soluble NKp46, a soluble DNAM-1, a scMHC I, a scMHC II, a scTCR, a soluble CD155, or a soluble CD28. In some embodiments, the soluble tissue factor domain is a soluble human tissue factor domain. In some embodiments, the soluble human tissue factor domain comprises a

30 sequence that is at least 80% identical to SEQ ID NO: 9. In some embodiments, the soluble human tissue factor domain comprises a sequence that is at least 90% identical to

SEQ ID NO: 9. In some embodiments, the soluble human tissue factor domain comprises a sequence that is at least 95% identical to SEQ ID NO: 9. In some embodiments, the single-chain chimeric polypeptide of the soluble human tissue factor domain does not comprise one or more of: a lysine at an amino acid position that corresponds to amino acid position 20 of mature wildtype human tissue factor protein; an isoleucine at an amino acid position that corresponds to amino acid position 22 of mature wildtype human tissue factor protein; a tryptophan at an amino acid position that corresponds to amino acid position 45 of mature wildtype human tissue factor protein; an aspartic acid at an amino acid position that corresponds to amino acid position 58 of mature wildtype human tissue factor protein; a tyrosine at an amino acid position that corresponds to amino acid position 94 of mature wildtype human tissue factor protein; an arginine at an amino acid position that corresponds to amino acid position 135 of mature wildtype human tissue factor protein; and a phenylalanine at an amino acid position that corresponds to amino acid position 140 of mature wildtype human tissue factor protein. In some embodiments, the soluble human tissue factor domain does not comprise any of: a lysine at an amino acid position that corresponds to amino acid position 20 of mature wildtype human tissue factor protein; an isoleucine at an amino acid position that corresponds to amino acid position 22 of mature wildtype human tissue factor protein; a tryptophan at an amino acid position that corresponds to amino acid position 45 of mature wildtype human tissue factor protein; an aspartic acid at an amino acid position that corresponds to amino acid position 58 of mature wildtype human tissue factor protein; a tyrosine at an amino acid position that corresponds to amino acid position 94 of mature wildtype human tissue factor protein; an arginine at an amino acid position that corresponds to amino acid position 135 of mature wildtype human tissue factor protein; and a phenylalanine at an amino acid position that corresponds to amino acid position 140 of mature wildtype human tissue factor protein. In some embodiments, the soluble tissue factor domain is not capable of binding Factor VIIa. In some embodiments, the soluble tissue factor domain does not convert inactive Factor X into Factor Xa. In some embodiments, the single-chain chimeric polypeptide does not stimulate blood coagulation in a mammal. In some embodiments, the single-chain chimeric polypeptide further comprises one or more additional target-binding domains at its N- and/or C-terminus. In some embodiments, the

single-chain chimeric polypeptide comprises one or more additional target-binding domains at its N-terminus. In some embodiments, one or more additional target-binding domains directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain. In some embodiments, the single-chain chimeric polypeptide further comprises a linker sequence between one of the at least one additional target-binding domains and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain. In some embodiments, the single-chain chimeric polypeptide comprises one or more additional target-binding domains at its C-terminus. In some embodiments, one of the one or more additional target-binding domains directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain. In some embodiments, the single-chain chimeric polypeptide further comprises a linker sequence between one of the at least one additional target-binding domains and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain. In some embodiments, the single-chain chimeric polypeptide comprises one or more additional target binding domains at its N-terminus and the C-terminus. In some embodiments, one of the one or more additional antigen binding domains at the N-terminus directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain. In some embodiments, the single-chain chimeric polypeptide further comprises a linker sequence between one of the one or more additional antigen-binding domains at the N-terminus and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain. In some embodiments, one of the one or more additional antigen binding domains at the C-terminus directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain. In some embodiments, the single-chain chimeric polypeptide further comprises a linker sequence between one of the one or more additional antigen-binding domains at the C-terminus and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain. In some embodiments, two or more of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains bind specifically to the same antigen. In some embodiments, two or more of the first target-binding domain, the second target-binding domain, and the one or more

additional target-binding domains bind specifically to the same epitope. In some embodiments, two or more of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains comprise the same amino acid sequence. In some embodiments, the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains each bind specifically to the same antigen. In some embodiments, the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains each bind specifically to the same epitope. In some embodiments, the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains each comprise the same amino acid sequence. In some embodiments, the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains bind specifically to different antigens. In some embodiments, one or more of the first target-binding domain, the second target-binding domain, and the one or more target-binding domains is an antigen-binding domain. In some embodiments, the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains are each an antigen-binding domain. In some embodiments, the antigen-binding domain comprises a scFv or a single domain antibody. In some embodiments, one or more of the first target-binding domain, the second target-binding domain, and the one or more target-binding domains bind specifically to a target selected from the group consisting of: CD16a, CD28, CD3, CD33, CD20, CD19, CD22, CD123, IL-1R, IL-1, VEGF, IL-6R, IL-4, IL-10, PDL-1, TIGIT, PD-1, TIM3, CTLA4, MICA, MICB, IL-6, IL-8, TNF α , CD26a, CD36, ULBP2, CD30, CD200, IGF-1R, MUC4AC, MUC5AC, Trop-2, CMET, EGFR, HER1, HER2, HER3, PSMA, CEA, B7H3, EPCAM, BCMA, P-cadherin, CEACAM5, a UL16-binding protein, HLA-DR, DLL4, TYRO3, AXL, MER, CD122, CD155, PDGF-DD, a ligand of TGF- β receptor II (TGF- β RII), a ligand of TGF- β RIII, a ligand of DNAM-1, a ligand of NKp46, a ligand of NKp44, a ligand of NKG2D, a ligand of NKp30, a ligand for a scMHCI, a ligand for a scMHCII, a ligand for a scTCR, a receptor for IL-1, a receptor for IL-2, a receptor for IL-3, a receptor for IL-7, a receptor for IL-8, a receptor for IL-10, a receptor for IL-12, a receptor for IL-15, a receptor for IL-17, a receptor for IL-18, a receptor for IL-21, a receptor for PDGF-D, a receptor for stem cell factor (SCF), a receptor for stem

cell-like tyrosine kinase 3 ligand (FLT3L), a receptor for MICA, a receptor for MICB, a receptor for a ULP16-binding protein, a receptor for CD155, a receptor for CD122, and a receptor for CD28. In some embodiments, one or more of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains is a soluble interleukin or cytokine protein. In some embodiments, the soluble interleukin or cytokine protein is selected from the group consisting of: IL-1, IL-2, IL-3, IL-7, IL-8, IL-10, IL-12, IL-15, IL-17, IL-18, IL-21, PDGF-DD, SCF, FLT3L, MICA, MICB, and a ULP16-binding protein. In some embodiments, one or more of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains is a soluble interleukin or cytokine receptor. In some embodiments, the soluble receptor is a soluble TGF- β receptor II (TGF- β RII), a soluble TGF- β RIII, a soluble NKG2D, a soluble Nkp30, a soluble Nkp44, a soluble Nkp46, a soluble DNAM-1, a scMHCI, a scMHCII, a scTCR, a soluble CD155, a soluble CD122, a soluble CD3, or a soluble CD28. In some embodiments, the single-chain chimeric polypeptide further comprises a signal sequence at its N-terminal end. In some embodiments, the single-chain chimeric polypeptide further comprises a peptide tag positioned at the N-terminal end or the C-terminal end of the single-chain chimeric polypeptide.

Also provided herein is a composition comprising any of the single-chain chimeric polypeptides discussed above. In some embodiments, the composition is a pharmaceutical composition. In some embodiments, the single-chain chimeric polypeptides comprise at least one dose of the composition within a kit.

Also provided herein is a method of stimulating an immune cell, the method comprising: contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides or compositions discussed above. In some embodiments, the method comprises contacting the immune in vitro. In some embodiments, the method comprises obtaining the immune cell from a subject. In some embodiments, the method further comprises obtaining the immune cell from the subject prior to the contacting step. In some embodiments, the method comprises contacting the immune cell in vivo. In some embodiments, the immune cell is selected from the group consisting of: an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell

precursor, a lymphoid progenitor cell, a Treg cell, a memory T cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T cell, a CD4⁺ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, and a natural killer cell. In some embodiments, the immune cell has previously been genetically modified to express a chimeric antigen receptor or a recombinant T-cell receptor. In some embodiments, the method further comprises introducing into the immune cell a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor after the contacting step. In some embodiments, the method further comprises administering the immune cell to a subject in need thereof. In some embodiments, the method comprises identifying or diagnosing the subject as having an age-related disease or condition. In some embodiments, the age-related disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction. In some embodiments, the method comprises identifying or diagnosing the subject as having a cancer. In some embodiments, the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast

cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma. In some embodiments, the method comprises diagnosing or identifying the subject as having an infectious disease. In some embodiments, the
5 infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Also provided herein is a method of inducing or increasing proliferation of an immune cell, the method comprising: contacting an immune cell with an effective
10 amount of any of the single-chain chimeric polypeptides or compositions discussed herein. In some embodiments, the method comprises contacting the immune cell in vitro. In some embodiments, the method comprises obtaining the immune cell from a subject. In some embodiments, method further comprises obtaining the immune cell from the subject prior to the contacting step. In some embodiments, the method comprises
15 contacting the immune cell in vivo. In some embodiments, the immune cell is selected from the group consisting of: an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a memory T cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T cell, a CD4⁺ T cell, a natural
20 killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, and a natural killer cell. In some embodiments, the immune cell has previously been genetically modified to express a chimeric antigen receptor or a recombinant T-cell receptor. In some embodiments, the method further comprises, after the contacting step, introducing into the immune cell a nucleic acid encoding a chimeric
25 antigen-receptor or a recombinant T-cell receptor. In some embodiments, the method further comprises administering the immune cell to a subject in need thereof. In some embodiments, the method comprises identifying or diagnosing the subject as having an age-related disease or condition. In some embodiments, the age-related disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic
30 fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration,

osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction. In some embodiments, the method comprises identifying or diagnosing the subject as having a cancer. In some embodiments, the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma. In some embodiments, the method comprises diagnosing or identifying the subject as having an infectious disease. In some embodiments, the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Also provided herein is a method of inducing differentiation of an immune cell into a memory or memory-like immune cell, the method comprising: contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides or compositions discussed above. In some embodiments, the method comprises contacting the immune cell in vitro. In some embodiments, the method comprises obtaining the immune cell from a subject. In some embodiments, the method further comprises obtaining the immune cell from the subject prior to the contacting step. In some

embodiments, the method comprises contacting the immune cell in vivo. In some embodiments, the immune cell is selected from the group consisting of: an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8+ T cell, a CD4+ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, and a natural killer cell. In some embodiments, the method comprises genetically modifying the immune cell to express a chimeric antigen receptor or a recombinant T-cell receptor. In some embodiments, the method further comprises introducing into the immune cell a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor after the contacting step. In some embodiments, the method further comprises administering the immune cell to a subject in need thereof. In some embodiments, the method comprises identifying or diagnosing the subject as having an age-related disease or condition. In some embodiments, the age-related disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction. In some embodiments, the method comprises identifying or diagnosing the subject as having a cancer. In some embodiments, the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic

syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma. In some embodiments, the method comprises diagnosing or identifying the subject as having an infectious disease. In some embodiments, the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Also provided herein is a method of killing a cancer cell, an infected cell, or a senescent cell in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of any of the single-chain chimeric polypeptides or compositions discussed above. In some embodiments, the method comprises identifying or diagnosing the subject as having a cancer. In some embodiments, the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma. In some embodiments, the method identifying or diagnosing the subject as having an aging-related disease or condition. In some embodiments, the aging-related disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose

atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

Also provided herein is a method of treating a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of any of the single-chain chimeric polypeptides or compositions discussed above. In some embodiments, the method comprises identifying or diagnosing the subject as having a cancer. In some embodiments, the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma. In some embodiments, the method comprises identifying or diagnosing the subject as having an aging-related disease or condition. In some embodiments, the aging-related disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of

lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction. In some embodiments, the method comprises diagnosing or identifying the subject as having an infectious
5 disease. In some embodiments, the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Also provided herein are nucleic acids encoding any of the single-chain chimeric
10 polypeptides discussed above. Some embodiments include a vector comprising the nucleic acid discussed above. In some embodiments, the vector is an expression vector. Some embodiments include a cell comprising the nucleic acid or the vector discussed above.

Also provided herein is a method of producing a single-chain chimeric
15 polypeptide, the method comprising: culturing the cell in a culture medium under conditions sufficient to result in the production of the single-chain chimeric polypeptide; and recovering the single-chain chimeric polypeptide from the cell and/or the culture medium. Some embodiments comprise single-chain chimeric polypeptide produced by the discussed above. In some embodiments of any of the single-chain chimeric
20 polypeptides provided herein, the human soluble tissue factor domain does not initiate blood coagulation. In some embodiments of any of the single-chain chimeric polypeptides provided herein, the soluble tissue factor domain comprises or consists of a soluble wildtype human tissue factor.

In some embodiments, the mutant soluble human tissue factor domain comprises
25 a sequence that is at least 80% identical to SEQ ID NO: 96. In some embodiments, the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 96. In some embodiments, the soluble human tissue factor domain comprises a sequence that is at least 95% identical to SEQ ID NO: 96. In some
30 embodiments, the soluble human tissue factor domain comprises a sequence that is 100% identical to SEQ ID NO: 96. In some embodiments, the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 97. In some

embodiments, the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 97. In some embodiments, the soluble human tissue factor domain comprises a sequence that is at least 95% identical to SEQ ID NO: 97. In some embodiments, the soluble human tissue factor domain comprises a sequence that is
5 100% identical to SEQ ID NO: 97.

As used herein, the term “chimeric” refers to a polypeptide that includes amino acid sequences (e.g., domains) originally derived from two different sources (e.g., two different naturally-occurring proteins, e.g., from the same or different species). For example, a chimeric polypeptide can include domains from at least two different
10 naturally occurring human proteins. In some examples, a chimeric polypeptide can include a domain that is a synthetic sequence (e.g., a scFv) and a domain that is derived from a naturally-occurring protein (e.g., a naturally-occurring human protein). In some embodiments, a chimeric polypeptide can include at least two different domains that are synthetic sequences (e.g., two different scFvs).

15 An “antigen-binding domain” is one or more protein domain(s) (e.g., formed from amino acids from a single polypeptide or formed from amino acids from two or more polypeptides (e.g., the same or different polypeptides) that is capable of specifically binding to one or more different antigen(s). In some examples, an antigen-binding domain can bind to an antigen or epitope with specificity and affinity similar to that of
20 naturally-occurring antibodies. In some embodiments, the antigen-binding domain can be an antibody or a fragment thereof. In some embodiments, an antigen-binding domain can include an alternative scaffold. Non-limiting examples of antigen-binding domains are described herein. Additional examples of antigen-binding domains are known in the art.

25 A “soluble tissue factor domain” refers to a polypeptide having at least 70% identity (e.g., at least 75% identity, at least 80% identity, at least 85% identity, at least 90% identity, at least 95% identity, at least 99% identity, or 100% identical) to a segment of a wildtype mammalian tissue factor protein (e.g., a wildtype human tissue factor protein) that lacks the transmembrane domain and the intracellular domain. Non-limiting
30 examples of soluble tissue factor domains are described herein.

The term “soluble interleukin protein” is used herein to refer to a mature and secreted interleukin protein or a biologically active fragment thereof. In some examples, a soluble interleukin protein can include a sequence that is at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical to a wildtype mature and secreted mammalian interleukin protein (e.g., a wildtype human interleukin protein) and retains its biological activity. Non-limiting examples of soluble interleukin proteins are described herein.

The term “soluble cytokine protein” is used herein to refer to a mature and secreted cytokine protein or a biologically active fragment thereof. In some examples, a soluble cytokine protein can include a sequence that is at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical to a wildtype mature and secreted mammalian interleukin protein (e.g., a wildtype human interleukin protein) and retains its biological activity. Non-limiting examples of soluble cytokine proteins are described herein.

The term “soluble interleukin receptor” is used herein in the broadest sense to refer to a polypeptide that lacks a transmembrane domain (and optionally an intracellular domain) that is capable of binding one or more of its natural ligands (e.g., under physiological conditions, e.g., in phosphate buffered saline at room temperature). For example, a soluble interleukin receptor can include a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to an extracellular domain of wildtype interleukin receptor and retains its ability to specifically bind to one or more of its natural ligands, but lacks its transmembrane domain (and optionally, further lacks its intracellular domain). Non-limiting examples of soluble interleukin receptors are described herein.

The term “soluble cytokine receptor” is used herein in the broadest sense to refer to a polypeptide that lacks a transmembrane domain (and optionally an intracellular domain) that is capable of binding one or more of its natural ligands (e.g., under physiological conditions, e.g., in phosphate buffered saline at room temperature). For

example, a soluble cytokine receptor can include a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to an extracellular domain of wildtype cytokine receptor and retains its ability to specifically bind to one or more of its natural ligands, but lacks its transmembrane domain (and optionally, further lacks its intracellular domain). Non-limiting examples of soluble cytokine receptors are described herein.

The term “antibody” is used herein in its broadest sense and includes certain types of immunoglobulin molecules that include one or more antigen-binding domains that specifically bind to an antigen or epitope. An antibody specifically includes, e.g., intact antibodies (e.g., intact immunoglobulins), antibody fragments, and multi-specific antibodies. One example of an antigen-binding domain is an antigen-binding domain formed by a VH -VL dimer. Additional examples of an antibody are described herein. Additional examples of an antibody are known in the art.

“Affinity” refers to the strength of the sum total of non-covalent interactions between an antigen-binding site and its binding partner (e.g., an antigen or epitope). Unless indicated otherwise, as used herein, “affinity” refers to intrinsic binding affinity, which reflects a 1:1 interaction between members of an antigen-binding domain and an antigen or epitope. The affinity of a molecule X for its partner Y can be represented by the dissociation equilibrium constant (K_D). The kinetic components that contribute to the dissociation equilibrium constant are described in more detail below. Affinity can be measured by common methods known in the art, including those described herein. Affinity can be determined, for example, using surface plasmon resonance (SPR) technology (e.g., BIACORE®) or biolayer interferometry (e.g., FORTEBIO®). Additional methods for determining the affinity for an antigen-binding domain and its corresponding antigen or epitope are known in the art.

A “single-chain polypeptide” as used herein refers to a single protein chain.

The term “pair of affinity domains” is two different protein domain(s) that bind specifically to each other with a K_D of less than of less than 1×10^{-7} M (e.g., less than 1×10^{-8} M, less than 1×10^{-9} M, less than 1×10^{-10} M, or less than 1×10^{-11} M). In some examples, a pair of affinity domains can be a pair of naturally-occurring proteins. In

some embodiments, a pair of affinity domains can be a pair of synthetic proteins. Non-limiting examples of pairs of affinity domains are described herein.

The term “epitope” means a portion of an antigen that specifically binds to an antigen-binding domain. Epitopes can, e.g., consist of surface-accessible amino acid residues and/or sugar side chains and may have specific three-dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter may be lost in the presence of denaturing solvents. An epitope may comprise amino acid residues that are directly involved in the binding, and other amino acid residues, which are not directly involved in the binding. Methods for identifying an epitope to which an antigen-binding domain binds are known in the art.

An “immune effector cell” refers to a cell of the immune system of a mammal that is capable, directly or indirectly, of recognizing and/or causing cytolysis or cell death of a pathogenic cell (e.g., a cancer cell) in the mammal. Non-limiting examples of immune effector cells include macrophages, T-lymphocytes (e.g., cytotoxic T-lymphocytes and T-helper cells), natural killer cells, neutrophils, monocytes, and eosinophils. Additional examples of immune effector cells are known in the art.

The term “treatment” means to ameliorate at least one symptom of a disorder. In some examples, the disorder being treated is cancer and to ameliorate at least one symptom of cancer includes reducing aberrant proliferation, gene expression, signaling, translation, and/or secretion of factors. Generally, the methods of treatment include administering a therapeutically effective amount of composition that reduces at least one symptom of a disorder to a subject who is in need of, or who has been determined to be in need of such treatment.

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 invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other

references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

5

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 are schematic diagrams of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide.

Figure 2 is a chromatograph showing the elution of an exemplary
10 α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide from an anti-tissue factor antibody affinity column.

Figure 3 is a chromatograph showing the elution of a Superdex 200 Increase 10/300 GL gel filtration column loaded with an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide.

Figure 4 is a sodium dodecyl sulfate polyacrylamide gel (4-12% NuPage Bis-Tris gel) of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide
15 purified using an anti-tissue factor antibody affinity column.

Figure 5 is a graph showing the ELISA quantitation of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide performed using the
20 methods described in Example 1. Purified tissue factor was used as the control.

Figure 6 is a graph showing the ability of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide to stimulate CD25 expression in CD4⁺ T-cells isolated from blood from two donors. The experiments were performed as described in Example 2.

Figure 7 is a graph showing the ability of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide to stimulate CD25 expression in CD8⁺ T-cells isolated from blood from two donors. The experiments were
25 performed as described in Example 2.

Figure 8 is a graph showing the ability of an exemplary
30 α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide to stimulate CD69

expression in CD4⁺ T-cells isolated from blood from two donors. The experiments were performed as described in Example 2.

Figure 9 are schematic diagrams of an exemplary IL-2/TF/IL-2 single-chain chimeric polypeptide.

5 Figure 10 shows IL-2 activity in IL-2/TF/IL-2 as compared to recombinant IL-2 using a 32D β cell proliferation assay.

Figure 11 shows IL-2 activity in IL-2/TF/IL-2 as compared to recombinant IL-2 using a CTLL-2 cell proliferation assay.

10 Figure 12 shows the fasting blood glucose levels in ApoE^{-/-} mice fed with standard chow or a high fat diet and treated with a PBS control (untreated) or with IL-2/TF/IL-2.

Figure 13 shows the ratio of CD4⁺CD25⁺FoxP3⁺ T regulatory cells in blood lymphocytes from ApoE^{-/-} mice fed with standard chow or a high fat diet and treated with a PBS control (untreated) or with IL-2/TF/IL-2.

15 Figure 14 is a line graph showing the chromatographic profile of IL-2/TF/IL-2 protein containing cell culture supernatant following binding and elution on anti-TF antibody resin.

Figure 15 shows an analytical SEC profile of IL-2/TF/IL-2.

20 Figures 16A and 16B show reduced SDS-PAGE analysis of IL-2/TF/IL-2 before and after deglycosylation. Figure 16A shows reduced SDS-PAGE analysis of IL-2/TF/IL-2 before deglycosylation. Figure 16B shows reduced SDS-PAGE analysis of IL-2/TF/IL-2 after deglycosylation.

25 Figures 17A and 17B show results of immunostimulation in C57BL/6 mice using IL-2/TF/IL-2. Figure 17A shows spleen weight following treatment with IL-2/TF/IL-2. Figure 17B shows the percentages of immune cell types following IL-2/TF/IL-2 treatment.

Figure 18 shows upregulation of CD25 expression of CD4⁺ T cells in mice treated with IL-2/TF/IL-2.

Figure 19 shows the pharmacokinetics of IL-2/TF/IL-2 in C57BL/6 mice.

30 Figures 20A and 20B show effects of IL-2/TF/IL-2 in attenuating the formation of high fat-induced atherosclerotic plaques in ApoE^{-/-} mice. Figure 20A shows a

representative view of atherosclerotic plaques from ApoE^{-/-} mice fed with standard chow or a high fat diet and treated with either PBS control or IL-2/TF/IL-2. Figure 20B shows the results of quantitative analysis of atherosclerotic plaques of each group.

Figure 21 shows fasting glucose levels in IL-2/TF/IL-2 treated-mice as compared
5 to control-treated mice.

Figure 22 shows the percentage of CD4⁺CD25⁺FoxP3⁺ Tregs in blood lymphocytes from mice treated with IL-2/TF/IL-2 and control-treated mice.

Figure 23 are schematic diagrams of an exemplary IL-15/TF/IL-15 single-chain chimeric polypeptide.

Figure 24 shows the IL-15 activity of IL-15/TF/IL-15 as compared to
10 recombinant IL-15 in a 32Dβ cell proliferation assay.

Figure 25 is a line graph showing the chromatographic profile of IL-15/TF/IL-15 protein containing cell culture supernatant following binding and elution on anti-TF antibody resin.

Figures 26A and 26B show reduced SDS-PAGE analysis of IL-15/TF/IL-15
15 before and after deglycosylation. Figure 26A shows reduced SDS-PAGE analysis of IL-15/TF/IL-15 before deglycosylation. Figure 26B shows reduced SDS-PAGE analysis of IL-15/TF/IL-15 after deglycosylation.

Figures 27A-27C is a set of graphs showing immunostimulation in C57BL/6 mice
20 following treatment with 2t2.

Figures 28A-28C is a set of graphs showing *in vivo* stimulation of Tregs, NK cells, and CD8⁺ T cells in ApoE^{-/-} mice fed with a Western diet and treated with 2t2.

Figures 29A and 29B is a set of graphs showing induction of splenocyte proliferation by 2t2 in C57BL/6 mice.

Figures 30A and 30B is a set of graphs showing *in vivo* induction of proliferation
25 of NK cells and CD8⁺ T cells in ApoE^{-/-} mice fed with a Western diet and treated with 2t2.

Figures 31A-31C is a set of graphs showing amelioration of the Western diet-induced hyperglycemia in ApoE^{-/-} mice by 2t2.

Figure 32 shows upregulation of CD44 memory T cells upon treatment with 2t2.
30

Figure 33 shows a graph of Factor X (FX) activation following treatment with single-chain or multi-chain chimeric polypeptides.

Figure 34A-34C show human blood lymphocyte pStat5a responses in CD4⁺CD25^{hi}T_{reg} cells, CD4⁺CD25⁺T_{con} cells, or in CD8⁺ T_{con} cells in response to 2t2 or IL2 treatment. Figure 34A shows pSTAT5 responses in CD4⁺CD25^{hi}T_{reg} cells. Figure 34B shows pSTAT5 responses in CD4⁺CD25⁺T_{con} cells. Figure 34C shows pSTAT5 responses in CD8⁺ T_{con} cells.

DETAILED DESCRIPTION

Provided herein are single-chain chimeric polypeptides that include: (i) a first target-binding domain (e.g., any of the target-binding domains described herein or known in the art), (ii) a soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein or known in the art), and (iii) as second target-binding domain (e.g., any of the target-binding domains described herein or known in the art).

Also provided herein are compositions that include any of the single-chain chimeric polypeptides described herein, nucleic acids that encode any of the single-chain chimeric polypeptides described herein, and cells that include any of the nucleic acids that encode any of the single-chain chimeric polypeptides described herein. Also provided herein are methods of stimulating an immune cell and methods of treating a subject in need thereof that include the use of any of the single-chain chimeric polypeptides described herein. Also provided herein are methods of producing any of the single-chain chimeric polypeptides described herein.

In some examples of any of the single-chain chimeric polypeptides described herein, the single-chain chimeric polypeptide can have a total length of about 50 amino acids to about 3000 amino acids, about 50 amino acids to about 2500 amino acids, about 50 amino acids to about 2000 amino acids, about 50 amino acids to about 1500 amino acids, about 50 amino acids to about 1000 amino acids, about 50 amino acids to about 950 amino acids, about 50 amino acids to about 900 amino acids, about 50 amino acids to about 850 amino acids, about 50 amino acids to about 800 amino acids, about 50 amino acids to about 750 amino acids, about 50 amino acids to about 700 amino acids, about 50 amino acids to about 650 amino acids, about 50 amino acids to about 600 amino acids,

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[illegible]

[illegible]

[illegible]

28

amino acids to about 700 amino acids, about 340 amino acids to about 650 amino acids,
about 340 amino acids to about 600 amino acids, about 340 amino acids to about 550
amino acids, about 340 amino acids to about 500 amino acids, about 340 amino acids to
about 480 amino acids, about 340 amino acids to about 460 amino acids, about 340
5 amino acids to about 440 amino acids, about 340 amino acids to about 420 amino acids,
about 340 amino acids to about 400 amino acids, about 340 amino acids to about 380
amino acids, about 340 amino acids to about 360 amino acids, about 360 amino acids to
about 3000 amino acids, about 360 amino acids to about 2500 amino acids, about 360
amino acids to about 2000 amino acids, about 360 amino acids to about 1500 amino
10 acids, about 360 amino acids to about 1000 amino acids, about 360 amino acids to about
950 amino acids, about 360 amino acids to about 900 amino acids, about 360 amino acids
to about 850 amino acids, about 360 amino acids to about 800 amino acids, about 360
amino acids to about 750 amino acids, about 360 amino acids to about 700 amino acids,
about 360 amino acids to about 650 amino acids, about 360 amino acids to about 600
15 amino acids, about 360 amino acids to about 550 amino acids, about 360 amino acids to
about 500 amino acids, about 360 amino acids to about 480 amino acids, about 360
amino acids to about 460 amino acids, about 360 amino acids to about 440 amino acids,
about 360 amino acids to about 420 amino acids, about 360 amino acids to about 400
amino acids, about 360 amino acids to about 380 amino acids, about 380 amino acids to
20 about 3000 amino acids, about 380 amino acids to about 2500 amino acids, about 380
amino acids to about 2000 amino acids, about 380 amino acids to about 1500 amino
acids, about 380 amino acids to about 1000 amino acids, about 380 amino acids to about
950 amino acids, about 380 amino acids to about 900 amino acids, about 380 amino acids
to about 850 amino acids, about 380 amino acids to about 800 amino acids, about 380
25 amino acids to about 750 amino acids, about 380 amino acids to about 700 amino acids,
about 380 amino acids to about 650 amino acids, about 380 amino acids to about 600
amino acids, about 380 amino acids to about 550 amino acids, about 380 amino acids to
about 500 amino acids, about 380 amino acids to about 480 amino acids, about 380
amino acids to about 460 amino acids, about 380 amino acids to about 440 amino acids,
30 about 380 amino acids to about 420 amino acids, about 380 amino acids to about 400
amino acids, about 400 amino acids to about 3000 amino acids, about 400 amino acids to

[illegible]

amino acids, about 460 amino acids to about 2000 amino acids, about 460 amino acids to
about 1500 amino acids, about 460 amino acids to about 1000 amino acids, about 460
amino acids to about 950 amino acids, about 460 amino acids to about 900 amino acids,
about 460 amino acids to about 850 amino acids, about 460 amino acids to about 800
5 amino acids, about 460 amino acids to about 750 amino acids, about 460 amino acids to
about 700 amino acids, about 460 amino acids to about 650 amino acids, about 460
amino acids to about 600 amino acids, about 460 amino acids to about 550 amino acids,
about 460 amino acids to about 500 amino acids, about 460 amino acids to about 480
amino acids, about 480 amino acids to about 3000 amino acids, about 480 amino acids to
10 about 2500 amino acids, about 480 amino acids to about 2000 amino acids, about 480
amino acids to about 1500 amino acids, about 480 amino acids to about 1000 amino
acids, about 480 amino acids to about 950 amino acids, about 480 amino acids to about
900 amino acids, about 480 amino acids to about 850 amino acids, about 480 amino acids
to about 800 amino acids, about 480 amino acids to about 750 amino acids, about 480
15 amino acids to about 700 amino acids, about 480 amino acids to about 650 amino acids,
about 480 amino acids to about 600 amino acids, about 480 amino acids to about 550
amino acids, about 480 amino acids to about 500 amino acids, about 500 amino acids to
about 3000 amino acids, about 500 amino acids to about 2500 amino acids, about 500
amino acids to about 2000 amino acids, about 500 amino acids to about 1500 amino
20 acids, about 500 amino acids to about 1000 amino acids, about 500 amino acids to about
950 amino acids, about 500 amino acids to about 900 amino acids, about 500 amino acids
to about 850 amino acids, about 500 amino acids to about 800 amino acids, about 500
amino acids to about 750 amino acids, about 500 amino acids to about 700 amino acids,
about 500 amino acids to about 650 amino acids, about 500 amino acids to about 600
25 amino acids, about 500 amino acids to about 550 amino acids, about 550 amino acids to
about 3000 amino acids, about 550 amino acids to about 2500 amino acids, about 550
amino acids to about 2000 amino acids, about 550 amino acids to about 1500 amino
acids, about 550 amino acids to about 1000 amino acids, about 550 amino acids to about
950 amino acids, about 550 amino acids to about 900 amino acids, about 550 amino acids
30 to about 850 amino acids, about 550 amino acids to about 800 amino acids, about 550
amino acids to about 750 amino acids, about 550 amino acids to about 700 amino acids,

[illegible]

amino acids to about 2000 amino acids, about 850 amino acids to about 1500 amino acids, about 850 amino acids to about 1000 amino acids, about 850 amino acids to about 950 amino acids, about 850 amino acids to about 900 amino acids, about 900 amino acids to about 3000 amino acids, about 900 amino acids to about 2500 amino acids, about 900 amino acids to about 2000 amino acids, about 900 amino acids to about 1500 amino acids, about 900 amino acids to about 1000 amino acids, about 900 amino acids to about 950 amino acids, about 950 amino acids to about 3000 amino acids, about 950 amino acids to about 2500 amino acids, about 950 amino acids to about 2000 amino acids, about 950 amino acids to about 1500 amino acids, about 950 amino acids to about 1000 amino acids, about 1000 amino acids to about 3000 amino acids, about 1000 amino acids to about 2500 amino acids, about 1000 amino acids to about 2000 amino acids, about 1000 amino acids to about 1500 amino acids, about 1500 amino acids to about 3000 amino acids, about 1500 amino acids to about 2500 amino acids, about 1500 amino acids to about 2000 amino acids, about 2000 amino acids to about 3000 amino acids, about 2000 amino acids to about 2500 amino acids, or about 2500 amino acids to about 3000 amino acids. Diagrams of an exemplary single-chain chimeric polypeptide provided herein are depicted in Figure 1.

In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) and the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein) directly abut each other. In some embodiments of any of the single-chain chimeric polypeptides described herein, the single-chain chimeric polypeptide further comprises a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) and the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein). In some embodiments of any of the single-chain chimeric polypeptides described herein, the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein) and the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) directly abut each other. In some embodiments of any of the single-chain

chimeric polypeptides described herein, the single-chain chimeric polypeptide further comprises a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein) and the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art).

In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) and the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) directly abut each other. In some embodiments of any of the single-chain chimeric polypeptides described herein, the single-chain chimeric polypeptide further comprises a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) and the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art).

In some embodiments of any of the single-chain chimeric polypeptides described herein, the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) and the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein) directly abut each other. In some embodiments of any of the single-chain chimeric polypeptides described herein, the single-chain chimeric polypeptide further comprises a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) and the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein or known in the art).

In some embodiments, a single-chain chimeric polypeptide can include a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to

QIVLTQSPAIMASASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLA
SGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRGG

GGSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQ
RPGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAV
YYCARYYDDHYCLDYWGQGTTTLTVSSSGTTNTVAAYNLTWKSTNFKTILEWEP
KPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDKVKQTYLARVFSYPAG
5 NVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR
NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQ
AVIPSRTVNRKSTDSPVECMGQEKGEFREVQLQQSGPELVKPGASVKMSCKASG
YTFTSYVIQWVKQKPGQGLEWIGSINPYNDYTKYNEKFKGKATLTSDKSSITAY
MEFSSLTSEDSALYYCARWGDGNYWGRGTTTLTVSSGGGGSGGGGSGGGGSDIE
10 MTQSPAIMSASLGERVTMTCTASSSVSSSYFHWYQKPGSSPKLCIYSTSNLASG
VPPRFGSGGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR (SEQ ID
NO: 1).

In some embodiments, a single-chain chimeric polypeptide is encoded by a
nucleic acid that includes a sequence that is at least 70% identical (e.g., at least 75%
15 identical, at least 80% identical, at least 85% identical, at least 90% identical, at least
95% identical, at least 99% identical, or 100% identical) to

CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAAGTGGTAT
CAGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAG
20 CTGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCACCAGCTA
CTCTTTAACCATTTCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCC
AGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAAT
CAATCGTGGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAG
CCAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCGGCCTCC
25 GTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCA
TTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAAC
CCTTCCCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTT
TAACCACTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACC
AGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTG
30 TTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGCTCCGGCACC
ACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGA

CAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGAT
 CTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACC
 GAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGG
 CTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGC
 5 GAGCCTCTCTACGAGAATTCCCCCGAATTACCCCTTATTTAGAGACCAATTT
 AGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTC
 ACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCC
 TCCGGGATGTGTTTCGGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCC
 AGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTG
 10 ACGTGGACAAAGGCGAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTC
 TCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAA
 GAAAAGGGCGAGTTCCGGGAGGTCCAGCTGCAGCAGAGCGGACCCGAATC
 GTGAAACCCGGTGCTTCCGTGAAAATGTCTTGTAAGGCCAGCGGATACACCT
 TCACCTCCTATGTGATCCAGTGGGTCAAACAGAAGCCCGGACAAGGTCTCGA
 15 GTGGATCGGCAGCATCAACCCTTACAACGACTATACCAAATACAACGAGAAG
 TTTAAGGGAAAGGCTACTTTAACCTCCGACAAAAGCTCCATCACAGCCTACA
 TGGAGTTCAGCTCTTTAACATCCGAGGACAGCGCTCTGTACTATTGCGCCCGG
 TGGGGCGACGGCAATTACTGGGGACGGGGCACAACACTGACCGTGAGCAGC
 GGAGGCGGAGGCTCCGGCGGAGGCGGATCTGGCGGTGGCGGCTCCGACATC
 20 GAGATGACCCAGTCCCCCGCTATCATGTCCGCCTCTTTAGGCGAGCGGGTCA
 CAATGACTTGTACAGCCTCCTCCAGCGTCTCCTCCTCCTACTTCCATTGGTAC
 CAACAGAAACCCGGAAGCTCCCCTAAACTGTGCATCTACAGCACCAGCAATC
 TCGCCAGCGGCGTGCCCCCTAGGTTTTCCGGAAGCGGAAGCACCAGCTACTC
 TTTAACCATCTCCTCCATGGAGGCTGAGGATGCCGCCACCTACTTTTGTACC
 25 AGTACCACCGGTCCCCCACCTTCGGAGGCGGCACCAAACCTGGAGACAAAGA
 GG (SEQ ID NO: 2).

In some embodiments, a single-chain chimeric polypeptide can include a
 sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical,
 at least 85% identical, at least 90% identical, at least 95% identical, at least 99%
 30 identical, or 100% identical) to

MKWVTFISLLFLFSSAYSQIVLTQSPAIMASAPGEKVTMTCSASSSVSYMNWYQQ
 KSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWS
 SNPFTFGSGTKLEINRGGGGSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCK
 ASGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQFKDKATLTDDKSS
 5 STAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSSGTTNTVAAY
 NLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVK
 DVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVG
 TKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNE
 FLIDVDKGENYCFSVQAVIPSRVTNRKSTDSPVECMGQEKGEFREVQLQQSGPEL
 10 VKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYNDYTKYNEKF
 KGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRGTTLTVSSGGG
 GSGGGGSGGGGSDIEMTQSPAIMASALGERVTMTCTASSSVSSSYFHWYQQKPG
 SSPKLCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGG
 GTKLETKR (SEQ ID NO: 3).

15 In some embodiments, a single-chain chimeric polypeptide is encoded by a
 nucleic acid that includes a sequence that is at least 70% identical (e.g., at least 75%
 identical, at least 80% identical, at least 85% identical, at least 90% identical, at least
 95% identical, at least 99% identical, or 100% identical) to

ATGAAGTGGGTGACCTTCATCAGCTTATTATTTTATTTCAGCTCCGCCTATTCC
 20 CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
 AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACTGGTAT
 CAGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAG
 CTGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCACCAGCTA
 CTCTTTAACCATTTCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCC
 25 AGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAAT
 CAATCGTGGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAG
 CCAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCGGCGCCTCC
 GTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCA
 TTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAAC
 30 CCTTCCCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTT
 TAACCACTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACC

AGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTG
TTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGCTCCGGCACC
ACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGA
CAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGAT
5 CTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACC
GAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGG
CTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGC
GAGCCTCTCTACGAGAATTCCCCGAATTCACCCCTTATTTAGAGACCAATTT
AGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTC
10 ACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCC
TCCGGGATGTGTTTCGGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCC
AGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTG
ACGTGGACAAAGGCGAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTC
TCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAA
15 GAAAAGGGCGAGTTCCGGGAGGTCCAGCTGCAGCAGAGCGGACCCGAATC
GTGAAACCCGGTGCTTCCGTGAAAATGTCTTGTAAGGCCAGCGGATACACCT
TCACCTCCTATGTGATCCAGTGGGTCAAACAGAAGCCCGGACAAGGTCTCGA
GTGGATCGGCAGCATCAACCCTTACAACGACTATACCAAATACAACGAGAAG
TTTAAGGGAAAGGCTACTTTAACCTCCGACAAAAGCTCCATCACAGCCTACA
20 TGGAGTTCAGCTCTTTAACATCCGAGGACAGCGCTCTGTACTATTGCGCCCGG
TGGGGCGACGGCAATTACTGGGGACGGGGCACAACACTGACCGTGAGCAGC
GGAGGCGGAGGCTCCGGCGGAGGCGGATCTGGCGGTGGCGGCTCCGACATC
GAGATGACCCAGTCCCCCGCTATCATGTCCGCCTCTTTAGGCGAGCGGGTCA
CAATGACTTGTACAGCCTCCTCCAGCGTCTCCTCCTCCTACTTCCATTGGTAC
25 CAACAGAAACCCGGAAGCTCCCCTAAACTGTGCATCTACAGCACCAGCAATC
TCGCCAGCGGCGTGCCCCCTAGGTTTTCCGGAAGCGGAAGCACCAGCTACTC
TTTAACCATCTCCTCCATGGAGGCTGAGGATGCCGCCACCTACTTTTGTACC
AGTACCACCGGTCCCCACCTTCGGAGGCGGCACCAAACCTGGAGACAAAGA
GG (SEQ ID NO: 4).

30 In some embodiments, a single-chain chimeric polypeptide can include a
sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical,

at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to

VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYN
DYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRG
5 TTLTVSSGGGGSGGGGSGGGGSDIEMTQSPAIMSASLGERVTMTCTASSSVSSSY
FHWYQQKPGSSPKLCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCH
QYHRSPTFGGGTKLETKRSGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTV
QISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG
EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDV
10 FGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRK
STDSPVECMGQEKGEFREQIVLTQSPAIMSASPGKVTMTCSASSSVSYMNWYQ
QKSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSSYSLTISGMEAEDAATYYCQQW
SSNPFTFGSGTKLEINRGGGGSGGGGSGGGGSQVQLQQSGAELARPGASVKMSC
KASGYTFTRYTMHWVKQRPQGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKS
15 SSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTTLTVSS (SEQ ID NO:
5).

In some embodiments, a single-chain chimeric polypeptide is encoded by a nucleic acid that includes a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least
20 95% identical, at least 99% identical, or 100% identical) to

GTGCAGCTGCAGCAGTCCGGACCCGAAGTGGTCAAGCCCGGTGCCTCCGTGA
AAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCAATGG
GTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAATCCCT
ACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTCTGAC
25 AAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACTTCTG
AGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTGGGGC
CGGGGAACACTTTAACAGTGAGCTCCGGCGGCGGCGGAAGCGGAGGTGGA
GGATCTGGCGGTGGAGGCAGCGACATCGAGATGACACAGTCCCCCGCTATCA
TGAGCGCCTCTTTAGGAGAACGTGTGACCATGACTTGTACAGCTTCCTCCAGC
30 GTGAGCAGCTCCTATTTCCACTGGTACCAGCAGAAACCCGGCTCCTCCCCTAA
ACTGTGTATCTACTCCACAAGCAATTTAGCTAGCGGCGTGCCTCCTCGTTTTA

GCGGCTCCGGCAGCACCTCTTACTCTTTAACCATTAGCTCTATGGAGGCCGAA
GATGCCGCCACATACTTTTGCCATCAGTACCACCGGTCCCCTACCTTTGGCGG
AGGCACAAAGCTGGAGACCAAGCGGAGCGGCACCACCAACACAGTGGCCGC
CTACAATCTGACTTGGAATCCACCAACTTCAAGACCATCCTCGAGTGGGAG
5 CCCAAGCCCGTTAATCAAGTTTATACCGTGCAGATTTCCACCAAGAGCGGCG
ACTGGAAATCCAAGTGCTTCTATACCACAGACACCGAGTGCGATCTCACCGA
CGAGATCGTCAAAGACGTGAAGCAGACATATTTAGCTAGGGTGTTCTCCTAC
CCCGCTGGAAACGTGGAGAGCACCGGATCCGCTGGAGAGCCTTTATACGAGA
ACTCCCCCGAATTCACCCCCTATCTGGAAACCAATTTAGGCCAGCCCACCATC
10 CAGAGCTTCGAACAAGTTGGCACAAAGGTGAACGTCACCGTCGAAGATGAG
AGGACTTTAGTGCGGAGGAACAATACATTTTTATCCTTACGTGACGTCTTCGG
CAAGGATTTAATCTACACACTGTATTACTGGAAGTCTAGCTCCTCCGGCAAGA
AGACCGCCAAGACCAATACCAACGAATTTTAATTGACGTGGACAAGGGCGA
GAACTACTGCTTCTCCGTGCAAGCTGTGATCCCCCTCCCGGACAGTGAACCGG
15 AAGTCCACCGACTCCCCCGTGGAGTGCATGGGCCAAGAGAAGGGAGAGTTTC
GTGAGCAGATCGTGCTGACCCAGTCCCCCGCTATTATGAGCGCTAGCCCCGG
TGAAAAGGTGACTATGACATGCAGCGCCAGCTCTTCCGTGAGCTACATGAAC
TGGTATCAGCAGAAGTCCGGCACCAGCCCTAAAAGGTGGATCTACGACACCA
GCAAGCTGGCCAGCGGCGTCCCCGCTCACTTTCGGGGCTCCGGCTCCGGAAC
20 AAGTACTCTCTGACCATCAGCGGCATGGAAGCCGAGGATGCCGCTACCTAT
TACTGTCAGCAGTGGAGCTCCAACCCCTTCACCTTTGGATCCGGCACCAAGCT
CGAGATTAATCGTGGAGGCGGAGGTAGCGGAGGAGGCGGATCCGGCGGTGG
AGGTAGCCAAGTTCAGCTCCAGCAAAGCGGCGCCGAACTCGCTCGGCCCCGGC
GCTTCCGTGAAGATGTCTTGTAAGGCCTCCGGCTATACCTTCACCCGGTACAC
25 AATGCACTGGGTCAAGCAACGGCCCCGGTCAAGGTTTAGAGTGGATTGGCTAT
ATCAACCCCTCCCGGGGCTATACCAACTACAACCAGAAGTTCAAGGACAAAG
CCACCCTCACCACCGACAAGTCCAGCAGCACCGCTTACATGCAGCTGAGCTC
TTTAACATCCGAGGATTCCGCCGTGTACTACTGCGCTCGGTACTACGACGATC
ATTACTGCCTCGATTACTGGGGCCAAGGTACCACCTTAACAGTCTCCTCC
30 (SEQ ID NO: 6).

In some embodiments, a single-chain chimeric polypeptide can include a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to

5 MKWVTFISLLFLFSSAYSVQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWV
KQKPGQGLEWIGSINPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSA
LYYCARWGDGNYWGRGTTLTVSSGGGGSGGGGSGGGGSDIEMTQSPAIMSASL
GERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYSTSNLASGVPPRFSGSGSTSY
SLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKRSGTTNTVAAYNLTWKSTN
10 FKILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDKVQTYL
ARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTV
EDERTLVRRNNTFLSLRDVFGKDLIYLYYWKSSSSGKKTAKTNTNEFLIDVDKG
ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFREQIVLTQSPAIMSASPGEK
VTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSL
15 TISGMEAEDAATYYCQWSSNPFTFGSGTKLEINRGGGGSGGGGSGGGGSQVQL
QQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPQGQLEWIGYINPSRGY
TNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWG
QGTTTLTVSS (SEQ ID NO: 7).

In some embodiments, a single-chain chimeric polypeptide is encoded by a
20 nucleic acid that includes a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to

ATGAAATGGGTCACCTTCATCTCTTTACTGTTTTTATTTAGCAGCGCCTACAG
CGTGCAGCTGCAGCAGTCCGGACCCGAAGTGGTCAAGCCCGGTGCCTCCGTG
25 AAAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCAATG
GGTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAATCCC
TACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTCTGA
CAAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACTTCT
GAGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTGGGG
30 CCGGGGAAGTACTTTAACAGTGAGCTCCGGCGGCGGCGGAAGCGGAGGTGG
AGGATCTGGCGGTGGAGGCAGCGACATCGAGATGACACAGTCCCCCGCTATC

ATGAGCGCCTCTTTAGGAGAACGTGTGACCATGACTTGTACAGCTTCCTCCAG
CGTGAGCAGCTCCTATTTCCACTGGTACCAGCAGAAACCCGGCTCCTCCCCTA
AACTGTGTATCTACTCCACAAGCAATTTAGCTAGCGGCGTGCCTCCTCGTTTT
AGCGGCTCCGGCAGCACCTCTTACTCTTTAACCATTAGCTCTATGGAGGCCGA
5 AGATGCCGCCACATACTTTTGCCATCAGTACCACCGGTCCCCTACCTTTGGCG
GAGGCACAAAGCTGGAGACCAAGCGGAGCGGCACCACCAACACAGTGGCCG
CCTACAATCTGACTTGGAATCCACCAACTTCAAGACCATCCTCGAGTGGGA
GCCCAAGCCCGTTAATCAAGTTTATACCGTGCAGATTTCCACCAAGAGCGGC
GACTGGAAATCCAAGTGCTTCTATACCACAGACACCGAGTGCGATCTCACCG
10 ACGAGATCGTCAAAGACGTGAAGCAGACATATTTAGCTAGGGTGTTCCTTA
CCCCGCTGGAAACGTGGAGAGCACC GGATCCGCTGGAGAGCCTTTATACGAG
AACTCCCCCGAATTCACCCCCTATCTGGAAACCAATTTAGGCCAGCCCACCAT
CCAGAGCTTCGAACAAGTTGGCACAAAGGTGAACGTCACCGTCGAAGATGAG
AGGACTTTAGTGCGGAGGAACAATACATTTTTATCCTTACGTGACGTCTTCGG
15 CAAGGATTTAATCTACACACTGTATTACTGGAAGTCTAGCTCCTCCGGCAAGA
AGACCGCCAAGACCAATACCAACGAATTTTAAATTGACGTGGACAAGGGCGA
GAACTACTGCTTCTCCGTGCAAGCTGTGATCCCCCTCCCGGACAGTGAACCGG
AAGTCCACCGACTCCCCCGTGGAGTGCATGGGCCAAGAGAAGGGGAGAGTTTC
GTGAGCAGATCGTGCTGACCCAGTCCCCCGCTATTATGAGCGCTAGCCCCGG
20 TGAAAAGGTGACTATGACATGCAGCGCCAGCTCTTCCGTGAGCTACATGAAC
TGGTATCAGCAGAAGTCCGGCACCAGCCCTAAAAGGTGGATCTACGACACCA
GCAAGCTGGCCAGCGGCGTCCCCGCTCACTTTCGGGGCTCCGGCTCCGGAAC
AAGCTACTCTCTGACCATCAGCGGCATGGAAGCCGAGGATGCCGCTACCTAT
TACTGTCAGCAGTGGAGCTCCAACCCCTTACCTTTGGATCCGGCACCAAGCT
25 CGAGATTAATCGTGGAGGCGGAGGTAGCGGAGGAGGCGGATCCGGCGGTGG
AGGTAGCCAAGTTCAGCTCCAGCAAAGCGGCGCCGAACCTCGCTCGGCCCGGC
GCTTCCGTGAAGATGTCTTGTAAGGCCTCCGGCTATACCTTCACCCGGTACAC
AATGCACTGGGTCAAGCAACGGCCCCGGTCAAGGTTTAGAGTGGATTGGCTAT
ATCAACCCCTCCCGGGGCTATACCAACTACAACCAGAAGTTCAAGGACAAAG
30 CCACCCTCACCACCGACAAGTCCAGCAGCACC GCTTACATGCAGCTGAGCTC
TTTAACATCCGAGGATTCCGCCGTGTACTACTGCGCTCGGTACTACGACGATC

ATTACTGCCTCGATTACTGGGGCCAAGGTACCACCTTAACAGTCTCCTCC
(SEQ ID NO: 8).

Non-limiting aspects of these chimeric polypeptides, nucleic acids, vectors, cells, and methods are described below, and can be used in any combination without limitation.

5 Additional aspects of these chimeric polypeptides, nucleic acids, vectors, cells, and methods are known in the art.

Tissue Factor

Human tissue factor is a 263 amino-acid transmembrane protein containing three
10 domains: (1) a 219-amino acid N-terminal extracellular domain (residues 1-219); (2) a 22-amino acid transmembrane domain (residues 220-242); and (3) a 21-amino acid cytoplasmic C-terminal tail (residues 242-263) ((UniProtKB Identifier Number: P13726). The cytoplasmic tail contains two phosphorylation sites at Ser253 and Ser258, and one S-palmitoylation site at Cys245. Deletion or mutation of the cytoplasmic domain was not
15 found to affect tissue factor coagulation activity. Tissue factor has one S-palmitoylation site in the intracellular domain of the protein at Cys245. The Cys245 is located at the amino acid terminus of the intracellular domain and close to the membrane surface. The tissue factor transmembrane domain is composed of a single-spanning α -helix.

The extracellular domain of tissue factor, composed of two fibronectin type III
20 domains, is connected to the transmembrane domain through a six-amino acid linker. This linker provides conformational flexibility to decouple the tissue factor extracellular domain from its transmembrane and cytoplasmic domains. Each tissue factor fibronectin type III module is composed of two overlapping β sheets with the top sheet domain containing three antiparallel β -strands and the bottom sheet containing four β -strands.
25 The β -strands are connected by β -loops between strand β A and β B, β C and β D, and β E and β F, all of which are conserved in conformation in the two modules. There are three short α -helix segments connecting the β -strands. A unique feature of tissue factor is a 17-amino acid β -hairpin between strand β 10 and strand β 11, which is not a common element of the fibronectin superfamily. The N-terminal domain also contains a 12 amino acid
30 loop between β 6F and β 7G that is not present in the C-terminal domain and is unique to tissue factor. Such a fibronectin type III domain structure is a feature of the

immunoglobulin-like family of protein folds and is conserved among a wide variety of extracellular proteins.

The zymogen FVII is rapidly converted to FVIIa by limited proteolysis once it binds to tissue to form the active tissue factor-FVIIa complex. The FVIIa, which
5 circulates as an enzyme at a concentration of approximately 0.1 nM (1% of plasma FVII), can also bind directly to tissue factor. The allosteric interaction between tissue factor and FVIIa on the tissue factor-FVIIa complex greatly increases the enzymatic activity of FVIIa: an approximate 20- to 100-fold increase in the rate of hydrolysis of small, chromogenic peptidyl substrates, and nearly a million-fold increase in the rate of
10 activation of the natural macromolecular substrates FIX and FX. In concert with allosteric activation of the active site of FVIIa upon binding to tissue factor, the formation of tissue factor-FVIIa complex on phospholipid bilayer (i.e., upon exposure of phosphatidyl-L-serine on membrane surfaces) increases the rate of FIX or FX activation, in a Ca^{2+} -dependent manner, an additional 1,000-fold. The roughly million-fold overall
15 increase in FX activation by tissue factor-FVIIa-phospholipid complex relative to free FVIIa is a critical regulatory point for the coagulation cascade.

FVII is a ~50 kDa, single-chain polypeptide consisting of 406 amino acid residues, with an N-terminal γ -carboxyglutamate-rich (GLA) domain, two epidermal growth factor-like domains (EGF1 and EGF2), and a C-terminal serine protease domain.
20 FVII is activated to FVIIa by a specific proteolytic cleavage of the Ile¹⁵⁴-Arg¹⁵² bond in the short linker region between the EGF2 and the protease domain. This cleavage results in the light and heavy chains being held together by a single disulfide bond of Cys¹³⁵ and Cys²⁶². FVIIa binds phospholipid membrane in a Ca^{2+} -dependent manner through its N-terminal GLA-domain. Immediately C-terminal to the GLA domain is an aromatic stack
25 and two EGF domains. The aromatic stack connects the GLA to EGF1 domain which binds a single Ca^{2+} ion. Occupancy of this Ca^{2+} -binding site increases FVIIa amidolytic activity and tissue factor association. The catalytic triad consist of His¹⁹³, Asp²⁴², and Ser³⁴⁴, and binding of a single Ca^{2+} ion within the FVIIa protease domain is critical for its catalytic activity. Proteolytic activation of FVII to FVIIa frees the newly formed amino
30 terminus at Ile¹⁵³ to fold back and be inserted into the activation pocket forming a salt bridge with the carboxylate of Asp³⁴³ to generate the oxyanion hole. Formation of this

salt bridge is critical for FVIIa activity. However, oxyanion hole formation does not occur in free FVIIa upon proteolytic activation. As a result, FVIIa circulates in a zymogen-like state that is poorly recognized by plasma protease inhibitors, allowing it to circulate with a half-life of approximately 90 minutes.

5 Tissue factor-mediated positioning of the FVIIa active site above the membrane surface is important for FVIIa towards cognate substrates. Free FVIIa adopts a stable, extended structure when bound to the membrane with its active site positioned $\sim 80\text{\AA}$ above the membrane surface. Upon FVIIa binding to tissue factor, the FVIIa active site is repositioned $\sim 6\text{\AA}$ closer to the membrane. This modulation may aid in a proper
10 alignment of the FVIIa catalytic triad with the target substrate cleavage site. Using GLA-domainless FVIIa, it has been shown that the active site was still positioned a similar distance above the membrane, demonstrating that tissue factor is able to fully support FVIIa active site positioning even in the absence of FVIIa-membrane interaction. Additional data showed that tissue factor supported full FVIIa proteolytic activity as long
15 as the tissue factor extracellular domain was tethered in some way to the membrane surface. However, raising the active site of FVIIa greater than $80\text{\AA}$ above the membrane surface greatly reduced the ability of the tissue factor-FVIIa complex to activate FX but did not diminish tissue factor-FVIIa amidolytic activity.

Alanine scanning mutagenesis has been used to assess the role of specific amino
20 acid side chains in the tissue factor extracellular domain for interaction with FVIIa (Gibbs et al., *Biochemistry* 33(47): 14003-14010, 1994; Schullek et al., *J Biol Chem* 269(30): 19399-19403, 1994). Alanine substitution identified a limited number of residue positions at which alanine replacements cause 5- to 10-fold lower affinity for FVIIa binding. Most of these residue side chains were found to be well-exposed to
25 solvent in the crystal structure, concordant with macromolecular ligand interaction. The FVIIa ligand-binding site is located over an extensive region at the boundary between the two modules. In the C-module, residues Arg¹³⁵ and Phe¹⁴⁰ located on the protruding B-C loop provide an independent contact with FVIIa. Leu¹³³ is located at the base of the fingerlike structure and packed into the cleft between the two modules. This provides
30 continuity to a major cluster of important binding residues consisting of Lys²⁰, Thr⁶⁰, Asp⁵⁸, and Ile²². Thr⁶⁰ is only partially solvent-exposed and may play a local structural

role rather than making a significant contact with ligand. The binding site extends onto the concave side of the intermodule angle involving Glu²⁴ and Gln¹¹⁰, and potentially the more distant residue Val²⁰⁷. The binding region extends from Asp⁵⁸ onto a convex surface area formed by Lys⁴⁸, Lys⁴⁶, Gln³⁷, Asp⁴⁴, and Trp⁴⁵. Trp⁴⁵ and Asp⁴⁴ do not
5 interact independently with FVIIa, indicating that the mutational effect at the Trp⁴⁵ position may reflect a structural importance of this side chain for the local packing of the adjacent Asp⁴⁴ and Gln³⁷ side chain. The interactive area further includes two surface-exposed aromatic residues, Phe⁷⁶ and Tyr⁷⁸, which form part of the hydrophobic cluster in the N-module.

10 The known physiologic substrates of tissue factor-FVIIa are FVII, FIX, and FX and certain proteinase-activated receptors. Mutational analysis has identified a number of residues that, when mutated, support full FVIIa amidolytic activity towards small peptidyl substrates but are deficient in their ability to support macromolecular substrate (i.e., FVII, FIX, and FX) activation (Ruf et al., *J Biol Chem* 267(31): 22206-22210, 1992;
15 Ruf et al., *J Biol Chem* 267(9): 6375-6381, 1992; Huang et al., *J Biol Chem* 271(36): 21752-21757, 1996; Kirchhofer et al., *Biochemistry* 39(25): 7380-7387, 2000). The tissue factor loop region at residues 159-165, and residues in or adjacent to this flexible loop have been shown to be critical for the proteolytic activity of the tissue factor-FVIIa complex. This defines the proposed substrate-binding exosite region of tissue factor that
20 is quite distant from the FVIIa active site. A substitution of the glycine residue by a marginally bulkier residue alanine, significantly impairs tissue factor-FVIIa proteolytic activity. This suggests that the flexibility afforded by glycine is critical for the loop of residues 159-165 for tissue factor macromolecular substrate recognition.

The residues Lys¹⁶⁵ and Lys¹⁶⁶ have also been demonstrated to be important for
25 substrate recognition and binding. Mutation of either of these residues to alanine results in a significant decrease in the tissue factor co-factor function. Lys¹⁶⁵ and Lys¹⁶⁶ face away from each other, with Lys¹⁶⁵ pointing towards FVIIa in most tissue factor-FVIIa structures, and Lys¹⁶⁶ pointing into the substrate binding exosite region in the crystal structure. Putative salt bridge formation between Lys¹⁶⁵ of and Gla³⁵ of FVIIa would
30 support the notion that tissue factor interaction with the GLA domain of FVIIa modulates substrate recognition. These results suggest that the C-terminal portion of the tissue

factor ectodomain directly interacts with the GLA-domain, the possible adjacent EGF1 domains, of FIX and FX, and that the presence of the FVIIa GLA-domain may modulate these interactions either directly or indirectly.

5 Soluble Tissue Factor Domain

In some embodiments of any of the single-chain chimeric polypeptides, compositions, or methods described herein, the soluble tissue factor domain can be a wildtype tissue factor polypeptide lacking the signal sequence, the transmembrane domain, and the intracellular domain. In some examples, the soluble tissue factor domain can be a tissue factor mutant, wherein a wildtype tissue factor polypeptide lacking the signal sequence, the transmembrane domain, and the intracellular domain, and has been further modified at selected amino acids. In some examples, the soluble tissue factor domain can be a soluble human tissue factor domain. In some examples, the soluble tissue factor domain can be a soluble mouse tissue factor domain. In some examples, the soluble tissue factor domain can be a soluble rat tissue factor domain. Non-limiting examples of soluble human tissue factor domains, a mouse soluble tissue factor domain, a rat soluble tissue factor domain, and mutant soluble tissue factor domains are shown below.

20 Exemplary Soluble Human Tissue Factor Domain (SEQ ID NO: 9)

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTD
TECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQ
PTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGK
KTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE

25

Exemplary Nucleic Acid Encoding Soluble Human Tissue Factor Domain (SEQ ID NO: 10)

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAGCACCA
ACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTAAACCAAGTTTACACC
30 GTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCTATACCAC
CGACACCGAGTGCGATCTCACCGATGAGATCGTGAAAGATGTGAAACAGACC

TACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGCACTGGTTC
 CGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTACCTCGAGA
 CCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGCACAAAGGT
 GAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAACAACACCTTT
 5 CTCAGCCTCCGGGATGTGTTCGGCAAAGATTTAATCTACACACTGTATTACTGG
 AAGTCCTCTTCTCCGGCAAGAAGACAGCTAAAACCAACACAAACGAGTTTT
 TAATCGACGTGGATAAAGGCGAAAACACTACTGTTTCAGCGTGCAAGCTGTGATC
 CCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGTTGAGTGCATGG
 GCCAAGAAAAGGGCGAGTTCCGGGAG

10

Exemplary Soluble Mouse Tissue Factor Domain (SEQ ID NO: 11)

agipekafnltwistdfktilwqpkptnytytvqisdrsrnwknkcfstt
 dtecdltdeivkdvtwayeakvlsvprnsvhgdgdlvihgeppftnap
 kflpyrdtnlgqpviqqfeqdgklnvvvkdsftlvrkngtftlrlqvfgk
 15 dlgyiityrkgsstgkktntntnefsidveegvsycffvqamifsrktnq
 nspgsstvcetqwkswflge

Exemplary Soluble Rat Tissue Factor Domain (SEQ ID NO: 12)

Agtppgkafnltwistdfktilwqpkptnytytvqisdrsrnwkykctgt
 20 ttecdltdeivkdvnwtyearvlsvpwrnsthgketlfgthgeppftna
 rkflpyrdtkigqpviqkyeqggtklkvtvkdsftlvrkngtftlrlqvfg
 ndlgyiltyrkdsstgrktnthtneflidvekgvsycffaqavifsrktn
 hkspesitkcteqwkswlge

25 Exemplary Mutant Soluble Human Tissue Factor Domain (SEQ ID NO: 96)

SGTTNTVAAYNLWKSTNFATALEWEPKPVNQVYTVQISTKSGDWKSKCFYTT
 DTECALTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNL
 GQPTIQSFEQVGTKVNVTVEDERTLVARNNTALSLRDVFGKDLYTYLWYKSSSS
 GKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEF

30 RE

Exemplary Mutant Soluble Human Tissue Factor Domain (SEQ ID NO: 97)

SGTTNTVAAYNLWKSTNFATALEWEPKPVNQVYTVQISTKSGDAKSKCFYTTD
 TECALTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLAENSPEFTPYLETNLG

OPTIQSFEQVGTKVNVTVEDERTLVARNNTALSLRDVFGKDLIYTLYYWKSSSSG
 KKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFR
 E

5 In some embodiments, a soluble tissue factor domain can include a sequence that is at least 70% identical, at least 72% identical, at least 74% identical, at least 76% identical, at least 78% identical, at least 80% identical, at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical,
 10 at least 99% identical, or 100% identical to SEQ ID NO: 9, 11, 12, 96, or 97. In some embodiments, a soluble tissue factor domain can include a sequence of SEQ ID NO: 9, 11, 12, 96, or 97 with one to twenty amino acids (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) amino acids removed from its N-terminus and/or one to twenty amino acids (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20)
 15 amino acids removed from its C-terminus.

 As can be appreciated in the art, one skilled in the art would understand that mutation of amino acids that are conserved between different mammalian species is more likely to decrease the activity and/or structural stability of the protein, while mutation of amino acids that are not conserved between different mammalian species is less likely to
 20 decrease the activity and/or structural stability of the protein.

 In some examples of any of the single-chain chimeric polypeptides described herein, the soluble tissue factor domain is not capable of binding to Factor VIIa. In some examples of any of the single-chain chimeric polypeptides described herein, the soluble tissue factor domain does not convert inactive Factor X into Factor Xa. In some
 25 embodiments of any of the single-chain chimeric polypeptides described herein, the single-chain chimeric polypeptide does not stimulate blood coagulation in a mammal.

 In some examples, the soluble tissue factor domain can be a soluble human tissue factor domain. In some embodiments, the soluble tissue factor domain can be a soluble mouse tissue factor domain. In some embodiments, the soluble tissue factor domain can
 30 be a soluble rat tissue factor domain.

In some examples, the soluble tissue factor domain does not include one or more (e.g., two, three, four, five, six or seven) of: a lysine at an amino acid position that corresponds to amino acid position 20 of mature wildtype human tissue factor protein; an isoleucine at an amino acid position that corresponds to amino acid position 22 of mature
5 wildtype human tissue factor protein; a tryptophan at an amino acid position that corresponds to amino acid position 45 of mature wildtype human tissue factor protein; an aspartic acid at an amino acid position that corresponds to amino acid position 58 of mature wildtype human tissue factor protein; a tyrosine at an amino acid position that corresponds to amino acid position 94 of mature wildtype human tissue factor protein; an
10 arginine at an amino acid position that corresponds to amino acid position 135 of mature wildtype human tissue factor protein; and a phenylalanine at an amino acid position that corresponds to amino acid position 140 of mature wildtype human tissue factor protein. In some embodiments, the mutant soluble tissue factor possesses the amino acid sequence of SEQ ID NO: 96 or SEQ ID NO: 97.

15 In some examples, the soluble tissue factor domain can be encoded by a nucleic acid including a sequence that is at least 70% identical, at least 72% identical, at least 74% identical, at least 76% identical, at least 78% identical, at least 80% identical, at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96%
20 identical, at least 98% identical, at least 99% identical, or 100% identical to SEQ ID NO: 10.

In some embodiments, the soluble tissue factor domain can have a total length of about 20 amino acids to about 220 amino acids, about 20 amino acids to about 215 amino acids, about 20 amino acids to about 210 amino acids, about 20 amino acids to about 205
25 amino acids, about 20 amino acids to about 200 amino acids, about 20 amino acids to about 195 amino acids, about 20 amino acids to about 190 amino acids, about 20 amino acids to about 185 amino acids, about 20 amino acids to about 180 amino acids, about 20 amino acids to about 175 amino acids, about 20 amino acids to about 170 amino acids, about 20 amino acids to about 165 amino acids, about 20 amino acids to about 160 amino
30 acids, about 20 amino acids to about 155 amino acids, about 20 amino acids to about 150 amino acids, about 20 amino acids to about 145 amino acids, about 20 amino acids to

[illegible]

[illegible]

amino acids to about 80 amino acids, about 50 amino acids to about 75 amino acids,
about 50 amino acids to about 70 amino acids, about 50 amino acids to about 60 amino
acids, about 60 amino acids to about 220 amino acids, about 60 amino acids to about 215
amino acids, about 60 amino acids to about 210 amino acids, about 60 amino acids to
5 about 205 amino acids, about 60 amino acids to about 200 amino acids, about 60 amino
acids to about 195 amino acids, about 60 amino acids to about 190 amino acids, about 60
amino acids to about 185 amino acids, about 60 amino acids to about 180 amino acids,
about 60 amino acids to about 175 amino acids, about 60 amino acids to about 170 amino
acids, about 60 amino acids to about 165 amino acids, about 60 amino acids to about 160
10 amino acids, about 60 amino acids to about 155 amino acids, about 60 amino acids to
about 150 amino acids, about 60 amino acids to about 145 amino acids, about 60 amino
acids to about 140 amino acids, about 60 amino acids to about 135 amino acids, about 60
amino acids to about 130 amino acids, about 60 amino acids to about 125 amino acids,
about 60 amino acids to about 120 amino acids, about 60 amino acids to about 115 amino
15 acids, about 60 amino acids to about 110 amino acids, about 60 amino acids to about 105
amino acids, about 60 amino acids to about 100 amino acids, about 60 amino acids to
about 95 amino acids, about 60 amino acids to about 90 amino acids, about 60 amino
acids to about 85 amino acids, about 60 amino acids to about 80 amino acids, about 60
amino acids to about 75 amino acids, about 60 amino acids to about 70 amino acids,
20 about 70 amino acids to about 220 amino acids, about 70 amino acids to about 215 amino
acids, about 70 amino acids to about 210 amino acids, about 70 amino acids to about 205
amino acids, about 70 amino acids to about 200 amino acids, about 70 amino acids to
about 195 amino acids, about 70 amino acids to about 190 amino acids, about 70 amino
acids to about 185 amino acids, about 70 amino acids to about 180 amino acids, about 70
25 amino acids to about 175 amino acids, about 70 amino acids to about 170 amino acids,
about 70 amino acids to about 165 amino acids, about 70 amino acids to about 160 amino
acids, about 70 amino acids to about 155 amino acids, about 70 amino acids to about 150
amino acids, about 70 amino acids to about 145 amino acids, about 70 amino acids to
about 140 amino acids, about 70 amino acids to about 135 amino acids, about 70 amino
30 acids to about 130 amino acids, about 70 amino acids to about 125 amino acids, about 70
amino acids to about 120 amino acids, about 70 amino acids to about 115 amino acids,

[illegible]

acids, about 90 amino acids to about 105 amino acids, about 90 amino acids to about 100 amino acids, about 100 amino acids to about 220 amino acids, about 100 amino acids to about 215 amino acids, about 100 amino acids to about 210 amino acids, about 100 amino acids to about 205 amino acids, about 100 amino acids to about 200 amino acids, 5 about 100 amino acids to about 195 amino acids, about 100 amino acids to about 190 amino acids, about 100 amino acids to about 185 amino acids, about 100 amino acids to about 180 amino acids, about 100 amino acids to about 175 amino acids, about 100 amino acids to about 170 amino acids, about 100 amino acids to about 165 amino acids, about 100 amino acids to about 160 amino acids, about 100 amino acids to about 155 10 amino acids, about 100 amino acids to about 150 amino acids, about 100 amino acids to about 145 amino acids, about 100 amino acids to about 140 amino acids, about 100 amino acids to about 135 amino acids, about 100 amino acids to about 130 amino acids, about 100 amino acids to about 125 amino acids, about 100 amino acids to about 120 amino acids, about 100 amino acids to about 115 amino acids, about 100 amino acids to 15 about 110 amino acids, about 110 amino acids to about 220 amino acids, about 110 amino acids to about 215 amino acids, about 110 amino acids to about 210 amino acids, about 110 amino acids to about 205 amino acids, about 110 amino acids to about 200 amino acids, about 110 amino acids to about 195 amino acids, about 110 amino acids to about 190 amino acids, about 110 amino acids to about 185 amino acids, about 110 amino acids 20 to about 180 amino acids, about 110 amino acids to about 175 amino acids, about 110 amino acids to about 170 amino acids, about 110 amino acids to about 165 amino acids, about 110 amino acids to about 160 amino acids, about 110 amino acids to about 155 amino acids, about 110 amino acids to about 150 amino acids, about 110 amino acids to about 145 amino acids, about 110 amino acids to about 140 amino acids, about 110 25 amino acids to about 135 amino acids, about 110 amino acids to about 130 amino acids, about 110 amino acids to about 125 amino acids, about 110 amino acids to about 120 amino acids, about 110 amino acids to about 115 amino acids, about 115 amino acids to about 220 amino acids, about 115 amino acids to about 215 amino acids, about 115 amino acids to about 210 amino acids, about 115 amino acids to about 205 amino acids, 30 about 115 amino acids to about 200 amino acids, about 115 amino acids to about 195 amino acids, about 115 amino acids to about 190 amino acids, about 115 amino acids to

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[illegible]

[illegible]

amino acids to about 195 amino acids, about 165 amino acids to about 190 amino acids,
about 165 amino acids to about 185 amino acids, about 165 amino acids to about 180
amino acids, about 165 amino acids to about 175 amino acids, about 165 amino acids to
about 170 amino acids, about 170 amino acids to about 220 amino acids, about 170
5 amino acids to about 215 amino acids, about 170 amino acids to about 210 amino acids,
about 170 amino acids to about 205 amino acids, about 170 amino acids to about 200
amino acids, about 170 amino acids to about 195 amino acids, about 170 amino acids to
about 190 amino acids, about 170 amino acids to about 185 amino acids, about 170
amino acids to about 180 amino acids, about 170 amino acids to about 175 amino acids,
10 about 175 amino acids to about 220 amino acids, about 175 amino acids to about 215
amino acids, about 175 amino acids to about 210 amino acids, about 175 amino acids to
about 205 amino acids, about 175 amino acids to about 200 amino acids, about 175
amino acids to about 195 amino acids, about 175 amino acids to about 190 amino acids,
about 175 amino acids to about 185 amino acids, about 175 amino acids to about 180
15 amino acids, about 180 amino acids to about 220 amino acids, about 180 amino acids to
about 215 amino acids, about 180 amino acids to about 210 amino acids, about 180
amino acids to about 205 amino acids, about 180 amino acids to about 200 amino acids,
about 180 amino acids to about 195 amino acids, about 180 amino acids to about 190
amino acids, about 180 amino acids to about 185 amino acids, about 185 amino acids to
20 about 220 amino acids, about 185 amino acids to about 215 amino acids, about 185
amino acids to about 210 amino acids, about 185 amino acids to about 205 amino acids,
about 185 amino acids to about 200 amino acids, about 185 amino acids to about 195
amino acids, about 185 amino acids to about 190 amino acids, about 190 amino acids to
about 220 amino acids, about 190 amino acids to about 215 amino acids, about 190
25 amino acids to about 210 amino acids, about 190 amino acids to about 205 amino acids,
about 190 amino acids to about 200 amino acids, about 190 amino acids to about 195
amino acids, about 195 amino acids to about 220 amino acids, about 195 amino acids to
about 215 amino acids, about 195 amino acids to about 210 amino acids, about 195
amino acids to about 205 amino acids, about 195 amino acids to about 200 amino acids,
30 about 200 amino acids to about 220 amino acids, about 200 amino acids to about 215
amino acids, about 200 amino acids to about 210 amino acids, about 200 amino acids to

about 205 amino acids, about 205 amino acids to about 220 amino acids, about 205 amino acids to about 215 amino acids, about 205 amino acids to about 210 amino acids, about 210 amino acids to about 220 amino acids, about 210 amino acids to about 215 amino acids, or about 215 amino acids to about 220 amino acids.

- 5 In some embodiments, the soluble tissue factor domain can comprise or consist of a soluble wildtype human tissue factor (or any sequence therefrom).

Linker Sequences

In some embodiments, the linker sequence can be a flexible linker sequence.

- 10 Non-limiting examples of linker sequences that can be used are described in Klein et al., Protein Engineering, Design & Selection Vol. 27, No. 10, pp. 325–330, 2014; Priyanka et al., Protein Sci., 2013 Feb; 22(2):153–167. In some examples, the linker sequence is a synthetic linker sequence.

- 15 In some embodiments of any of the single-chain chimeric polypeptides described herein can include one, two, three, four, five, six, seven, eight, nine, or ten linker sequence(s) (e.g., the same or different linker sequences, e.g., any of the exemplary linker sequences described herein or known in the art). In some embodiments of any of the single-chain chimeric polypeptides described herein can include one, two, three, four, five, six, seven, eight, nine, or ten linker sequence(s) (e.g., the same or different linker
20 sequences, e.g., any of the exemplary linker sequences described herein or known in the art).

- In some embodiments, a linker sequence can have a total length of 1 amino acid to about 100 amino acids, 1 amino acid to about 90 amino acids, 1 amino acid to about 80 amino acids, 1 amino acid to about 70 amino acids, 1 amino acid to about 60 amino acids,
25 1 amino acid to about 50 amino acids, 1 amino acid to about 45 amino acids, 1 amino acid to about 40 amino acids, 1 amino acid to about 35 amino acids, 1 amino acid to about 30 amino acids, 1 amino acid to about 25 amino acids, 1 amino acid to about 24 amino acids, 1 amino acid to about 22 amino acids, 1 amino acid to about 20 amino acids, 1 amino acid to about 18 amino acids, 1 amino acid to about 16 amino acids, 1 amino
30 acid to about 14 amino acids, 1 amino acid to about 12 amino acids, 1 amino acid to about 10 amino acids, 1 amino acid to about 8 amino acids, 1 amino acid to about 6

amino acids, 1 amino acid to about 4 amino acids, about 2 amino acids to about 100
amino acids, about 2 amino acids to about 90 amino acids, about 2 amino acids to about
80 amino acids, about 2 amino acids to about 70 amino acids, about 2 amino acids to
about 60 amino acids, about 2 amino acids to about 50 amino acids, about 2 amino acids
5 to about 45 amino acids, about 2 amino acids to about 40 amino acids, about 2 amino
acids to about 35 amino acids, about 2 amino acids to about 30 amino acids, about 2
amino acids to about 25 amino acids, about 2 amino acids to about 24 amino acids, about
2 amino acids to about 22 amino acids, about 2 amino acids to about 20 amino acids,
about 2 amino acids to about 18 amino acids, about 2 amino acids to about 16 amino
10 acids, about 2 amino acids to about 14 amino acids, about 2 amino acids to about 12
amino acids, about 2 amino acids to about 10 amino acids, about 2 amino acids to about 8
amino acids, about 2 amino acids to about 6 amino acids, about 2 amino acids to about 4
amino acids, about 4 amino acids to about 100 amino acids, about 4 amino acids to about
90 amino acids, about 4 amino acids to about 80 amino acids, about 4 amino acids to
15 about 70 amino acids, about 4 amino acids to about 60 amino acids, about 4 amino acids
to about 50 amino acids, about 4 amino acids to about 45 amino acids, about 4 amino
acids to about 40 amino acids, about 4 amino acids to about 35 amino acids, about 4
amino acids to about 30 amino acids, about 4 amino acids to about 25 amino acids, about
4 amino acids to about 24 amino acids, about 4 amino acids to about 22 amino acids,
20 about 4 amino acids to about 20 amino acids, about 4 amino acids to about 18 amino
acids, about 4 amino acids to about 16 amino acids, about 4 amino acids to about 14
amino acids, about 4 amino acids to about 12 amino acids, about 4 amino acids to about
10 amino acids, about 4 amino acids to about 8 amino acids, about 4 amino acids to about
6 amino acids, about 6 amino acids to about 100 amino acids, about 6 amino acids to
25 about 90 amino acids, about 6 amino acids to about 80 amino acids, about 6 amino acids
to about 70 amino acids, about 6 amino acids to about 60 amino acids, about 6 amino
acids to about 50 amino acids, about 6 amino acids to about 45 amino acids, about 6
amino acids to about 40 amino acids, about 6 amino acids to about 35 amino acids, about
6 amino acids to about 30 amino acids, about 6 amino acids to about 25 amino acids,
30 about 6 amino acids to about 24 amino acids, about 6 amino acids to about 22 amino
acids, about 6 amino acids to about 20 amino acids, about 6 amino acids to about 18

[illegible]

[illegible]

acids to about 25 amino acids, about 20 amino acids to about 24 amino acids, about 20
amino acids to about 22 amino acids, about 22 amino acids to about 100 amino acids,
about 22 amino acids to about 90 amino acids, about 22 amino acids to about 80 amino
acids, about 22 amino acids to about 70 amino acids, about 22 amino acids to about 60
5 amino acids, about 22 amino acids to about 50 amino acids, about 22 amino acids to
about 45 amino acids, about 22 amino acids to about 40 amino acids, about 22 amino
acids to about 35 amino acids, about 22 amino acids to about 30 amino acids, about 22
amino acids to about 25 amino acids, about 22 amino acids to about 24 amino acids,
about 25 amino acids to about 100 amino acids, about 25 amino acids to about 90 amino
10 acids, about 25 amino acids to about 80 amino acids, about 25 amino acids to about 70
amino acids, about 25 amino acids to about 60 amino acids, about 25 amino acids to
about 50 amino acids, about 25 amino acids to about 45 amino acids, about 25 amino
acids to about 40 amino acids, about 25 amino acids to about 35 amino acids, about 25
amino acids to about 30 amino acids, about 30 amino acids to about 100 amino acids,
15 about 30 amino acids to about 90 amino acids, about 30 amino acids to about 80 amino
acids, about 30 amino acids to about 70 amino acids, about 30 amino acids to about 60
amino acids, about 30 amino acids to about 50 amino acids, about 30 amino acids to
about 45 amino acids, about 30 amino acids to about 40 amino acids, about 30 amino
acids to about 35 amino acids, about 35 amino acids to about 100 amino acids, about 35
20 amino acids to about 90 amino acids, about 35 amino acids to about 80 amino acids,
about 35 amino acids to about 70 amino acids, about 35 amino acids to about 60 amino
acids, about 35 amino acids to about 50 amino acids, about 35 amino acids to about 45
amino acids, about 35 amino acids to about 40 amino acids, about 40 amino acids to
about 100 amino acids, about 40 amino acids to about 90 amino acids, about 40 amino
25 acids to about 80 amino acids, about 40 amino acids to about 70 amino acids, about 40
amino acids to about 60 amino acids, about 40 amino acids to about 50 amino acids,
about 40 amino acids to about 45 amino acids, about 45 amino acids to about 100 amino
acids, about 45 amino acids to about 90 amino acids, about 45 amino acids to about 80
amino acids, about 45 amino acids to about 70 amino acids, about 45 amino acids to
30 about 60 amino acids, about 45 amino acids to about 50 amino acids, about 50 amino
acids to about 100 amino acids, about 50 amino acids to about 90 amino acids, about 50

amino acids to about 80 amino acids, about 50 amino acids to about 70 amino acids, about 50 amino acids to about 60 amino acids, about 60 amino acids to about 100 amino acids, about 60 amino acids to about 90 amino acids, about 60 amino acids to about 80 amino acids, about 60 amino acids to about 70 amino acids, about 70 amino acids to about 100 amino acids, about 70 amino acids to about 90 amino acids, about 70 amino acids to about 80 amino acids, about 80 amino acids to about 100 amino acids, about 80 amino acids to about 90 amino acids, or about 90 amino acids to about 100 amino acids.

In some embodiments, the linker is rich in glycine (Gly or G) residues. In some embodiments, the linker is rich in serine (Ser or S) residues. In some embodiments, the linker is rich in glycine and serine residues. In some embodiments, the linker has one or more glycine-serine residue pairs (GS), e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GS pairs. In some embodiments, the linker has one or more Gly-Gly-Gly-Ser (GGGS) sequences, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GGGS sequences. In some embodiments, the linker has one or more Gly-Gly-Gly-Gly-Ser (GGGGS) sequences, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GGGGS sequences. In some embodiments, the linker has one or more Gly-Gly-Ser-Gly (GGSG) sequences, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GGSG sequences.

In some embodiments, the linker sequence can comprise or consist of GGGGSGGGGSGGGGS (SEQ ID NO: 13). In some embodiments, the linker sequence can be encoded by a nucleic acid comprising or consisting of: GGCGGTGGAGGATCCGGAGGAGGTGGCTCCGGCGGCGGAGGATCT (SEQ ID NO: 14). In some embodiments, the linker sequence can comprise or consist of: GGGSGGGS (SEQ ID NO: 15).

25 Target-Binding Domains

In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain, the second target-binding domain, and/or the additional one or more target-binding domains can be an antigen-binding domain (e.g., any of the exemplary antigen-binding domains described herein or known in the art), a soluble interleukin or cytokine protein (e.g., any of the exemplary soluble interleukin proteins or soluble cytokine proteins described herein), and a soluble interleukin or

cytokine receptor (e.g., any of the exemplary soluble interleukin receptors or soluble cytokine receptors described herein).

In some embodiments of any of the single-chain chimeric polypeptides described herein, one or more of the first target-binding domain (e.g., any of the exemplary first target binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary second target binding domains described herein or known in the art), and the one or more additional target binding domains can each, independently, bind specifically to a target selected from the group of: bind specifically to a target selected from the group consisting of: CD16a, CD28, CD3 (e.g., one or more of CD3 α , CD3 β , CD3 δ , CD3 ϵ , and CD3 γ), CD33, CD20, CD19, CD22, CD123, IL-1R, IL-1, VEGF, IL-6R, IL-4, IL-10, PDL-1, TIGIT, PD-1, TIM3, CTLA4, MICA, MICB, IL-6, IL-8, TNF α , CD26a, CD36, ULBP2, CD30, CD200, IGF-1R, MUC4AC, MUC5AC, Trop-2, CMET, EGFR, HER1, HER2, HER3, PSMA, CEA, B7H3, EPCAM, BCMA, P-cadherin, CEACAM5, a UL16-binding protein (e.g., ULBP1, ULBP2, ULBP3, ULBP4, ULBP5, and ULBP6), HLA-DR, DLL4, TYRO3, AXL, MER, CD122, CD155, PDGF-DD, a ligand of TGF- β receptor II (TGF- β RII), a ligand of TGF- β RIII, a ligand of DNAM1, a ligand of NKp46, a ligand of NKp44, a ligand of NKG2D, a ligand of NKp30, a ligand for a scMHCI, a ligand for a scMHCII, a ligand for a scTCR, a receptor for IL-1, a receptor for IL-2, a receptor for IL-3, a receptor for IL-7, a receptor for IL-8, a receptor for IL-10, a receptor for IL-12, a receptor for IL-15, a receptor for IL-17, a receptor for IL-18, a receptor for IL-21, a receptor for PDGF-DD, a receptor for stem cell factor (SCF), a receptor for stem cell-like tyrosine kinase 3 ligand (FLT3L), a receptor for MICA, a receptor for MICB, a receptor for a ULP16-binding protein, a receptor for CD155, a receptor for CD122, and a receptor for CD28.

In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain, the second target-binding domain, and/or the one or more additional target-binding domains can each independent have a total number of amino acids of about 5 amino acids to about 1000 amino acids, about 5 amino acids to about 950 amino acids, about 5 amino acids to about 900 amino acids, about 5 amino acids to about 850 amino acids, about 5 amino acids to about 800 amino acids, about 5 amino acids to about 750 amino acids, about 5 amino acids to about 700 amino acids,

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amino acids to about 400 amino acids, about 30 amino acids to about 350 amino acids,
about 30 amino acids to about 300 amino acids, about 30 amino acids to about 280 amino
acids, about 30 amino acids to about 260 amino acids, about 30 amino acids to about 240
amino acids, about 30 amino acids to about 220 amino acids, about 30 amino acids to
5 about 200 amino acids, about 30 amino acids to about 195 amino acids, about 30 amino
acids to about 190 amino acids, about 30 amino acids to about 185 amino acids, about 30
amino acids to about 180 amino acids, about 30 amino acids to about 175 amino acids,
about 30 amino acids to about 170 amino acids, about 30 amino acids to about 165 amino
acids, about 30 amino acids to about 160 amino acids, about 30 amino acids to about 155
10 amino acids, about 30 amino acids to about 150 amino acids, about 30 amino acids to
about 145 amino acids, about 30 amino acids to about 140 amino acids, about 30 amino
acids to about 135 amino acids, about 30 amino acids to about 130 amino acids, about 30
amino acids to about 125 amino acids, about 30 amino acids to about 120 amino acids,
about 30 amino acids to about 115 amino acids, about 30 amino acids to about 110 amino
15 acids, about 30 amino acids to about 105 amino acids, about 30 amino acids to about 100
amino acids, about 30 amino acids to about 95 amino acids, about 30 amino acids to
about 90 amino acids, about 30 amino acids to about 85 amino acids, about 30 amino
acids to about 80 amino acids, about 30 amino acids to about 75 amino acids, about 30
amino acids to about 70 amino acids, about 30 amino acids to about 65 amino acids,
20 about 30 amino acids to about 60 amino acids, about 30 amino acids to about 55 amino
acids, about 30 amino acids to about 50 amino acids, about 30 amino acids to about 45
amino acids, about 30 amino acids to about 40 amino acids, about 30 amino acids to
about 35 amino acids, about 35 amino acids to about 1000 amino acids, about 35 amino
acids to about 950 amino acids, about 35 amino acids to about 900 amino acids, about 35
25 amino acids to about 850 amino acids, about 35 amino acids to about 800 amino acids,
about 35 amino acids to about 750 amino acids, about 35 amino acids to about 700 amino
acids, about 35 amino acids to about 650 amino acids, about 35 amino acids to about 600
amino acids, about 35 amino acids to about 550 amino acids, about 35 amino acids to
about 500 amino acids, about 35 amino acids to about 450 amino acids, about 35 amino
30 acids to about 400 amino acids, about 35 amino acids to about 350 amino acids, about 35
amino acids to about 300 amino acids, about 35 amino acids to about 280 amino acids,

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[illegible]

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[illegible]

amino acids to about 850 amino acids, about 65 amino acids to about 800 amino acids,
about 65 amino acids to about 750 amino acids, about 65 amino acids to about 700 amino
acids, about 65 amino acids to about 650 amino acids, about 65 amino acids to about 600
amino acids, about 65 amino acids to about 550 amino acids, about 65 amino acids to
5 about 500 amino acids, about 65 amino acids to about 450 amino acids, about 65 amino
acids to about 400 amino acids, about 65 amino acids to about 350 amino acids, about 65
amino acids to about 300 amino acids, about 65 amino acids to about 280 amino acids,
about 65 amino acids to about 260 amino acids, about 65 amino acids to about 240 amino
acids, about 65 amino acids to about 220 amino acids, about 65 amino acids to about 200
10 amino acids, about 65 amino acids to about 195 amino acids, about 65 amino acids to
about 190 amino acids, about 65 amino acids to about 185 amino acids, about 65 amino
acids to about 180 amino acids, about 65 amino acids to about 175 amino acids, about 65
amino acids to about 170 amino acids, about 65 amino acids to about 165 amino acids,
about 65 amino acids to about 160 amino acids, about 65 amino acids to about 155 amino
15 acids, about 65 amino acids to about 150 amino acids, about 65 amino acids to about 145
amino acids, about 65 amino acids to about 140 amino acids, about 65 amino acids to
about 135 amino acids, about 65 amino acids to about 130 amino acids, about 65 amino
acids to about 125 amino acids, about 65 amino acids to about 120 amino acids, about 65
amino acids to about 115 amino acids, about 65 amino acids to about 110 amino acids,
20 about 65 amino acids to about 105 amino acids, about 65 amino acids to about 100 amino
acids, about 65 amino acids to about 95 amino acids, about 65 amino acids to about 90
amino acids, about 65 amino acids to about 85 amino acids, about 65 amino acids to
about 80 amino acids, about 65 amino acids to about 75 amino acids, about 65 amino
acids to about 70 amino acids, about 70 amino acids to about 1000 amino acids, about 70
25 amino acids to about 950 amino acids, about 70 amino acids to about 900 amino acids,
about 70 amino acids to about 850 amino acids, about 70 amino acids to about 800 amino
acids, about 70 amino acids to about 750 amino acids, about 70 amino acids to about 700
amino acids, about 70 amino acids to about 650 amino acids, about 70 amino acids to
about 600 amino acids, about 70 amino acids to about 550 amino acids, about 70 amino
30 acids to about 500 amino acids, about 70 amino acids to about 450 amino acids, about 70
amino acids to about 400 amino acids, about 70 amino acids to about 350 amino acids,

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amino acids to about 900 amino acids, about 105 amino acids to about 850 amino acids,
about 105 amino acids to about 800 amino acids, about 105 amino acids to about 750
amino acids, about 105 amino acids to about 700 amino acids, about 105 amino acids to
about 650 amino acids, about 105 amino acids to about 600 amino acids, about 105
5 amino acids to about 550 amino acids, about 105 amino acids to about 500 amino acids,
about 105 amino acids to about 450 amino acids, about 105 amino acids to about 400
amino acids, about 105 amino acids to about 350 amino acids, about 105 amino acids to
about 300 amino acids, about 105 amino acids to about 280 amino acids, about 105
amino acids to about 260 amino acids, about 105 amino acids to about 240 amino acids,
10 about 105 amino acids to about 220 amino acids, about 105 amino acids to about 200
amino acids, about 105 amino acids to about 195 amino acids, about 105 amino acids to
about 190 amino acids, about 105 amino acids to about 185 amino acids, about 105
amino acids to about 180 amino acids, about 105 amino acids to about 175 amino acids,
about 105 amino acids to about 170 amino acids, about 105 amino acids to about 165
15 amino acids, about 105 amino acids to about 160 amino acids, about 105 amino acids to
about 155 amino acids, about 105 amino acids to about 150 amino acids, about 105
amino acids to about 145 amino acids, about 105 amino acids to about 140 amino acids,
about 105 amino acids to about 135 amino acids, about 105 amino acids to about 130
amino acids, about 105 amino acids to about 125 amino acids, about 105 amino acids to
20 about 120 amino acids, about 105 amino acids to about 115 amino acids, about 105
amino acids to about 110 amino acids, about 110 amino acids to about 1000 amino acids,
about 110 amino acids to about 950 amino acids, about 110 amino acids to about 900
amino acids, about 110 amino acids to about 850 amino acids, about 110 amino acids to
about 800 amino acids, about 110 amino acids to about 750 amino acids, about 110
25 amino acids to about 700 amino acids, about 110 amino acids to about 650 amino acids,
about 110 amino acids to about 600 amino acids, about 110 amino acids to about 550
amino acids, about 110 amino acids to about 500 amino acids, about 110 amino acids to
about 450 amino acids, about 110 amino acids to about 400 amino acids, about 110
amino acids to about 350 amino acids, about 110 amino acids to about 300 amino acids,
30 about 110 amino acids to about 280 amino acids, about 110 amino acids to about 260
amino acids, about 110 amino acids to about 240 amino acids, about 110 amino acids to

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amino acids to about 700 amino acids, about 135 amino acids to about 650 amino acids,
about 135 amino acids to about 600 amino acids, about 135 amino acids to about 550
amino acids, about 135 amino acids to about 500 amino acids, about 135 amino acids to
about 450 amino acids, about 135 amino acids to about 400 amino acids, about 135
5 amino acids to about 350 amino acids, about 135 amino acids to about 300 amino acids,
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amino acids to about 195 amino acids, about 135 amino acids to about 190 amino acids,
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amino acids, about 140 amino acids to about 160 amino acids, about 140 amino acids to

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amino acids to about 195 amino acids, about 170 amino acids to about 190 amino acids,
about 170 amino acids to about 185 amino acids, about 170 amino acids to about 180
amino acids, about 170 amino acids to about 175 amino acids, about 175 amino acids to
about 1000 amino acids, about 175 amino acids to about 950 amino acids, about 175
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amino acids, about 175 amino acids to about 700 amino acids, about 175 amino acids to
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amino acids to about 700 amino acids, about 180 amino acids to about 650 amino acids,
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about 450 amino acids, about 180 amino acids to about 400 amino acids, about 180
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amino acids to about 195 amino acids, about 180 amino acids to about 190 amino acids,
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amino acids, about 185 amino acids to about 950 amino acids, about 185 amino acids to

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amino acids to about 850 amino acids, about 350 amino acids to about 800 amino acids,
about 350 amino acids to about 750 amino acids, about 350 amino acids to about 700
amino acids, about 350 amino acids to about 650 amino acids, about 350 amino acids to
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amino acids, about 600 amino acids to about 950 amino acids, about 600 amino acids to

about 900 amino acids, about 600 amino acids to about 850 amino acids, about 600 amino acids to about 800 amino acids, about 600 amino acids to about 750 amino acids, about 600 amino acids to about 700 amino acids, about 600 amino acids to about 650 amino acids, about 650 amino acids to about 1000 amino acids, about 650 amino acids to about 950 amino acids, about 650 amino acids to about 900 amino acids, about 650 amino acids to about 850 amino acids, about 650 amino acids to about 800 amino acids, about 650 amino acids to about 750 amino acids, about 650 amino acids to about 700 amino acids, about 700 amino acids to about 1000 amino acids, about 700 amino acids to about 950 amino acids, about 700 amino acids to about 900 amino acids, about 700 amino acids to about 850 amino acids, about 700 amino acids to about 800 amino acids, about 700 amino acids to about 750 amino acids, about 750 amino acids to about 1000 amino acids, about 750 amino acids to about 950 amino acids, about 750 amino acids to about 900 amino acids, about 750 amino acids to about 850 amino acids, about 750 amino acids to about 800 amino acids, about 800 amino acids to about 1000 amino acids, about 800 amino acids to about 950 amino acids, about 800 amino acids to about 900 amino acids, about 800 amino acids to about 850 amino acids, about 850 amino acids to about 1000 amino acids, about 850 amino acids to about 950 amino acids, about 850 amino acids to about 900 amino acids, about 900 amino acids to about 1000 amino acids, about 900 amino acids to about 950 amino acids, or about 950 amino acids to about 1000 amino acids.

Any of the target-binding domains described herein can bind to its target with a dissociation equilibrium constant (K_D) of less than 1×10^{-7} M, less than 1×10^{-8} M, less than 1×10^{-9} M, less than 1×10^{-10} M, less than 1×10^{-11} M, less than 1×10^{-12} M, or less than 1×10^{-13} M. In some embodiments, the antigen-binding protein construct provided herein can bind to an identifying antigen with a K_D of about 1×10^{-3} M to about 1×10^{-5} M, about 1×10^{-4} M to about 1×10^{-6} M, about 1×10^{-5} M to about 1×10^{-7} M, about 1×10^{-6} M to about 1×10^{-8} M, about 1×10^{-7} M to about 1×10^{-9} M, about 1×10^{-8} M to about 1×10^{-10} M, or about 1×10^{-9} M to about 1×10^{-11} M (inclusive).

Any of the target-binding domains described herein can bind to its target with a K_D of between about 1 pM to about 30 nM (e.g., about 1 pM to about 25 nM, about 1 pM to about 20 nM, about 1 pM to about 15 nM, about 1 pM to about 10 nM, about 1 pM to

about 5 nM, about 1 pM to about 2 nM, about 1 pM to about 1 nM, about 1 pM to about 950 pM, about 1 pM to about 900 pM, about 1 pM to about 850 pM, about 1 pM to about 800 pM, about 1 pM to about 750 pM, about 1 pM to about 700 pM, about 1 pM to about 650 pM, about 1 pM to about 600 pM, about 1 pM to about 550 pM, about 1 pM to about 500 pM, about 1 pM to about 450 pM, about 1 pM to about 400 pM, about 1 pM to about 350 pM, about 1 pM to about 300 pM, about 1 pM to about 250 pM, about 1 pM to about 200 pM, about 1 pM to about 150 pM, about 1 pM to about 100 pM, about 1 pM to about 90 pM, about 1 pM to about 80 pM, about 1 pM to about 70 pM, about 1 pM to about 60 pM, about 1 pM to about 50 pM, about 1 pM to about 40 pM, about 1 pM to about 30 pM, about 1 pM to about 20 pM, about 1 pM to about 10 pM, about 1 pM to about 5 pM, about 1 pM to about 4 pM, about 1 pM to about 3 pM, about 1 pM to about 2 pM, about 2 pM to about 30 nM, about 2 pM to about 25 nM, about 2 pM to about 20 nM, about 2 pM to about 15 nM, about 2 pM to about 10 nM, about 2 pM to about 5 nM, about 2 pM to about 2 nM, about 2 pM to about 1 nM, about 2 pM to about 950 pM, about 2 pM to about 900 pM, about 2 pM to about 850 pM, about 2 pM to about 800 pM, about 2 pM to about 750 pM, about 2 pM to about 700 pM, about 2 pM to about 650 pM, about 2 pM to about 600 pM, about 2 pM to about 550 pM, about 2 pM to about 500 pM, about 2 pM to about 450 pM, about 2 pM to about 400 pM, about 2 pM to about 350 pM, about 2 pM to about 300 pM, about 2 pM to about 250 pM, about 2 pM to about 200 pM, about 2 pM to about 150 pM, about 2 pM to about 100 pM, about 2 pM to about 90 pM, about 2 pM to about 80 pM, about 2 pM to about 70 pM, about 2 pM to about 60 pM, about 2 pM to about 50 pM, about 2 pM to about 40 pM, about 2 pM to about 30 pM, about 2 pM to about 20 pM, about 2 pM to about 10 pM, about 2 pM to about 5 pM, about 2 pM to about 4 pM, about 2 pM to about 3 pM, about 5 pM to about 30 nM, about 5 pM to about 25 nM, about 5 pM to about 20 nM, about 5 pM to about 15 nM, about 5 pM to about 10 nM, about 5 pM to about 5 nM, about 5 pM to about 2 nM, about 5 pM to about 1 nM, about 5 pM to about 950 pM, about 5 pM to about 900 pM, about 5 pM to about 850 pM, about 5 pM to about 800 pM, about 5 pM to about 750 pM, about 5 pM to about 700 pM, about 5 pM to about 650 pM, about 5 pM to about 600 pM, about 5 pM to about 550 pM, about 5 pM to about 500 pM, about 5 pM to about 450 pM, about 5 pM to about 400 pM, about 5 pM to about 350 pM, about 5 pM to about 300 pM, about 5 pM to about 250 pM,

about 5 pM to about 200 pM, about 5 pM to about 150 pM, about 5 pM to about 100 pM,
about 5 pM to about 90 pM, about 5 pM to about 80 pM, about 5 pM to about 70 pM,
about 5 pM to about 60 pM, about 5 pM to about 50 pM, about 5 pM to about 40 pM,
about 5 pM to about 30 pM, about 5 pM to about 20 pM, about 5 pM to about 10 pM,
5 about 10 pM to about 30 nM, about 10 pM to about 25 nM, about 10 pM to about 20 nM,
about 10 pM to about 15 nM, about 10 pM to about 10 nM, about 10 pM to about 5 nM,
about 10 pM to about 2 nM, about 10 pM to about 1 nM, about 10 pM to about 950 pM,
about 10 pM to about 900 pM, about 10 pM to about 850 pM, about 10 pM to about 800
pM, about 10 pM to about 750 pM, about 10 pM to about 700 pM, about 10 pM to about
10 650 pM, about 10 pM to about 600 pM, about 10 pM to about 550 pM, about 10 pM to
about 500 pM, about 10 pM to about 450 pM, about 10 pM to about 400 pM, about 10
pM to about 350 pM, about 10 pM to about 300 pM, about 10 pM to about 250 pM, about
10 pM to about 200 pM, about 10 pM to about 150 pM, about 10 pM to about 100 pM,
about 10 pM to about 90 pM, about 10 pM to about 80 pM, about 10 pM to about 70 pM,
15 about 10 pM to about 60 pM, about 10 pM to about 50 pM, about 10 pM to about 40 pM,
about 10 pM to about 30 pM, about 10 pM to about 20 pM, about 15 pM to about 30 nM,
about 15 pM to about 25 nM, about 15 pM to about 20 nM, about 15 pM to about 15 nM,
about 15 pM to about 10 nM, about 15 pM to about 5 nM, about 15 pM to about 2 nM,
about 15 pM to about 1 nM, about 15 pM to about 950 pM, about 15 pM to about 900
20 pM, about 15 pM to about 850 pM, about 15 pM to about 800 pM, about 15 pM to about
750 pM, about 15 pM to about 700 pM, about 15 pM to about 650 pM, about 15 pM to
about 600 pM, about 15 pM to about 550 pM, about 15 pM to about 500 pM, about 15
pM to about 450 pM, about 15 pM to about 400 pM, about 15 pM to about 350 pM, about
15 pM to about 300 pM, about 15 pM to about 250 pM, about 15 pM to about 200 pM,
25 about 15 pM to about 150 pM, about 15 pM to about 100 pM, about 15 pM to about 90
pM, about 15 pM to about 80 pM, about 15 pM to about 70 pM, about 15 pM to about 60
pM, about 15 pM to about 50 pM, about 15 pM to about 40 pM, about 15 pM to about 30
pM, about 15 pM to about 20 pM, about 20 pM to about 30 nM, about 20 pM to about 25
nM, about 20 pM to about 20 nM, about 20 pM to about 15 nM, about 20 pM to about 10
30 nM, about 20 pM to about 5 nM, about 20 pM to about 2 nM, about 20 pM to about 1
nM, about 20 pM to about 950 pM, about 20 pM to about 900 pM, about 20 pM to about

850 pM, about 20 pM to about 800 pM, about 20 pM to about 750 pM, about 20 pM to about 700 pM, about 20 pM to about 650 pM, about 20 pM to about 600 pM, about 20 pM to about 550 pM, about 20 pM to about 500 pM, about 20 pM to about 450 pM, about 20 pM to about 400 pM, about 20 pM to about 350 pM, about 20 pM to about 300 pM, about 20 pM to about 250 pM, about 20 pM to about 20 pM, about 200 pM to about 150 pM, about 20 pM to about 100 pM, about 20 pM to about 90 pM, about 20 pM to about 80 pM, about 20 pM to about 70 pM, about 20 pM to about 60 pM, about 20 pM to about 50 pM, about 20 pM to about 40 pM, about 20 pM to about 30 pM, about 30 pM to about 30 nM, about 30 pM to about 25 nM, about 30 pM to about 30 nM, about 30 pM to about 15 nM, about 30 pM to about 10 nM, about 30 pM to about 5 nM, about 30 pM to about 2 nM, about 30 pM to about 1 nM, about 30 pM to about 950 pM, about 30 pM to about 900 pM, about 30 pM to about 850 pM, about 30 pM to about 800 pM, about 30 pM to about 750 pM, about 30 pM to about 700 pM, about 30 pM to about 650 pM, about 30 pM to about 600 pM, about 30 pM to about 550 pM, about 30 pM to about 500 pM, about 30 pM to about 450 pM, about 30 pM to about 400 pM, about 30 pM to about 350 pM, about 30 pM to about 300 pM, about 30 pM to about 250 pM, about 30 pM to about 200 pM, about 30 pM to about 150 pM, about 30 pM to about 100 pM, about 30 pM to about 90 pM, about 30 pM to about 80 pM, about 30 pM to about 70 pM, about 30 pM to about 60 pM, about 30 pM to about 50 pM, about 30 pM to about 40 pM, about 40 pM to about 30 nM, about 40 pM to about 25 nM, about 40 pM to about 30 nM, about 40 pM to about 15 nM, about 40 pM to about 10 nM, about 40 pM to about 5 nM, about 40 pM to about 2 nM, about 40 pM to about 1 nM, about 40 pM to about 950 pM, about 40 pM to about 900 pM, about 40 pM to about 850 pM, about 40 pM to about 800 pM, about 40 pM to about 750 pM, about 40 pM to about 700 pM, about 40 pM to about 650 pM, about 40 pM to about 600 pM, about 40 pM to about 550 pM, about 40 pM to about 500 pM, about 40 pM to about 450 pM, about 40 pM to about 400 pM, about 40 pM to about 350 pM, about 40 pM to about 300 pM, about 40 pM to about 250 pM, about 40 pM to about 200 pM, about 40 pM to about 150 pM, about 40 pM to about 100 pM, about 40 pM to about 90 pM, about 40 pM to about 80 pM, about 40 pM to about 70 pM, about 40 pM to about 60 pM, about 40 pM to about 50 pM, about 50 pM to about 30 nM, about 50 pM to about 25 nM, about 50 pM to about 30 nM, about 50 pM to about 15 nM, about 50 pM to about

10 nM, about 50 pM to about 5 nM, about 50 pM to about 2 nM, about 50 pM to about 1
nM, about 50 pM to about 950 pM, about 50 pM to about 900 pM, about 50 pM to about
850 pM, about 50 pM to about 800 pM, about 50 pM to about 750 pM, about 50 pM to
about 700 pM, about 50 pM to about 650 pM, about 50 pM to about 600 pM, about 50
5 pM to about 550 pM, about 50 pM to about 500 pM, about 50 pM to about 450 pM, about
50 pM to about 400 pM, about 50 pM to about 350 pM, about 50 pM to about 300 pM,
about 50 pM to about 250 pM, about 50 pM to about 200 pM, about 50 pM to about 150
pM, about 50 pM to about 100 pM, about 50 pM to about 90 pM, about 50 pM to about
80 pM, about 50 pM to about 70 pM, about 50 pM to about 60 pM, about 60 pM to about
10 30 nM, about 60 pM to about 25 nM, about 60 pM to about 30 nM, about 60 pM to about
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900 pM, about 60 pM to about 850 pM, about 60 pM to about 800 pM, about 60 pM to
about 750 pM, about 60 pM to about 700 pM, about 60 pM to about 650 pM, about 60
15 pM to about 600 pM, about 60 pM to about 550 pM, about 60 pM to about 500 pM, about
60 pM to about 450 pM, about 60 pM to about 400 pM, about 60 pM to about 350 pM,
about 60 pM to about 300 pM, about 60 pM to about 250 pM, about 60 pM to about 200
pM, about 60 pM to about 150 pM, about 60 pM to about 100 pM, about 60 pM to about
90 pM, about 60 pM to about 80 pM, about 60 pM to about 70 pM, about 70 pM to about
20 30 nM, about 70 pM to about 25 nM, about 70 pM to about 30 nM, about 70 pM to about
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pM, about 70 pM to about 150 pM, about 70 pM to about 100 pM, about 70 pM to about
90 pM, about 70 pM to about 80 pM, about 80 pM to about 30 nM, about 80 pM to about
30 25 nM, about 80 pM to about 30 nM, about 80 pM to about 15 nM, about 80 pM to about
10 nM, about 80 pM to about 5 nM, about 80 pM to about 2 nM, about 80 pM to about 1

nM, about 80 pM to about 950 pM, about 80 pM to about 900 pM, about 80 pM to about 850 pM, about 80 pM to about 800 pM, about 80 pM to about 750 pM, about 80 pM to about 700 pM, about 80 pM to about 650 pM, about 80 pM to about 600 pM, about 80 pM to about 550 pM, about 80 pM to about 500 pM, about 80 pM to about 450 pM, about 80 pM to about 400 pM, about 80 pM to about 350 pM, about 80 pM to about 300 pM, about 80 pM to about 250 pM, about 80 pM to about 200 pM, about 80 pM to about 150 pM, about 80 pM to about 100 pM, about 80 pM to about 90 pM, about 90 pM to about 30 nM, about 90 pM to about 25 nM, about 90 pM to about 30 nM, about 90 pM to about 15 nM, about 90 pM to about 10 nM, about 90 pM to about 5 nM, about 90 pM to about 2 nM, about 90 pM to about 1 nM, about 90 pM to about 950 pM, about 90 pM to about 900 pM, about 90 pM to about 850 pM, about 90 pM to about 800 pM, about 90 pM to about 750 pM, about 90 pM to about 700 pM, about 90 pM to about 650 pM, about 90 pM to about 600 pM, about 90 pM to about 550 pM, about 90 pM to about 500 pM, about 90 pM to about 450 pM, about 90 pM to about 400 pM, about 90 pM to about 350 pM, about 90 pM to about 300 pM, about 90 pM to about 250 pM, about 90 pM to about 200 pM, about 90 pM to about 150 pM, about 90 pM to about 100 pM, about 100 pM to about 30 nM, about 100 pM to about 25 nM, about 100 pM to about 30 nM, about 100 pM to about 15 nM, about 100 pM to about 10 nM, about 100 pM to about 5 nM, about 100 pM to about 2 nM, about 100 pM to about 1 nM, about 100 pM to about 950 pM, about 100 pM to about 900 pM, about 100 pM to about 850 pM, about 100 pM to about 800 pM, about 100 pM to about 750 pM, about 100 pM to about 700 pM, about 100 pM to about 650 pM, about 100 pM to about 600 pM, about 100 pM to about 550 pM, about 100 pM to about 500 pM, about 100 pM to about 450 pM, about 100 pM to about 400 pM, about 100 pM to about 350 pM, about 100 pM to about 300 pM, about 100 pM to about 250 pM, about 100 pM to about 200 pM, about 100 pM to about 150 pM, about 150 pM to about 30 nM, about 150 pM to about 25 nM, about 150 pM to about 30 nM, about 150 pM to about 15 nM, about 150 pM to about 10 nM, about 150 pM to about 5 nM, about 150 pM to about 2 nM, about 150 pM to about 1 nM, about 150 pM to about 950 pM, about 150 pM to about 900 pM, about 150 pM to about 850 pM, about 150 pM to about 800 pM, about 150 pM to about 750 pM, about 150 pM to about 700 pM, about 150 pM to about 650 pM, about 150 pM to about 600 pM, about 150 pM to about 550

pM, about 150 pM to about 500 pM, about 150 pM to about 450 pM, about 150 pM to about 400 pM, about 150 pM to about 350 pM, about 150 pM to about 300 pM, about 150 pM to about 250 pM, about 150 pM to about 200 pM, about 200 pM to about 30 nM, about 200 pM to about 25 nM, about 200 pM to about 30 nM, about 200 pM to about 15 nM, about 200 pM to about 10 nM, about 200 pM to about 5 nM, about 200 pM to about 2 nM, about 200 pM to about 1 nM, about 200 pM to about 950 pM, about 200 pM to about 900 pM, about 200 pM to about 850 pM, about 200 pM to about 800 pM, about 200 pM to about 750 pM, about 200 pM to about 700 pM, about 200 pM to about 650 pM, about 200 pM to about 600 pM, about 200 pM to about 550 pM, about 200 pM to about 500 pM, about 200 pM to about 450 pM, about 200 pM to about 400 pM, about 200 pM to about 350 pM, about 200 pM to about 300 pM, about 200 pM to about 250 pM, about 300 pM to about 30 nM, about 300 pM to about 25 nM, about 300 pM to about 30 nM, about 300 pM to about 15 nM, about 300 pM to about 10 nM, about 300 pM to about 5 nM, about 300 pM to about 2 nM, about 300 pM to about 1 nM, about 300 pM to about 950 pM, about 300 pM to about 900 pM, about 300 pM to about 850 pM, about 300 pM to about 800 pM, about 300 pM to about 750 pM, about 300 pM to about 700 pM, about 300 pM to about 650 pM, about 300 pM to about 600 pM, about 300 pM to about 550 pM, about 300 pM to about 500 pM, about 300 pM to about 450 pM, about 300 pM to about 400 pM, about 300 pM to about 350 pM, about 400 pM to about 30 nM, about 400 pM to about 25 nM, about 400 pM to about 30 nM, about 400 pM to about 15 nM, about 400 pM to about 10 nM, about 400 pM to about 5 nM, about 400 pM to about 2 nM, about 400 pM to about 1 nM, about 400 pM to about 950 pM, about 400 pM to about 900 pM, about 400 pM to about 850 pM, about 400 pM to about 800 pM, about 400 pM to about 750 pM, about 400 pM to about 700 pM, about 400 pM to about 650 pM, about 400 pM to about 600 pM, about 400 pM to about 550 pM, about 400 pM to about 500 pM, about 400 pM to about 450 pM, about 400 pM to about 400 pM, about 500 pM to about 30 nM, about 500 pM to about 25 nM, about 500 pM to about 30 nM, about 500 pM to about 15 nM, about 500 pM to about 10 nM, about 500 pM to about 5 nM, about 500 pM to about 2 nM, about 500 pM to about 1 nM, about 500 pM to about 950 pM, about 500 pM to about 900 pM, about 500 pM to about 850 pM, about 500 pM to about 800 pM, about 500 pM to about 750 pM, about 500 pM to about 700 pM, about 500 pM to about 650 pM, about 500 pM to about 600 pM, about

500 pM to about 550 pM, about 600 pM to about 30 nM, about 600 pM to about 25 nM, about 600 pM to about 30 nM, about 600 pM to about 15 nM, about 600 pM to about 10 nM, about 600 pM to about 5 nM, about 600 pM to about 2 nM, about 600 pM to about 1 nM, about 600 pM to about 950 pM, about 600 pM to about 900 pM, about 600 pM to about 850 pM, about 600 pM to about 800 pM, about 600 pM to about 750 pM, about 600 pM to about 700 pM, about 600 pM to about 650 pM, about 700 pM to about 30 nM, about 700 pM to about 25 nM, about 700 pM to about 30 nM, about 700 pM to about 15 nM, about 700 pM to about 10 nM, about 700 pM to about 5 nM, about 700 pM to about 2 nM, about 700 pM to about 1 nM, about 700 pM to about 950 pM, about 700 pM to about 900 pM, about 700 pM to about 850 pM, about 700 pM to about 800 pM, about 700 pM to about 750 pM, about 800 pM to about 30 nM, about 800 pM to about 25 nM, about 800 pM to about 30 nM, about 800 pM to about 15 nM, about 800 pM to about 10 nM, about 800 pM to about 5 nM, about 800 pM to about 2 nM, about 800 pM to about 1 nM, about 800 pM to about 950 pM, about 800 pM to about 900 pM, about 800 pM to about 850 pM, about 900 pM to about 30 nM, about 900 pM to about 25 nM, about 900 pM to about 30 nM, about 900 pM to about 15 nM, about 900 pM to about 10 nM, about 900 pM to about 5 nM, about 900 pM to about 2 nM, about 900 pM to about 1 nM, about 900 pM to about 950 pM, about 1 nM to about 30 nM, about 1 nM to about 25 nM, about 1 nM to about 20 nM, about 1 nM to about 15 nM, about 1 nM to about 10 nM, about 1 nM to about 5 nM, about 2 nM to about 30 nM, about 2 nM to about 25 nM, about 2 nM to about 20 nM, about 2 nM to about 15 nM, about 2 nM to about 10 nM, about 2 nM to about 5 nM, about 4 nM to about 30 nM, about 4 nM to about 25 nM, about 4 nM to about 20 nM, about 4 nM to about 15 nM, about 4 nM to about 10 nM, about 4 nM to about 5 nM, about 5 nM to about 30 nM, about 5 nM to about 25 nM, about 5 nM to about 20 nM, about 5 nM to about 15 nM, about 5 nM to about 10 nM, about 10 nM to about 30 nM, about 10 nM to about 25 nM, about 10 nM to about 20 nM, about 10 nM to about 15 nM, about 15 nM to about 30 nM, about 15 nM to about 25 nM, about 15 nM to about 20 nM, about 20 nM to about 30 nM, and about 20 nM to about 25 nM).

Any of the target-binding domains described herein can bind to its target with a K_D of between about 1 nM to about 10 nM (e.g., about 1 nM to about 9 nM, about 1 nM to about 8 nM, about 1 nM to about 7 nM, about 1 nM to about 6 nM, about 1 nM to

about 5 nM, about 1 nM to about 4 nM, about 1 nM to about 3 nM, about 1 nM to about 2 nM, about 2 nM to about 10 nM, about 2 nM to about 9 nM, about 2 nM to about 8 nM, about 2 nM to about 7 nM, about 2 nM to about 6 nM, about 2 nM to about 5 nM, about 2 nM to about 4 nM, about 2 nM to about 3 nM, about 3 nM to about 10 nM, about 3 nM to about 9 nM, about 3 nM to about 8 nM, about 3 nM to about 7 nM, about 3 nM to about 6 nM, about 3 nM to about 5 nM, about 3 nM to about 4 nM, about 4 nM to about 10 nM, about 4 nM to about 9 nM, about 4 nM to about 8 nM, about 4 nM to about 7 nM, about 4 nM to about 6 nM, about 4 nM to about 5 nM, about 5 nM to about 10 nM, about 5 nM to about 9 nM, about 5 nM to about 8 nM, about 5 nM to about 7 nM, about 5 nM to about 6 nM, about 6 nM to about 10 nM, about 6 nM to about 9 nM, about 6 nM to about 8 nM, about 6 nM to about 7 nM, about 7 nM to about 10 nM, about 7 nM to about 9 nM, about 7 nM to about 8 nM, about 8 nM to about 10 nM, about 8 nM to about 9 nM, and about 9 nM to about 10 nM).

A variety of different methods known in the art can be used to determine the K_D values of any of the antigen-binding protein constructs described herein (e.g., an electrophoretic mobility shift assay, a filter binding assay, surface plasmon resonance, and a biomolecular binding kinetics assay, etc.).

Antigen-Binding Domains

In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain and the second target-binding domain bind specifically to the same antigen. In some embodiments of these single-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain bind specifically to the same epitope. In some embodiments of these single-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain include the same amino acid sequence.

In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain and the second target-binding domain bind specifically to different antigens.

In some embodiments of any of the single-chain chimeric polypeptides described herein, one or both of the first target-binding domain and the second target-binding

domain is an antigen-binding domain. In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain and the second target-binding domain are each an antigen-binding domain.

In some embodiments of any of the single-chain chimeric polypeptides described
5 herein, the antigen-binding domain includes or is a scFv or a single domain antibody (e.g., a V_HH or a V_{NAR} domain).

In some examples, an antigen-binding domain (e.g., any of the antigen-binding domains described herein) can bind specifically to any one of CD16a (see, e.g., those described in U.S. Patent No. 9,035,026), CD28 (see, e.g., those described in U.S. Patent
10 No. 7,723,482), CD3 (see, e.g., those described in U.S. Patent No. 9,226,962), CD33 (see, e.g., those described in U.S. Patent No. 8,759,494), CD20 (see, e.g., those described in WO 2014/026054), CD19 (see, e.g., those described in U.S. Patent No. 9,701,758), CD22 (see, e.g., those described in WO 2003/104425), CD123 (see, e.g., those described in WO 2014/130635), IL-1R (see, e.g., those described in U.S. Patent No. 8,741,604), IL-
15 1 (see, e.g., those described in WO 2014/095808), VEGF (see, e.g., those described in U.S. Patent No. 9,090,684), IL-6R (see, e.g., those described in U.S. Patent No. 7,482,436), IL-4 (see, e.g., those described in U.S. Patent Application Publication No. 2012/0171197), IL-10 (see, e.g., those described in U.S. Patent Application Publication No. 2016/0340413), PDL-1 (see, e.g., those described in Drees et al., *Protein Express.*
20 *Purif.* 94:60-66, 2014), TIGIT (see, e.g., those described in U.S. Patent Application Publication No. 2017/0198042), PD-1 (see, e.g., those described in U.S. Patent No. 7,488,802), TIM3 (see, e.g., those described in U.S. Patent No. 8,552,156), CTLA4 (see, e.g., those described in WO 2012/120125), MICA (see, e.g., those described in WO 2016/154585), MICB (see, e.g., those described in U.S. Patent No. 8,753,640), IL-6 (see,
25 e.g., those described in Gejima et al., *Human Antibodies* 11(4):121-129, 2002), IL-8 (see, e.g., those described in U.S. Patent No. 6,117,980), TNF α (see, e.g., those described in Geng et al., *Immunol. Res.* 62(3):377-385, 2015), CD26a (see, e.g., those described in WO 2017/189526), CD36 (see, e.g., those described in U.S. Patent Application Publication No. 2015/0259429), ULBP2 (see, e.g., those described in U.S. Patent No.
30 9,273,136), CD30 (see, e.g., those described in Homach et al., *Scand. J. Immunol.* 48(5):497-501, 1998), CD200 (see, e.g., those described in U.S. Patent No. 9,085,623),

IGF-1R (see, e.g., those described in U.S. Patent Application Publication No. 2017/0051063), MUC4AC (see, e.g., those described in WO 2012/170470), MUC5AC (see, e.g., those described in U.S. Patent No. 9,238,084), Trop-2 (see, e.g., those described in WO 2013/068946), CMET (see, e.g., those described in Edwardraja et al., *Biotechnol. Bioeng.* 106(3):367-375, 2010), EGFR (see, e.g., those described in Akbari et al., *Protein Expr. Purif.* 127:8-15, 2016), HER1 (see, e.g., those described in U.S. Patent Application Publication No. 2013/0274446), HER2 (see, e.g., those described in Cao et al., *Biotechnol. Lett.* 37(7):1347-1354, 2015), HER3 (see, e.g., those described in U.S. Patent No. 9,505,843), PSMA (see, e.g., those described in Parker et al., *Protein Expr. Purif.* 89(2):136-145, 2013), CEA (see, e.g., those described in WO 1995/015341), B7H3 (see, e.g., those described in U.S. Patent No. 9,371,395), EPCAM (see, e.g., those described in WO 2014/159531), BCMA (see, e.g., those described in Smith et al., *Mol. Ther.* 26(6):1447-1456, 2018), P-cadherin (see, e.g., those described in U.S. Patent No. 7,452,537), CEACAM5 (see, e.g., those described in U.S. Patent No. 9,617,345), a UL16-binding protein (see, e.g., those described in WO 2017/083612), HLA-DR (see, e.g., Pistillo et al., *Exp. Clin. Immunogenet.* 14(2):123-130, 1997), DLL4 (see, e.g., those described in WO 2014/007513), TYRO3 (see, e.g., those described in WO 2016/166348), AXL (see, e.g., those described in WO 2012/175692), MER (see, e.g., those described in WO 2016/106221), CD122 (see, e.g., those described in U.S. Patent Application Publication No. 2016/0367664), CD155 (see, e.g., those described in WO 2017/149538), or PDGFDD (see, e.g., those described in U.S. Patent No. 9,441,034).

The antigen-binding domains present in any of the single-chain chimeric polypeptides described herein are each independently selected from the group consisting of: a VHH domain, a VNAR domain, and a scFv.

In some embodiments, the first target binding domain, the second target-binding domain, and/or one or more of the one or more additional antigen-binding domains can be an anti-CD3 scFv. In some embodiments, the anti-CD3 scFv can include a heavy chain variable domain including a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLEWIGYINP

SRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCL
DYWGQGTTTLTVSS (SEQ ID NO: 16) and/or a light chain variable domain including a
sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical,
at least 85% identical, at least 90% identical, at least 95% identical, at least 99%
5 identical, or 100% identical) to
QIVLTQSPAIMASAPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLA
SGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINR
(SEQ ID NO: 17). In some embodiments, a scFv (e.g., any of the scFvs described herein)
can include a linker sequence (e.g., any of the exemplary linker sequences described
10 herein or known in the art) between the heavy chain variable domain and the light chain
variable domain. In some embodiments, the anti-CD3 scFv can include a heavy chain
variable domain encoded by a nucleic acid including a sequence that is at least 70%
identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at
least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to
15 CAAGTTCAGCTCCAGCAAAGCGGCGCCGAACCTCGCTCGGCCCCGGCGCTTCCG
TGAAGATGTCTTGTAAGGCCTCCGGCTATACCTTCACCCGGTACACAATGCAC
TGGGTCAAGCAACGGCCCGGTCAAGGTTTAGAGTGGATTGGCTATATCAACC
CCTCCCGGGGCTATACCAACTACAACCAGAAGTTCAAGGACAAAGCCACCCT
CACCACCGACAAGTCCAGCAGCACCGCTTACATGCAGCTGAGCTCTTTAACA
20 TCCGAGGATTCCGCCGTGTACTACTGCGCTCGGTACTACGACGATCATTACTG
CCTCGATTACTGGGGCCAAGGTACCACCTTAACAGTCTCCTCC (SEQ ID NO:
18), and/or a light chain variable domain encoded by a nucleic acid including a sequence
that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least
85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or
25 100% identical) to
CAGATCGTGCTGACCCAGTCCCCCGCTATTATGAGCGCTAGCCCCGGTGAAA
AGGTGACTATGACATGCAGCGCCAGCTCTTCCGTGAGCTACATGAACTGGTA
TCAGCAGAAGTCCGGCACCAAGCCCTAAAAGGTGGATCTACGACACCAGCAA
GCTGGCCAGCGGCGTCCCCGCTCACTTTCGGGGCTCCGGCTCCGGAACAAGC
30 TACTCTCTGACCATCAGCGGCATGGAAGCCGAGGATGCCGCTACCTATTACT

GTCAGCAGTGGAGCTCCAACCCCTTCACCTTTGGATCCGGCACCAAGCTCGA
GATTAATCGT (SEQ ID NO: 19).

In some embodiments, an anti-CD3 scFv can include a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to
5 QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLA
SGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRGG
GGSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQ
RPGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAV
10 YYCARYYDDHYCLDYWGQGTTLVSS (SEQ ID NO: 20). In some embodiments,
an anti-CD3 scFv can include the six CDRs present in SEQ ID NO: 20.

In some embodiments, an anti-CD3 scFv can include a sequence encoded by a nucleic acid sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to
15 CAGATCGTGCTGACCCAGTCCCCCGCTATTATGAGCGCTAGCCCCGGTGAAA
AGGTGACTATGACATGCAGCGCCAGCTCTTCCGTGAGCTACATGAACTGGTA
TCAGCAGAAGTCCGGCACCCAGCCCTAAAAGGTGGATCTACGACACCAGCAA
GCTGGCCAGCGGCGTCCCCGCTCACTTTCGGGGCTCCGGCTCCGGAACAAGC
20 TACTCTCTGACCATCAGCGGCATGGAAGCCGAGGATGCCGCTACCTATTACT
GTCAGCAGTGGAGCTCCAACCCCTTCACCTTTGGATCCGGCACCAAGCTCGA
GATTAATCGTGGAGGCGGAGGTAGCGGAGGAGGCGGATCCGGCGGTGGAGG
TAGCCAAGTTCAGCTCCAGCAAAGCGGCGCCGAACCTCGCTCGGCCCCGGCGCT
TCCGTGAAGATGTCTTGTAAGGCCTCCGGCTATACCTTCACCCGGTACACAAT
25 GCACTGGGTCAAGCAACGGCCCCGGTCAAGGTTTAGAGTGGATTGGCTATATC
AACCCCTCCCGGGGCTATACCAACTACAACCAGAAGTTCAAGGACAAAGCCA
CCCTCACCACCGACAAGTCCAGCAGCACCCTTACATGCAGCTGAGCTCTTT
AACATCCGAGGATTCCGCCGTGTACTACTGCGCTCGGTACTACGACGATCATT
ACTGCCTCGATTACTGGGGCCAAGGTACCACCTTAACAGTCTCCTCC (SEQ ID
30 NO: 21).

In some embodiments, the first target binding domain, the second target-binding domain, and/or one or more of the one or more additional antigen-binding domains can be an anti-CD28 scFv. In some embodiments, the anti-CD28 scFv can include a heavy chain variable domain including a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to
DIEMTQSPAIMSASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYSTSNLA
SGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR (SEQ
ID NO: 22) and/or a light chain variable domain including a sequence that is at least 70%
identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at
least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to
VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYN
DYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRG
TTLTVSS (SEQ ID NO: 23). In some embodiments, the anti-CD28 scFv can include a
heavy chain variable domain encoded by a nucleic acid including a sequence that is at
least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85%
identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100%
identical) to
GACATCGAGATGACACAGTCCCCCGCTATCATGAGCGCCTCTTTAGGAGAAC
GTGTGACCATGACTTGTACAGCTTCCTCCAGCGTGAGCAGCTCCTATTTCAC
TGGTACCAGCAGAAACCCGGCTCCTCCCCTAAACTGTGTATCTACTCCACAA
GCAATTTAGCTAGCGGCGTGCCCTCCTCGTTTTAGCGGCTCCGGCAGCACCTCT
TACTCTTTAACCATTAGCTCTATGGAGGCCGAAGATGCCGCCACATACTTTTG
CCATCAGTACCACCGGTCCCCTACCTTTGGCGGAGGCACAAAGCTGGAGACC
AAGCGG (SEQ ID NO: 24), and/or a light chain variable domain encoded by a nucleic
acid including a sequence that is at least 70% identical (e.g., at least 75% identical, at
least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical,
at least 99% identical, or 100% identical) to
GTGCAGCTGCAGCAGTCCGGACCCGAAGTGGTCAAGCCCGGTGCCTCCGTGA
AAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCAATGG
GTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAATCCCT

ACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTCTGAC
 AAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACTTCTG
 AGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTGGGGC
 CGGGGAAGTACTTTAACAGTGAGCTCC (SEQ ID NO: 25).

5 In some embodiments, an anti-CD28 scFv can include a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYN
 DYTKEYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRG
 10 TTLTVSSGGGSGGGGSGGGGSDIEMTQSPAIMSASLGERVTMTCTASSSVSSSY
 FHWYQQKPGSSPKLCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCH
 QYHRSPFTFGGGTKLETKR (SEQ ID NO: 26). In some embodiments, an anti-CD28 scFv can include the six CDRs present in SEQ ID NO: 26.

In some embodiments, an anti-CD28 scFv can include a sequence encoded by a
 15 nucleic acid sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to
 GTGCAGCTGCAGCAGTCCGGACCCGAAGTGGTCAAGCCCGGTGCCTCCGTGA
 AAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCAATGG
 20 GTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAATCCCT
 ACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTCTGAC
 AAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACTTCTG
 AGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTGGGGC
 CGGGGAAGTACTTTAACAGTGAGCTCCGGCGGCGGCGGAAGCGGAGGTGGA
 25 GGATCTGGCGGTGGAGGCAGCGACATCGAGATGACACAGTCCCCCGCTATCA
 TGAGCGCCTCTTTAGGAGAACGTGTGACCATGACTTGTACAGCTTCCTCCAGC
 GTGAGCAGCTCCTATTTCCACTGGTACCAGCAGAAACCCGGCTCCTCCCCTAA
 ACTGTGTATCTACTCCACAAGCAATTTAGCTAGCGGCGTGCCTCCTCGTTTTA
 GCGGCTCCGGCAGCACCTCTTACTCTTTAACCATTAGCTCTATGGAGGCCGAA
 30 GATGCCGCCACATACTTTTGCCATCAGTACCACCGGTCCCCTACCTTTGGCGG
 AGGCACAAAGCTGGAGACCAAGCGG (SEQ ID NO: 27).

In some embodiments, any of the antigen-binding domains described herein is a BiTe, a (scFv)₂, a nanobody, a nanobody-HSA, a DART, a TandAb, a scDiabody, a scDiabody-CH3, scFv-CH-CL-scFv, a HSAbody, scDiabody-HAS, or a tandem-scFv. Additional examples of antigen-binding domains that can be used in any of the single-chain chimeric polypeptide are known in the art.

A VHH domain is a single monomeric variable antibody domain that can be found in camelids. A V_{NAR} domain is a single monomeric variable antibody domain that can be found in cartilaginous fish. Non-limiting aspects of VHH domains and V_{NAR} domains are described in, e.g., Cromie et al., *Curr. Top. Med. Chem.* 15:2543-2557, 2016; De Genst et al., *Dev. Comp. Immunol.* 30:187-198, 2006; De Meyer et al., *Trends Biotechnol.* 32:263-270, 2014; Kijanka et al., *Nanomedicine* 10:161-174, 2015; Kovaleva et al., *Expert. Opin. Biol. Ther.* 14:1527-1539, 2014; Krah et al., *Immunopharmacol. Immunotoxicol.* 38:21-28, 2016; Mujic-Delic et al., *Trends Pharmacol. Sci.* 35:247-255, 2014; Muyldermans, *J. Biotechnol.* 74:277-302, 2001; Muyldermans et al., *Trends Biochem. Sci.* 26:230-235, 2001; Muyldermans, *Ann. Rev. Biochem.* 82:775-797, 2013; Rahbarizadeh et al., *Immunol. Invest.* 40:299-338, 2011; Van Audenhove et al., *EBioMedicine* 8:40-48, 2016; Van Bockstaele et al., *Curr. Opin. Investig. Drugs* 10:1212-1224, 2009; Vincke et al., *Methods Mol. Biol.* 911:15-26, 2012; and Wesolowski et al., *Med. Microbiol. Immunol.* 198:157-174, 2009.

In some embodiments, each of the antigen-binding domains in the single-chain chimeric polypeptides described herein are both VHH domains, or at least one antigen-binding domain is a VHH domain. In some embodiments, each of the antigen-binding domains in the single-chain chimeric polypeptides described herein are both V_{NAR} domains, or at least one antigen-binding domain is a V_{NAR} domain. In some embodiments, each of the antigen-binding domains in the single-chain chimeric polypeptides described herein are both scFv domains, or at least one antigen-binding domain is a scFv domain. DARTs are described in, e.g., Garber, *Nature Reviews Drug Discovery* 13:799-801, 2014.

In some embodiments of any of the antigen-binding domains described herein can bind to an antigen selected from the group consisting of: a protein, a carbohydrate, a lipid, and a combination thereof.

Additional examples and aspects of antigen-binding domains are known in the art.

Soluble Interleukin or Cytokine Protein

In some embodiments of any of the single-chain chimeric polypeptides described
 5 herein, one or both of the first target-binding domain and the second target-binding
 domain can be a soluble interleukin protein, soluble cytokine protein, or soluble cell
 surface protein. In some embodiments, the soluble interleukin or soluble cytokine
 protein, is selected from the group of: IL-2, IL-3, IL-7, IL-8, IL-10, IL-12, IL-15, IL-17,
 IL-18, IL-21, PDGF-DD, SCF, FLT3L. Non-limiting examples of soluble IL-2, IL-3, IL-
 10 7, IL-8, IL-10, IL-15, IL-17, IL-18, IL-21, PDGF-DD, SCF, and FLT3L are provided
 below.

Human Soluble IL-2 (SEQ ID NO: 28)

aptssstkkk qlqlchllld lqmilnginn yknpkltrml tfkfympkka
 15 telkhlqcle eelkpleevl nlaqsknfhl rprdlisnin vivlelkgse
 ttfmceyade tativeflnr witfcqsiis tlt

Human Soluble IL-3 (SEQ ID NO: 29)

apmtqtptlkt swvnscnmid eiithlkqpp lplldfnnln gedqdilmen
 20 nlrrpnleaf nraavkslqna saiesilkn1 lpclplataa ptrhpihikd
 gdwnefrrkl tfylktlena qaqqttls1a if

Human Soluble IL-7 (SEQ ID NO: 30)

dcdiegkdgkqyesv lmvsidqlld smkeigsnc1 nnefnffkrh icdankegmf
 25 lfraarklrq flkmnstgdf dlhllkvseg ttillnctgq vkgrkpaalg
 eaqptkslee nkslkeqkkl ndlcflkrll qeiktcwnki lmgtkch

Human Soluble IL-8 (SEQ ID NO: 31)

egavlprsak elrcqikty skpfhpkfik elrviesgph canteiivkl
 30 sdgrelcldp kenwvqrvve kflkraens

Human Soluble IL-10 (SEQ ID NO: 32)

spgqgtqsensc thfpgnlpnm lrdlrdafrs vktffqmkdq ldnlllkesl
ledfkgylgc qalsemiqfy leevmpqaen qdpdikahvn slgenlktlr
lrlrrchrfl pcenkskave qvknafnklq ekgiykamse fdifinyiea
5 ymtmkirn

Human Soluble IL-12 β (p40) (SEQ ID NO: 33)

IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLT
IQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNKTFLR
10 CEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERVGRDNK
EYEYSVEQCQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIKPDPPKNL
QLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKTS
ATVICRKNASISVRAQDRYSSSWSEWASVPCS

15 Nucleic Acid Encoding Human Soluble IL-12 β (p40) (SEQ ID NO: 34)

ATTTGGGAACTGAAGAAGGACGTCTACGTGGTTCGAACTGGACTGGTATCCCGA
TGCTCCCGGCGAAATGGTGGTGCTCACTTGTGACACCCCCGAAGAAGACGGC
ATCACTTGGACCCTCGATCAGAGCAGCGAGGTGCTGGGCTCCGGAAAGACCC
TCACAATCCAAGTTAAGGAGTTCGGAGACGCTGGCCAATACACATGCCACAA
20 GGGAGGCGAGGTGCTCAGCCATTCTTATTATTATTACACAAGAAGGAAGACG
GAATCTGGTCCACCGACATTTTAAAAGATCAGAAGGAGCCCAAGAATAAGAC
CTTTTAAAGGTGTGAGGCCAAAACTACAGCGGTCTTTCACTTGTGGTGGC
TGACCACCATTTCACCGATTTAACCTTCTCCGTGAAAAGCAGCCGGGGAAGC
TCCGACCCTCAAGGTGTGACATGTGGAGCCGCTACCCTCAGCGCTGAGAGGG
25 TTCGTGGCGATAACAAGGAATACGAGTACAGCGTGGAGTGCCAAGAAGATAG
CGCTTGTCCTCCGCTGCCGAAGAATCTTTACCCATTGAGGTGATGGTGGACGCCG
TGCACAAACTCAAGTACGAGAACTACACCTCCTCCTTTATCCGGGACATCA
TTAAGCCCGATCCTCCTAAGAATTTACAGCTGAAGCCTCTCAAAAATAGCCGG
CAAGTTGAGGTCTCTTGGGAATATCCCGACACTTGGAGCACACCCACAGCTA
30 CTTCTCTTTAACCTTTTGTGTGCAAGTTCAAGGTAAAAGCAAGCGGGAGAAGA
AAGACCGGGTGTTTACCGACAAAACCAGCGCCACCGTCATCTGTTCGGAAGAA

CGCCTCCATCAGCGTGAGGGCTCAAGATCGTTACTCCAGCAGCTGGTCCG
AGTGGGCCAGCGTGCCTTGTTC

Human Soluble IL-12 α (p35) (SEQ ID NO: 35)

5 RNLPVATPDPGMFPC LHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKD
KTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKM
YQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPD
FYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS

10 **Nucleic Acid Encoding Human Soluble IL-12 α (p35) (SEQ ID NO: 36)**

CGTAACCTCCCCGTGGCTACCCCGATCCCGGAATGTTCCCTTGTTTACACCAC
AGCCAGAATTTACTGAGGGCCGTGAGCAACATGCTGCAGAAAGCTAGGCAGA
CTTTAGAATTTTACCCTTGCACCAGCGAGGAGATCGACCATGAAGATATCACCA
AGGACAAGACATCCACCGTGGAGGCTTGTTTACCTCTGGAGCTGACAAAGAA
15 CGAGTCTTGTCTCAACTCTCGTGAAACCAGCTTCATCACAAATGGCTCTTGTTT
AGCTTCCCGGAAGACCTCCTTTATGATGGCTTTATGCCTCAGCTCCATCTACGA
GGATTAAAGATGTACCAAGTGGAGTTCAAGACCATGAACGCCAAGCTGCTCA
TGGACCTAAACGGCAGATCTTTTATAGACCAGAACATGCTGGCTGTGATTGAT
GAGCTGATGCAAGCTTTAAACTTCAACTCCGAGACCGTCCCTCAGAAGTCCTC
20 CCTCGAGGAGCCCGATTTTACAAGACAAAGATCAAACGTGCATTTTACTCC
ACGCCTTTAGGATCCGGGCCGTGACCATTGACCGGGTCATGAGCTATTTAAAC
GCCAGC

Exemplary Human Soluble IL-12 (SEQ ID NO: 37)

25 IWELKKDVYVVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLT
IQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNKTF LR
CEAKNYSGRFTCWWLTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERVGRDN
KEYEYSVEQCEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIIKPDPPKN
LQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKT
30 SATVICRKNASISVRAQDRYYSSSWSEWASVPCSGGGGSGGGGSGGGGSRNLPV
ATPDPGMFPC LHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKDKTSTV

EACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEF
KTMNAKLLMDPKRQIFLDQNMMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTK
IKLCILLHAFRIRAVTIDRVMSYLNAS

5 **Nucleic Acid Encoding Exemplary Human Soluble IL-12 (SEQ ID NO: 38)**

ATTTGGGAACTGAAGAAGGACGTCTACGTGGTCGAACTGGACTGGTATCCCG
ATGCTCCCGGCGAAATGGTGGTGCTCACTTGTGACACCCCGAAGAAGACGG
CATCACTTGGACCCTCGATCAGAGCAGCGAGGTGCTGGGCTCCGGAAAGACC
CTCACAATCCAAGTTAAGGAGTTCGGAGACGCTGGCCAATACACATGCCACA
10 AGGGAGGCGAGGTGCTCAGCCATTCCTTATTATTATTACACAAGAAGGAAGA
CGGAATCTGGTCCACCGACATTTTAAAAGATCAGAAGGAGCCCAAGAATAAG
ACCTTTTTAAGGTGTGAGGCCAAAACTACAGCGGTCTGTTTCACTTGTGGTG
GCTGACCACCATTTCACCGATTTAACCTTCTCCGTGAAAAGCAGCCGGGGA
AGCTCCGACCCTCAAGGTGTGACATGTGGAGCCGCTACCCCTCAGCGCTGAGA
15 GGGTTCGTGGCGATAACAAGGAATACGAGTACAGCGTGGAGTGCCAAGAAG
ATAGCGCTTGTCCCGCTGCCGAAGAATCTTTACCCATTGAGGTGATGGTGGAC
GCCGTGCACAACTCAAGTACGAGAACTACACCTCCTCCTTCTTTATCCGGGA
CATCATTAAGCCCGATCCTCCTAAGAATTTACAGCTGAAGCCTCTCAAAAATA
GCCGGCAAGTTGAGGTCTCTTGGAATATCCCGACACTTGAGACACACCCCA
20 CAGCTACTTCTCTTTAACCTTTTGTGTGCAAGTTCAAGGTAAAAGCAAGCGGG
AGAAGAAAGACCGGGTGTTTACCGACAAAACCAGCGCCACCGTCATCTGTCTG
GAAGAACGCCTCCATCAGCGTGAGGGCTCAAGATCGTTATTACTCCAGCAGC
TGGTCCGAGTGGGCCAGCGTGCCTTGTTCGGCGGTGGAGGATCCGGAGGAG
GTGGCTCCGGCGGCGGAGGATCTCGTAACCTCCCGTGGCTACCCCGATCC
25 CGGAATGTTCCCTTGTTTACACCACAGCCAGAATTTACTGAGGGCCGTGAGC
AACATGCTGCAGAAAGCTAGGCAGACTTTAGAATTTTACCCTTGCACCAGCG
AGGAGATCGACCATGAAGATATCACCAAGGACAAGACATCCACCGTGGAGG
CTTGTTTACCTCTGGAGCTGACAAAGAACGAGTCTTGTCTCAACTCTCGTGAA
ACCAGCTTCATCACAAATGGCTCTTGTTTAGCTTCCCGGAAGACCTCCTTTAT
30 GATGGCTTTATGCCTCAGCTCCATCTACGAGGATTTAAAGATGTACCAAGTGG
AGTTCAAGACCATGAACGCCAAGCTGCTCATGGACCCTAAACGGCAGATCTT

TTTAGACCAGAACATGCTGGCTGTGATTGATGAGCTGATGCAAGCTTTAAACT
TCAACTCCGAGACCGTCCCTCAGAAGTCCTCCCTCGAGGAGCCCGATTTTAC
AAGACAAAGATCAAACGTGTCATTTTACTCCACGCCTTTAGGATCCGGGCCG
TGACCATTGACCGGGTCATGAGCTATTTAAACGCCAGC

5

Human Soluble IL-15 (SEQ ID NO: 39)

Nwvnvisdlkki edliqsmhid atlytesdvh psckvtamkc fllelqvisl
esgdasihdt venliilann slssngnvte sgckeceele eknikeflqs
fvhivqmfin ts

10

Human Soluble IL-17 (SEQ ID NO: 40)

gitiprn pgcpnsedkn fprrvmvnlh ihnrntntnp krssdyynrs
tspwnlhrne dperypsviw eakcrhlhci nadgnvdyhm nsvpigqeil
vrrrepphpc nsfrlekilv svgtcvtpi vhhva

15

Human Soluble IL-18 (SEQ ID NO: 41)

YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISMYKDSQPRG
MAVTISVKCEKISTLSCENKIISFKEMNPPDNIKDTKSDIIFQRSVPGHDNKMQFESS
SYEGYFLACEKERDLFKLILKKEDELGDRSIMFTVQNE

20

Nucleic Acid Encoding Human Soluble IL-18 (SEQ ID NO: 42)

TACTTCGGCAAACCTGGAATCCAAGCTGAGCGTGATCCGGAATTTAAACGACCA
AGTTCTGTTTATCGATCAAGGTAACCGGCCTCTGTTTCGAGGACATGACCGACT
CCGATTGCCGGGACAATGCCCCCGGACCATCTTCATTATCTCCATGTACAAGG
25 ACAGCCAGCCCCGGGGCATGGCTGTGACAATTAGCGTGAAGTGTGAGAAAAT
CAGCACTTTATCTTGTGAGAACAAAGATCATCTCCTTTAAGGAAATGAACCCCC
CCGATAACATCAAGGACACCAAGTCCGATATCATCTTCTTCCAGCGGTCCGTGC
CCGGTCACGATAACAAGATGCAGTTCGAATCCTCCTCCTACGAGGGCTACTTTT
TAGCTTGTGAAAAGGAGAGGGATTTATTCAAGCTGATCCTCAAGAAGGAGGA
30 CGAGCTGGGCGATCGTTCCATCATGTTACCGTCCAAAACGAGGAT

Human Soluble IL-21 (SEQ ID NO: 43)

QGGDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWSAFSCFQKAQ
 LKSANTGNNERIINVSIKKLKRKPPSTNAGRRQKHRLTCPSCDSYEKKPPKEFLER
 FKSLQKMIHQHLSRTHGSEDS

5

Nucleic Acid Encoding Human Soluble IL-21 (SEQ ID NO: 44)

CAGGGCCAGGACAGGCACATGATCCGGATGAGGCAGCTCATCGACATCGTCG
 ACCAGCTGAAGAACTACGTGAACGACCTGGTGCCCGAGTTTCTGCCTGCCCC
 CGAGGACGTGGAGACCAACTGCGAGTGGTCCGCCTTCTCCTGCTTTTCAGAAG
 10 GCCCAGCTGAAGTCCGCCAACACCGGCAACAACGAGCGGATCATCAACGTGA
 GCATCAAGAAGCTGAAGCGGAAGCCTCCCTCCACAAACGCCGGCAGGAGGC
 AGAAGCACAGGCTGACCTGCCCCAGCTGTGACTCCTACGAGAAGAAGCCCCC
 CAAGGAGTTCCTGGAGAGGTTCAAGTCCCTGCTGCAGAAGATGATCCATCAG
 CACCTGTCCTCCAGGACCCACGGCTCCGAGGACTCC

15

Human Soluble PDGF-DD (SEQ ID NO: 45)

rdtsatpqsasi kalrnanlrr desnhltdly rrdetiqvkg ngyvqsprfp
 nsyprnlllt wrlhsqentr iqlvfdnqfg leeaendicr ydfvevedis
 etstiirgrw cghkevppri ksrtngikit fksddyfvak pgfkiiysll
 20 edfqpaase tnwsvtssi sgvsynspsv tdptliadal dkkiaefdtv
 edllkyfnpe swqedlenmy ldtpyrgrs yhdrkskvd1 drlnddakry
 sctprnysvn ireelklanv vffprcllvq rcggnccgct vnrwsctcns
 gktvkkyhev lqfepghikr rgraktmalv diqldhherc dcicssrppr

Human Soluble SCF (SEQ ID NO: 46)

egicrnrvtnnvkdv tkivanlpkd ymitlkyvpg mdvlpshcwi semvvqlsds
 ltdlldkfsn iseglsnysi idklvnivdd lvecvkeness kdlkksfksp
 eprlftpeef frifnrsida fkdfvvaset sdcvvsstls pekdsrvsvt
 kpfmlppvaa sslrndssss nrkaknppgd sslhwaamal palfsliigf
 30 afgalywkk rpslstraven iqineednei smlqekeref gev

Human Soluble FLT3L (SEQ ID NO: 47)

tqdcsfqhspissd favkirelsd yllqdypvtv asnlqdeelc gglwrlvlaq
 rwmerlktva gskmqgller vnteihfvtk cafqpppscl rfvqtnisrl
 lqetseqlva lkpwithrqnf srclelqcqp dsstlpppws prpleatapt
 5 apqpplllll llpvglllla aawclhwqrt rrrtprpgeq vppvpqpdl
 llveh

Exemplary soluble cell surface proteins include soluble MICA, MICB, and a
 ULP16 binding protein (e.g., ULBP1, ULBP2, ULBP3, ULBP4, ULBP5, or ULBP6).

10 Exemplary sequences for soluble MICA, MICB, ULBP1, ULBP2, ULBP3, ULBP4,
 ULBP5, and ULBP6 are listed below.

Human Soluble MICA (SEQ ID NO: 48)

ephslry nltvlswdgs vqsgfltevh ldgqpflrcl rpkcrakpqq
 15 qwaedvlgk twdretldt gngkdlrmtl ahikdqkegl hslqeirvce
 ihednstrss qhfyydgelf lsqnletkew tmpqssraqt lamnvrnflk
 edamktkthy hamhadclqe lrrylksgvv lrrtvppmvn vtrseasegn
 itvtcrasgf ypwnitlswr qdgvslshdt qqwgdvlpdg ngtyqtwvat
 ricqgeeqrfr tcymehsgnh sthvpvsgkv lvlqshwqtf hvsavaaaaai
 20 fviiiifyvrc ckkktsaaeg pelvslqvld qhpvgtsdhr datqlgfqpl
 msdlgstgst ega

Human Soluble MICB (SEQ ID NO: 49)

aephslry nlmvlsqdes vqsgflaegh ldgqpflryd rpkrrakpqq
 25 qwaedvlgak twdtetedlt engqdlrmtl thikdqkggl hslqeirvce
 ihedsstrgs rhfyydgelf lsqnletqes tvpqssraqt lamnvtnfwk
 edamktkthy ramqadclqk lqrylksgva irrtpvppmvn vtrseasegn
 itvtcrassf yprnitltwr qdgvslshnt qqwgdvlpdg ngtyqtwvat
 rirqgeeqrfr tcymehsgnh gthvpvsgkv lvlqsqrtdf pyvsaampcf
 30 viiiilcvpc ckkktsaaeg pelvslqvld qhpvgtdghr
 daaqlgfqpl msatgstgst ega

Human Soluble ULBP1 (SEQ ID NO: 50)

wvdthclcydfiit pksrpepqcw evqglvderp flhydcvnhk akafaslgkk
 vnvttktweeq tetlrdvvdv lkgqlldiqv enlipieplt lqarmscehe
 ahghgrgswq flfngqkflf fdsnnrkwt lhpgakkmte kweknrdvtm
 5 ffqkislgdc kmwleeflmy weqmldptkp pslapg

Human Soluble ULBP2 (SEQ ID NO: 51)

gradphslcyditvi pkfrpgprwc avqgqvdekt flhydcgnkt vtpv splgk
 lnvttawkaq npvlrevvdi lteqlrldiq enlytpkeplt lqarmsceq
 10 aeghssgswq fsfdgqifll fdsekrmwtt vhp garkmke kwendkvvam
 sfhyfsmgdc igwledflmg mdstlepszag aplams

Human Soluble ULBP3 (SEQ ID NO: 52)

dahslwynfti ihlprhgqgw cevqsqvdk nflsydcgsd kvlsmghee
 15 qlyatdawgk qlemlrevgq rlrleladte ledftpsgpl tlqvrmscec
 eadgyirgsw qfsfdgrkfl lfdsnrkwt vvhagarmk ekwekdsglt
 tffkmvsmrd ckswlrdflm hrkkrlpta pptmapg

Human Soluble ULBP4 (SEQ ID NO: 53)

hslcfnftik slsrpgqpw eaqvflnkn flqynsdnm vkplgllgk
 vyatstwgel tqtlgevgrd lrmllcdikp qiktsdpstl qvemfcqrea
 erctgaswqf atngeksllf damnmtwtvi nheaskiket wkkdrgleky
 frklsgdcd hwlreflghw eampeptvsp vnasdihwss sslpdrwiil
 gafillvlmg ivlicvwwqn gewqaglwpl rts

25

Human Soluble ULBP5 (SEQ ID NO: 54)

gladp hslcyditvi pkfrpgprwc avqgqvdekt flhydcgskt
 vtpv splgk lnvttawkaq npvlrevvdi lteqlldiq enlypkeplt
 lqarmsceq aeghgsgswq lsfdgqifll fdsenrmwtt vhp garkmke
 30 kwendkdmtm sfhyismgdc tgwledflmg mdstlepszag apptmssg

Human Soluble ULBP6 (SEQ ID NO: 55)

rrddp hslcyditvi pkfrpgprwc avqqqvdekt flhydcgnkt
 vtpv splgkk lnvtmawkaq npvlrevvdi lteqlldiql enytpkeplt
 lqarmsceqk aeghssgswq fsidggtfll fdsekrmwtt vhp garkmke
 5 kwendkdvam sfhyismgdc igwledflmg mdstlepszag aplanmssg

In some embodiments, a soluble IL-12 protein can include a first sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 33, and a second sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 35. In some embodiments, the soluble IL-12 can further include a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between the first
 15 sequence and the second sequence.

In some embodiments, a soluble IL-12 protein is encoded by a first nucleic acid encoding a first sequence at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 34, and a second nucleic acid sequence
 20 encoding a second sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 36. In some embodiments, the nucleic acid encoding a soluble IL-12 protein further includes a nucleic acid sequence encoding a linker sequence (e.g., any of the exemplary linker sequences described herein
 25 or known in the art) between the first nucleic acid and the second nucleic acid.

In some embodiments, a soluble IL-12 protein includes a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 37. In some embodiments, a soluble IL-12 protein is encoded by a nucleic
 30 acid including a sequence at least 70% identical (e.g., at least 75% identical, at least 80%

identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 38.

In some embodiments, a soluble IL-18 protein can include a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 41. In some embodiments, a soluble IL-18 protein is encoded by a nucleic acid including a sequence at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 42.

In some embodiments, a soluble IL-21 protein can include a sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 43. In some embodiments, a soluble IL-21 protein is encoded by a nucleic acid including a sequence at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to SEQ ID NO: 44.

Additional examples of soluble interleukin proteins and soluble cytokine proteins are known in the art.

Soluble Receptor

In some embodiments of any of the single-chain chimeric polypeptides described herein, one or both of the first target-binding domain and the second target-binding domain is a soluble interleukin receptor, a soluble cytokine receptor, or a soluble cell surface receptor. In some embodiments, the soluble receptor is a soluble TGF- β receptor II (TGF- β RII) (see, e.g., those described in Yung et al., *Am. J. Resp. Crit. Care Med.* 194(9):1140-1151, 2016), a soluble TGF- β RIII (see, e.g., those described in Heng et al., *Placenta* 57:320, 2017), or a soluble NKG2D (see, e.g., Cosman et al., *Immunity* 14(2):123-133, 2001; Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150). In some embodiments, the soluble cell surface receptor is a soluble NKp30 (see, e.g., Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150), a soluble NKp44 (see, e.g., those

described in Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150), a soluble NKp46 (see, e.g., Mandelboim et al., *Nature* 409:1055-1060, 2001; Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150), a soluble DNAM1 (see, e.g., those described in Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150), a scMHCI (see, e.g., those described in Washburn et al., *PLoS One* 6(3):e18439, 2011), a scMHCII (see, e.g., those described in Bishwajit et al., *Cellular Immunol.* 170(1):25-33, 1996), a scTCR (see, e.g., those described in Weber et al., *Nature* 356(6372):793-796, 1992), a soluble CD155 (see, e.g., those described in Tahara-Hanaoka et al., *Int. Immunol.* 16(4):533-538, 2004), or a soluble CD28 (see, e.g., Hebbar et al., *Clin. Exp. Immunol.* 136:388-392, 2004).

In some embodiments, a soluble TGF β RII receptor can include a first sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to:

IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICE
KPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGET
FFMCSCSSDECNDNIIFSEEYNTSNPD (SEQ ID NO: 56), and a second sequence that is at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to:

IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICE
KPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGET
FFMCSCSSDECNDNIIFSEEYNTSNPD (SEQ ID NO: 56). In some embodiments, the soluble TGF β RII receptor can further include a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between the first sequence and the second sequence.

In some embodiments, a soluble TGF β RII receptor is encoded by a first nucleic acid encoding a first sequence at least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to:

ATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACCGACA
 ACAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCAGGTTC
 AGCACCTGCGATAATCAGAAAGTCCTGCATGTCCAAGTGCACGATCACCTCCA
 TCTGCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAATGACG
 5 AGAACATCACCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATCACGA
 CTTCAATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAAGAAG
 AAGCCCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGTAACG
 ACAACATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGAT (SEQ ID NO:
 57), and a second nucleic acid sequence encoding a second sequence that is at least 70%
 10 identical (e.g., at least 75% identical, at least 80% identical, at least 85% identical, at
 least 90% identical, at least 95% identical, at least 99% identical, or 100% identical) to:
 ATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACCGACA
 ACAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCAGGTTC
 AGCACCTGCGATAATCAGAAAGTCCTGCATGTCCAAGTGCACGATCACCTCCA
 15 TCTGCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAATGACG
 AGAACATCACCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATCACGA
 CTTCAATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAAGAAG
 AAGCCCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGTAACG
 ACAACATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGAT (SEQ ID NO:
 20 57). In some embodiments, the nucleic acid encoding a soluble TGF β RII receptor further
 includes a nucleic acid sequence encoding a linker sequence (e.g., any of the exemplary
 linker sequences described herein or known in the art) between the first nucleic acid and
 the second nucleic acid.

In some embodiments, a soluble TGF β RII receptor includes a sequence that is at
 25 least 70% identical (e.g., at least 75% identical, at least 80% identical, at least 85%
 identical, at least 90% identical, at least 95% identical, at least 99% identical, or 100%
 identical) to:

IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICE
 KPQEVCAVWRKNNDENITLETVCHDPKLPYHDFILEDASPCKIMKEKKKPGET
 30 FFMCSRSSDECNDNIIFSEEYNTSNPDGGGGSGGGSGGGGSIPPHVQKSVNNDM
 IVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCAVWRK

NDENITLETVCHDPKLPYHDFILED AASPKCIMKEKKKPGETFFMCSCSSDECND
NIIFSEYNTSNPD (SEQ ID NO: 60). In some embodiments, a soluble TGFβRII

receptor is encoded by a nucleic acid including a sequence at least 70% identical (e.g., at
least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical,
5 at least 95% identical, at least 99% identical, or 100% identical) to:

ATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACCGACA
ACAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCAGGTTC
AGCACCTGCGATAATCAGAAGTCCTGCATGTCCAACGACGATCACCTCCA
TCTGCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAATGACG
10 AGAACATCACCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATCACGA
CTTCATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAAGAAG
AAGCCCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGTAACG
ACAACATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGATGGAGGTGG
CGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGTATTCCTCCCCACGTG
15 CAGAAGAGCGTGAATAATGACATGATCGTGACCGATAACAATGGCGCCGTGA
AATTTCCCCAGCTGTGCAAATTCTGCGATGTGAGGTTTTCCACCTGCGACAAC
CAGAAGTCCTGTATGAGCAACTGCACAATCACCTCCATCTGTGAGAAGCCTC
AGGAGGTGTGCGTGGCTGTCTGGCGGAAGAATGACGAGAATATCACCTGGA
AACCGTCTGCCACGATCCCAAGCTGCCCTACCACGATTTTCATCCTGGAAGAC
20 GCCGCCAGCCCTAAGTGCATCATGAAAGAGAAAAAGAAGCCTGGCGAGACC
TTTTTCATGTGCTCCTGCAGCAGCGACGAATGCAACGACAATATCATCTTTAG
CGAGGAATACAATACCAGCAACCCCGAC (SEQ ID NO: 61).

Additional examples of soluble interleukin receptors and soluble cytokine
receptors are known in the art.

25

Additional Antigen-Binding Domains

Some embodiments of any of the single-chain chimeric polypeptides described
herein can further include one or more (e.g., two, three, four, five, six, seven, eight, nine,
or ten) additional target-binding domains (e.g., any of the exemplary target-binding
30 domains described herein or known in the art) at its N- and/or C-terminus.

In some embodiments, the single-chain chimeric polypeptides can include one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) at its N-terminus. In some embodiments, one of the one or more additional
5 target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) at the N-terminus of the single-chain chimeric polypeptide can directly abut the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), or the
10 soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein). In some embodiments, the single-chain chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between one of the at least one additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art)
15 at the N-terminus of the single-chain chimeric polypeptide and the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), or the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein).

20 In some embodiments of any of the single-chain chimeric polypeptides described herein, the single-chain chimeric polypeptide includes one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) at its C-terminus. In some embodiments, one of the one or more additional target-binding domains (e.g.,
25 any of the exemplary target-binding domains described herein or known in the art) at the C-terminus of the single-chain chimeric polypeptide directly abuts the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), or the soluble tissue factor domain (e.g.,
30 any of the exemplary soluble tissue factor domains described herein or known in the art). In some embodiments, the single-chain chimeric polypeptide further comprises a linker

sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between one of the at least one additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) at the C-terminus of the single-chain chimeric polypeptide and the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), or the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein).

In some embodiments of any of the single-chain chimeric polypeptides described herein, the single-chain chimeric polypeptide comprises one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) additional target binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) at its N-terminus and its C-terminus. In some embodiments, one of the one or more additional antigen binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) at the N-terminus of the single-chain chimeric polypeptide directly abuts the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), or the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein). In some embodiments, the single-chain chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between one of the one or more additional antigen-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) at the N-terminus and the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), or the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains). In some embodiments, one of the one or more additional antigen binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) at the C-terminus directly abuts the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second

target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), or the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains). In some embodiments, the single-chain chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between one of the one or more additional antigen-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) at the C-terminus and the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), or the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein).

In some embodiments of any of the single-chain chimeric polypeptides described herein, two or more (e.g., three, four, five, six, seven, eight, nine, or ten) of the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), and the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) bind specifically to the same antigen. In some embodiments, two or more (e.g., three, four, five, six, seven, eight, nine, or ten) of the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), and the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) bind specifically to the same epitope. In some embodiments, two or more (e.g., three, four, five, six, seven, eight, nine, or ten) of the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), and the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) include the same amino acid sequence.

In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), and the one or
5 more (e.g., two, three, four, five, six, seven, eight, nine, or ten) additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) each bind specifically to the same antigen. In some embodiments, the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), and the one or more (e.g., two,
10 three, four, five, six, seven, eight, nine, or ten) additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) each bind specifically to the same epitope. In some embodiments, the first target-binding domain, the second target-binding domain, and the one or more (e.g., two, three, four,
15 five, six, seven, eight, nine, or ten) additional target-binding domains each comprise the same amino acid sequence.

In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the
20 exemplary target-binding domains described herein or known in the art), and the one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) bind specifically to different antigens.

In some embodiments of any of the single-chain chimeric polypeptides, one or
25 more of the first target-binding domain, the second target-binding domain, and the one or more target-binding domains is an antigen-binding domain (e.g., any of the exemplary antigen-binding domains described herein or known in the art). In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain, the second target-binding domain, and the one or more additional target-binding
30 domains are each an antigen-binding domain (e.g., any of the exemplary antigen-binding

domains described herein or known in the art). In some embodiments, the antigen-binding domain can include a scFv or a single domain antibody.

In some embodiments of any of the single-chain chimeric polypeptides described herein, one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) of the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), and the one or more target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) bind specifically to a target selected from the group consisting of:

CD16a, CD28, CD3, CD33, CD20, CD19, CD22, CD123, IL-1R, IL-1, VEGF, IL-6R, IL-4, IL-10, PDL-1, TIGIT, PD-1, TIM3, CTLA4, MICA, MICB, IL-6, IL-8, TNF α , CD26a, CD36, ULBP2, CD30, CD200, IGF-1R, MUC4AC, MUC5AC, Trop-2, CMET, EGFR, HER1, HER2, HER3, PSMA, CEA, B7H3, EPCAM, BCMA, P-cadherin, CEACAM5, a UL16-binding protein, HLA-DR, DLL4, TYRO3, AXL, MER, CD122, CD155, PDGF-DD, a ligand of TGF- β receptor II (TGF- β RII), a ligand of TGF- β RIII, a ligand of DNAM1, a ligand of NKp46, a ligand of NKp44, a ligand of NKG2D, a ligand of NKp30, a ligand for a scMHCI, a ligand for a scMHCII, a ligand for a scTCR, a receptor for IL-1, a receptor for IL-2, a receptor for IL-3, a receptor for IL-7, a receptor for IL-8, a receptor for IL-10, a receptor for IL-12, a receptor for IL-15, a receptor for IL-17, a receptor for IL-18, a receptor for IL-21, a receptor for PDGF-DD, a receptor for stem cell factor (SCF), a receptor for stem cell-like tyrosine kinase 3 ligand (FLT3L), a receptor for MICA, a receptor for MICB, a receptor for a ULP16-binding protein, a receptor for CD155, a receptor for CD122, and a receptor for CD28.

In some embodiments of any of the single-chain chimeric polypeptides described herein, one or more of the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), and the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) is a soluble interleukin, a soluble cytokine protein, or a soluble cell surface protein. Non-limiting examples of soluble interleukin proteins and soluble cytokine proteins include: IL-1, IL-2, IL-3, IL-7, IL-8,

IL-10, IL-12, IL-15, IL-17, IL-18, IL-21, PDGF-DD, SCF, and FLT3L. Non-limiting examples of soluble cell surface proteins include: MICA, MICB, and a ULP16-binding protein.

In some embodiments of any of the single-chain chimeric polypeptides described herein, one or more of the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art), and the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) is a soluble interleukin receptor, a soluble cytokine receptor, or a soluble cell surface receptor. Non-limiting examples of soluble interleukin receptors and soluble cytokine receptors include: a soluble TGF- β receptor II (TGF- β RII) and a soluble TGF- β RIII. Non-limiting examples of soluble cell surface receptors include: a soluble NKG2D, a soluble NK30, a soluble NKp44, a soluble NKp46, a soluble DNAM1, a scMHCI, a scMHCII, a scTCR, a soluble CD155, a soluble CD122, a soluble CD3, and a soluble CD28.

Signal Sequence

In some embodiments, a single-chain chimeric polypeptide includes a signal sequence at its N-terminal end. As will be understood by those of ordinary skill in the art, a signal sequence is an amino acid sequence that is present at the N-terminus of a number of endogenously produced proteins that directs the protein to the secretory pathway (e.g., the protein is directed to reside in certain intracellular organelles, to reside in the cell membrane, or to be secreted from the cell). Signal sequences are heterogeneous and differ greatly in their primary amino acid sequences. However, signal sequences are typically 16 to 30 amino acids in length and include a hydrophilic, usually positively charged N-terminal region, a central hydrophobic domain, and a C-terminal region that contains the cleavage site for signal peptidase.

In some embodiments, a single-chain chimeric polypeptide includes a signal sequence having an amino acid sequence MKWVTFISLLFLFSSAYS (SEQ ID NO: 62). In some embodiments, a single chain chimeric polypeptide includes a signal sequence encoded by the nucleic acid sequence

ATGAAATGGGTGACCTTTATTTCTTTACTGTTTCCTCTTTAGCAGCGCCTACTCC
(SEQ ID NO: 63),

ATGAAGTGGGTGACATTTATCTCTTTACTGTTTCCTCTTCTCCAGCGCCTACAGC
(SEQ ID NO: 64), or

5 ATGAAATGGGTGACCTTTATTTCTTTACTGTTTCCTCTTTAGCAGCGCCTACTCC
(SEQ ID NO: 65).

In some embodiments, a single-chain chimeric polypeptide includes a signal
sequence having an amino acid sequence MKCLLYLAFLFLGVNC (SEQ ID NO: 66).

In some embodiments, a single-chain chimeric polypeptide includes a signal sequence
10 having an amino acid sequence
MGQIVTMFEALPHIIDEVINIVILIIITSIKAVYNFATCGILALVSFLFLAGRSCG
(SEQ ID NO: 67). In some embodiments, a single-chain chimeric polypeptide includes a
signal sequence having an amino acid sequence

MPNHQSGSPTGSSDLLSGKKQRPHLALRRKRRREMRKINRKVRRMNLAPIKEK
15 TAWQHLQALISEAEVLKTSQTPQNSLTLFLALLSVLGPPVTG (SEQ ID NO: 68).

In some embodiments, a single-chain chimeric polypeptide includes a signal sequence
having an amino acid sequence MDSKGSSQKGSRLLLLLVVSNNLLCQGVVS (SEQ
ID NO: 69). Those of ordinary skill in the art will be aware of other appropriate signal
sequences for use in a single-chain chimeric polypeptide.

20 In some embodiments, a single-chain chimeric polypeptide includes a signal
sequence that is about 10 to 100 amino acids in length. For example, a signal sequence
can be about 10 to 100 amino acids in length, about 15 to 100 amino acids in length,
about 20 to 100 amino acids in length, about 25 to 100 amino acids in length, about 30 to
100 amino acids in length, about 35 to 100 amino acids in length, about 40 to 100 amino
25 acids in length, about 45 to 100 amino acids in length, about 50 to 100 amino acids in
length, about 55 to 100 amino acids in length, about 60 to 100 amino acids in length,
about 65 to 100 amino acids in length, about 70 to 100 amino acids in length, about 75 to
100 amino acids in length, about 80 to 100 amino acids in length, about 85 to 100 amino
acids in length, about 90 to 100 amino acids in length, about 95 to 100 amino acids in
30 length, about 10 to 95 amino acids in length, about 10 to 90 amino acids in length, about
10 to 85 amino acids in length, about 10 to 80 amino acids in length, about 10 to 75

amino acids in length, about 10 to 70 amino acids in length, about 10 to 65 amino acids in length, about 10 to 60 amino acids in length, about 10 to 55 amino acids in length, about 10 to 50 amino acids in length, about 10 to 45 amino acids in length, about 10 to 40 amino acids in length, about 10 to 35 amino acids in length, about 10 to 30 amino acids in length, about 10 to 25 amino acids in length, about 10 to 20 amino acids in length, about 10 to 15 amino acids in length, about 20 to 30 amino acids in length, about 30 to 40 amino acids in length, about 40 to 50 amino acids in length, about 50 to 60 amino acids in length, about 60 to 70 amino acids in length, about 70 to 80 amino acids in length, about 80 to 90 amino acids in length, about 90 to 100 amino acids in length, about 20 to 90 amino acids in length, about 30 to 80 amino acids in length, about 40 to 70 amino acids in length, about 50 to 60 amino acids in length, or any range in between. In some embodiments, a signal sequence is about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acids in length.

In some embodiments, any of the signal sequences disclosed herein can include one or more additional amino acids (e.g., 1, 2, 3, 5, 6, 7, 8, 9, 10, or more amino acids) at its N-terminus and/or C-terminus, so long as the function of the signal sequence remains intact. For example, a signal sequence having the amino acid sequence MKWVTFISLLFLFSSAYS (SEQ ID NO: 62) can include one or more additional amino acids at the N-terminus or C-terminus, while still retaining the ability to direct the single-chain chimeric polypeptide to the secretory pathway.

In some embodiments, a single-chain chimeric polypeptide includes a signal sequence that directs the single-chain chimeric polypeptide into the extracellular space. Such embodiments are useful in producing single-chain chimeric polypeptides that are relatively easy to be isolated and/or purified.

Peptide Tags

In some embodiments, a single-chain chimeric polypeptide includes a peptide tag (e.g., at the N-terminal end or the C-terminal end of the single-chain chimeric polypeptide). In some embodiments, a single-chain chimeric polypeptide includes two or more peptide tags.

Exemplary peptide tags that can be included in a single-chain chimeric polypeptide include, without limitation, AviTag (GLNDIFEAQKIEWHE; SEQ ID NO: 70), a calmodulin-tag (KRRWKKNFIAVSAANRFKKISSSGAL; SEQ ID NO: 71), a polyglutamate tag (EEEEEE; SEQ ID NO: 72), an E-tag (GAPVPYPDPLEPR; SEQ ID NO: 73), a FLAG-tag (DYKDDDDK; SEQ ID NO: 74), an HA-tag, a peptide from hemagglutinin (YPYDVPDYA; SEQ ID NO: 75), a his-tag (HHHHH (SEQ ID NO: 76); HHHHHH (SEQ ID NO: 77); HHHHHHH (SEQ ID NO: 78); HHHHHHHH (SEQ ID NO: 79); HHHHHHHHH (SEQ ID NO: 80); or HHHHHHHHHH (SEQ ID NO: 81)), a myc-tag (EQKLISEEDL; SEQ ID NO: 82), NE-tag (TKENPRSNQEESYDDNES; SEQ ID NO: 83), S-tag, (KETAAAKFERQHMDS; SEQ ID NO: 84), SBP-tag (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP; SEQ ID NO: 85), Softag 1 (SLAELLNAGLGGS; SEQ ID NO: 86), Softag 3 (TQDPSRVG; SEQ ID NO: 87), Spot-tag (PDRVRAVSHWSS; SEQ ID NO: 88), Strep-tag (WSHPQFEK; SEQ ID NO: 89), TC tag (CCPGCC; SEQ ID NO: 90), Ty tag (EVHTNQDPLD; SEQ ID NO: 91), V5 tag (GKPIPNPLLGLDST; SEQ ID NO: 92), VSV-tag (YTDIEMNRLGK; SEQ ID NO: 93), and Xpress tag (DLYDDDDK; SEQ ID NO: 94). In some embodiments, tissue factor protein is a peptide tag.

Peptide tags that can be included in a single-chain chimeric polypeptide can be used in any of a variety of applications related to the single-chain chimeric polypeptide. For example, a peptide tag can be used in the purification of a single-chain chimeric polypeptide. As one non-limiting example, a single-chain chimeric polypeptide can include a myc tag; and can be purified using an antibody that recognizes the myc tag(s). One non-limiting example of an antibody that recognizes a myc tag is 9E10, available from the non-commercial Developmental Studies Hybridoma Bank. As another non-limiting example, a single-chain chimeric polypeptide can include a histidine tag, and can be purified using a nickel or cobalt chelate. Those of ordinary skill in the art will be aware of other suitable tags and agent that bind those tags for use in purifying a single-chain chimeric polypeptide. In some embodiments, a peptide tag is removed from the single-chain chimeric polypeptide after purification. In some embodiments, a peptide tag is not removed from the single-chain chimeric polypeptide after purification.

Peptide tags that can be included in a single-chain chimeric polypeptide can be used, for example, in immunoprecipitation of the single-chain chimeric polypeptide, imaging of the single-chain chimeric polypeptide (e.g., via Western blotting, ELISA, flow cytometry, and/or immunocytochemistry), and/or solubilization of the single-chain
5 chimeric polypeptide.

In some embodiments, a single-chain chimeric polypeptide includes a peptide tag that is about 10 to 100 amino acids in length. For example, a peptide tag can be about 10 to 100 amino acids in length, about 15 to 100 amino acids in length, about 20 to 100 amino acids in length, about 25 to 100 amino acids in length, about 30 to 100 amino acids
10 in length, about 35 to 100 amino acids in length, about 40 to 100 amino acids in length, about 45 to 100 amino acids in length, about 50 to 100 amino acids in length, about 55 to 100 amino acids in length, about 60 to 100 amino acids in length, about 65 to 100 amino acids in length, about 70 to 100 amino acids in length, about 75 to 100 amino acids in
15 length, about 80 to 100 amino acids in length, about 85 to 100 amino acids in length, about 90 to 100 amino acids in length, about 95 to 100 amino acids in length, about 10 to 95 amino acids in length, about 10 to 90 amino acids in length, about 10 to 85 amino acids in length, about 10 to 80 amino acids in length, about 10 to 75 amino acids in
20 length, about 10 to 70 amino acids in length, about 10 to 65 amino acids in length, about 10 to 60 amino acids in length, about 10 to 55 amino acids in length, about 10 to 50 amino acids in length, about 10 to 45 amino acids in length, about 10 to 40 amino acids in length, about 10 to 35 amino acids in length, about 10 to 30 amino acids in length, about
25 10 to 25 amino acids in length, about 10 to 20 amino acids in length, about 10 to 15 amino acids in length, about 20 to 30 amino acids in length, about 30 to 40 amino acids in length, about 40 to 50 amino acids in length, about 50 to 60 amino acids in length, about
30 60 to 70 amino acids in length, about 70 to 80 amino acids in length, about 80 to 90 amino acids in length, about 90 to 100 amino acids in length, about 20 to 90 amino acids in length, about 30 to 80 amino acids in length, about 40 to 70 amino acids in length, about 50 to 60 amino acids in length, or any range in between. In some embodiments, a peptide tag is about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or
100 amino acids in length.

Peptide tags included in a single-chain chimeric polypeptide can be of any suitable length. For example, peptide tags can be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more amino acids in length. In embodiments in which a single-chain chimeric polypeptide includes two or more peptide tags, the two or more peptide tags can
5 be of the same or different lengths. In some embodiments, any of the peptide tags disclosed herein may include one or more additional amino acids (e.g., 1, 2, 3, 5, 6, 7, 8, 9, 10, or more amino acids) at the N-terminus and/or C-terminus, so long as the function of the peptide tag remains intact. For example, a myc tag having the amino acid sequence EQKLISEEDL (SEQ ID NO: 82) can include one or more additional amino
10 acids (e.g., at the N-terminus and/or the C-terminus of the peptide tag), while still retaining the ability to be bound by an antibody (e.g., 9E10).

Exemplary Embodiments of Single-Chain Chimeric Polypeptides- Type A

In some embodiments of any of the single-chain chimeric polypeptides described
15 herein, the first target-binding domain and/or the second target-binding domain can independently bind specifically to CD3 (e.g., human CD3) or CD28 (e.g., human CD28). In some embodiments, the first target-binding domain binds specifically to CD3 (e.g., human CD3) and the second target-binding domain binds specifically to CD28 (e.g., human CD28). In some embodiments, the first target-binding domain binds specifically
20 to CD28 (e.g., human CD28) and the second target-binding domain binds specifically to CD3 (e.g., human CD3).

In some embodiments of these single-chain chimeric polypeptides, the first target-binding domain and the soluble tissue factor domain directly abut each other. In some embodiments of these single-chain chimeric polypeptides, the single-chain chimeric
25 polypeptide further includes a linker sequence (e.g., any of the exemplary linkers described herein) between the first target-binding domain and the soluble tissue factor domain.

In some embodiments of these single-chain chimeric polypeptides, the soluble tissue factor domain and the second target-binding domain directly abut each other. In
30 some embodiments of these single-chain chimeric polypeptides, the single-chain chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linkers

described herein) between the soluble tissue factor domain and the second target-binding domain.

In some embodiments of these single-chain chimeric polypeptides, one or both of the first target-binding domain and the second target-binding domain is an antigen-binding domain. In some embodiments of these single-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain are each an antigen-binding domain (e.g., any of the exemplary antigen-binding domains described herein). In some embodiments of these single-chain chimeric polypeptides, the antigen-binding domain includes a scFv or a single domain antibody.

A non-limiting example of an scFv that binds specifically to CD3 can include a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLAS
GVP AHFRGSGSGTSYSLTISGMEAEDAATYYCQWSSNPFTFGSGTKLEINRGGG
GSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQR
PGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYY
CARYYDDHYCLDYWGQGTTTLTVSS (SEQ ID NO: 20).

In some embodiments, an scFv that binds specifically to CD3 can be encoded by a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAAGTGGTATC
AGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAGC
TGGCCTCCGGAGTGCCCGCTCATTCCGGGGCTCTGGATCCGGCACCAGCTAC
TCTTTAACCATTTCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCCAG
CAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAATCAA
TCGTGGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAGCCA

AGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCGGCCCGGCGCCTCCGTC
AAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCATTGG
GTCAAGCAGAGGGCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAACCCTTC
CCGGGGCTACACCAACTATAACCAAAAGTTCAAGGATAAAGCCACTTTAACCA
5 CTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACAGCGAG
GACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTGTTTAGAC
TATTGGGGACAAGGTACCACTTTAACCGTCAGCAGC (SEQ ID NO: 99).

A non-limiting example of an scFv that binds specifically to CD28 can include a
sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical,
10 at least 86% identical, at least 88% identical, at least 90% identical, at least 92%
identical, at least 94% identical, at least 96% identical, at least 98% identical, at least
99% identical, or 100% identical) to:

VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYN
DYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRGT
15 TLTVSSGGGGSGGGSGGGGSDIEMTQSPAIMSASLGERVTMTCTASSSVSSSYFH
WYQQKPGSSPKLCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQY
HRSPTFGGGTKLETKR (SEQ ID NO: 26).

In some embodiments, an scFv that binds specifically to CD28 can be encoded by
a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84%
20 identical, at least 86% identical, at least 88% identical, at least 90% identical, at least
92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at
least 99% identical, or 100% identical) to:

GTCCAGCTGCAGCAGAGCGGACCCGAACTCGTGAAACCCGGTGCTTCCGTGA
AAATGTCTTGTAAGGCCAGCGGATACACCTTCACCTCCTATGTGATCCAGTGGG
25 TCAAACAGAAGCCCGGACAAGGTCTCGAGTGGATCGGCAGCATCAACCCTTA
CAACGACTATACCAATAACAACGAGAAGTTTAAGGGAAAGGCTACTTTAACCT
CCGACAAAAGCTCCATCACAGCCTACATGGAGTTCAGCTCTTTAACATCCGAG
GACAGCGCTCTGTACTATTGCGCCCGGTGGGGCGACGGCAATTACTGGGGACG
GGGCACAACACTGACCGTGAGCAGCGGAGGCGGAGGCTCCGGCGGAGGCGG
30 ATCTGGCGGTGGCGGCTCCGACATCGAGATGACCCAGTCCCCGCTATCATGT
CCGCCTCTTTAGGCGAGCGGGTCACAATGACTTGTACAGCCTCCTCCAGCGTC

TCCTCCTCCTACTTCCATTGGTACCAACAGAAACCCGGAAGCTCCCCTAAACT
 GTGCATCTACAGCACCAGCAATCTCGCCAGCGGCGTGCCCCCTAGGTTTTCCG
 GAAGCGGAAGCACCAGCTACTCTTTAACCATCTCCTCCATGGAGGCTGAGGAT
 GCCGCCACCTACTTTTGTACCAGTACCACCGGTCCCCCACCTTCGGAGGCGG
 5 CACCAAAGTGGAGACAAAGAGG (SEQ ID NO: 101).

In some embodiments of these single-chain chimeric polypeptides, the first target-binding domain and/or the second target-binding domain is a soluble receptor (e.g., a soluble CD28 receptor or a soluble CD3 receptor). In some embodiments of these single-chain chimeric polypeptides, the soluble tissue factor domain can be any of the
 10 exemplary soluble tissue factor domains described herein.

In some embodiments, a single-chain chimeric polypeptide can include a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least
 15 99% identical, or 100% identical) to:

QIVLTQSPAIMASPGKEVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLAS
 GVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRGGG
 GSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQR
 PGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYY
 20 CARYYDDHYCLDYWGQGTTLTVSSSGTTNTVAAYNLTWKSTNFKTILEWEPKPV
 NQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVE
 STGSAGEPLYENSPEFTPYLETNLTGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTF
 LSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFVQAVIPSR
 TVNRKSTDSPVECMGQEKGEFREVQLQQSGPELVKPGASVKMSCKASGYTFTSY
 25 VIQWVKQKPGQGLEWIGSINPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTS
 EDSALYYCARWGDGNYWGRGTTTLTVSSGGGGSGGGGSGGGGSDIEMTQSPAIM
 SASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYSTSNLASGVPPRFSGSG
 STSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR (SEQ ID NO: 1).

In some embodiments, a single-chain chimeric polypeptide is encoded by a
 30 sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92%

identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACTGGTATC
5 AGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAGC
TGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCACCAGCTAC
TCTTTAACCATTTCCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCCAG
CAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAATCAA
TCGTGGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAGCCA
10 AGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCGGCGCCTCCGTC
AAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCATTGG
GTCAAGCAGAGGCCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAACCCTTC
CCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTTTAACCA
CTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACCAAGCGAG
15 GACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTGTTTAGAC
TATTGGGGACAAGGTACCACTTTAACCGTCAGCAGCTCCGGCACCACCAATAC
CGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGACAATTCTGG
AATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGATCTCCACCAAA
TCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACCGAGTGTGATT
20 TAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGGCTCGGGTCTTT
TCCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGCGAGCCTCTCTA
CGAGAATCCCCCGAATTCACCCCTTATTTAGAGACCAATTTAGGCCAGCCTAC
CATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTCACCGTCGAGGAT
GAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCCTCCGGGATGTGTTC
25 GGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCCAGCTCCTCCGGCAA
AAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTGACGTGGACAAAGGC
GAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTCTCGTACCGTCAACCG
GAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTC
CGGGAGGTCCAGCTGCAGCAGAGCGGACCCGAACCTCGTGAAACCCGGTGCTT
30 CCGTGAAAATGTCTTGTAAGGCCAGCGGATACACCTTCACCTCCTATGTGATCC
AGTGGGTCAAACAGAAGCCCGGACAAGGTCTCGAGTGGATCGGCAGCATCAA

CCCTTACAACGACTATACCAAATACAACGAGAAGTTTAAGGGAAAGGCTACTT
 TAACCTCCGACAAAAGCTCCATCACAGCCTACATGGAGTTCAGCTCTTTAACA
 TCCGAGGACAGCGCTCTGTACTATTGCGCCCGGTGGGGCGACGGCAATTACTG
 GGGACGGGGCACAACACTGACCGTGAGCAGCGGAGGCGGAGGCTCCGGCGG
 5 AGGCGGATCTGGCGGTGGCGGCTCCGACATCGAGATGACCCAGTCCCCCGCTA
 TCATGTCCGCCTCTTTAGGCGAGCGGGTCACAATGACTTGTACAGCCTCCTCC
 AGCGTCTCCTCCTCCTACTTCCATTGGTACCAACAGAAACCCGGAAGCTCCCC
 TAAACTGTGCATCTACAGCACCAGCAATCTCGCCAGCGGCGTGCCCCCTAGGT
 TTTCCGGAAGCGGAAGCACCAGCTACTCTTTAACCATCTCCTCCATGGAGGCT
 10 GAGGATGCCGCCACCTACTTTTGTACACCAGTACCACCGGTCCCCCACCTTCGG
 AGGCGGCACCAAAGTGGAGACAAAGAGG (SEQ ID NO: 2).

In some embodiments, a single-chain chimeric polypeptide can include a
 sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical,
 at least 86% identical, at least 88% identical, at least 90% identical, at least 92%
 15 identical, at least 94% identical, at least 96% identical, at least 98% identical, at least
 99% identical, or 100% identical) to:

MKWVTFISLLFLFSSAYSQIVLTQSPAIMASAPGEKVTMTCSASSSVSYMNWYQQ
 KSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSS
 NPFTFGSGTKLEINRGGGGSGGGSGGGGSQVQLQQSGAELARPGASVKMSCK
 20 ASGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSS
 TAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTTLTVSSSGTTNTVAAYNL
 TWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKD
 VKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTK
 VNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLI
 25 DVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFREVQLQQSGPELVK
 PGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYNDYTKYNEKFKG
 KATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRGTTTLTVSSGGGGSG
 GGGSGGGGSDIEMTQSPAIMASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPK
 LCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKL
 30 ETKR (SEQ ID NO: 3).

In some embodiments, a single-chain chimeric polypeptide is encoded by a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

```
5  ATGAAGTGGGTGACCTTCATCAGCTTATTATTTTATTCAGCTCCGCCTATTCCC
   AGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGAA
   GGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAAGTGGTATCA
   GCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAGCT
10  GGCCTCCGGAGTGCCCGCTCATTTCGGGGGCTCTGGATCCGGCACCAGCTACT
   CTTTAACCATTTCCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCCAG
   CAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAATCAA
   TCGTGGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAGCCA
   AGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCGCGCCTCCGTC
15  AAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCATTGG
   GTCAAGCAGAGGGCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAACCCTTC
   CCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTTTAACCA
   CTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACCAGCGAG
   GACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTGTTTAGAC
20  TATTGGGGACAAGGTACCACCTTTAACCGTCAGCAGCTCCGGCACCACCAATAC
   CGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGACAATTCTGG
   AATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGATCTCCACCAAA
   TCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACCGAGTGTGATT
   TAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGGCTCGGGTCTTT
25  TCCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGCGAGCCTCTCTA
   CGAGAATTCCCCCGAATTCACCCCTTATTTAGAGACCAATTTAGGCCAGCCTAC
   CATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTCACCGTCGAGGAT
   GAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCCTCCGGGATGTGTTT
   GGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCCAGCTCCTCCGGCAA
30  AAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTGACGTGGACAAAGGC
   GAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTCTCGTACCGTCAACCG
```

GAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTC
 CGGGAGGTCCAGCTGCAGCAGAGCGGACCCGAACTCGTGAAACCCGGTGCTT
 CCGTGAAAATGTCTTGTAAGGCCAGCGGATACACCTTCACCTCCTATGTGATCC
 AGTGGGTCAAACAGAAGCCCGGACAAGGTCTCGAGTGGATCGGCAGCATCAA
 5 CCCTTACAACGACTATACCAAATACAACGAGAAGTTTAAGGGAAAGGCTACTT
 TAACCTCCGACAAAAGCTCCATCACAGCCTACATGGAGTTCAGCTCTTTAACA
 TCCGAGGACAGCGCTCTGTACTATTGCGCCCGGTGGGGCGACGGCAATTACTG
 GGGACGGGGCACAACACTGACCGTGAGCAGCGGAGGCGGAGGCTCCGGCGG
 AGGCGGATCTGGCGGTGGCGGCTCCGACATCGAGATGACCCAGTCCCCCGCTA
 10 TCATGTCCGCCTCTTTAGGCGAGCGGGTCACAATGACTTGTACAGCCTCCTCC
 AGCGTCTCCTCCTCCTACTTCCATTGGTACCAACAGAAACCCGGAAGCTCCCC
 TAAACTGTGCATCTACAGCACCAGCAATCTCGCCAGCGGCGTGCCCCCTAGGT
 TTTCCGGAAGCGGAAGCACCAGCTACTCTTTAACCATCTCCTCCATGGAGGCT
 GAGGATGCCGCCACCTACTTTTGTACACAGTACCACCGGTCCCCCACCTTCGG
 15 AGGCGGCACCAAACCTGGAGACAAAGAGG (SEQ ID NO: 4).

Exemplary Embodiments of Single-Chain Chimeric Polypeptides- Type B

In some embodiments of any of the single-chain chimeric polypeptides described herein, the first target-binding domain and/or the second target-binding domain can
 20 independently bind specifically to an IL-2 receptor (e.g., human IL-2 receptor).

In some embodiments of these single-chain chimeric polypeptides, the first target-binding domain and the soluble tissue factor domain directly abut each other. In some embodiments of these single-chain chimeric polypeptides, the single-chain chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linkers
 25 described herein) between the first target-binding domain and the soluble tissue factor domain.

In some embodiments of these single-chain chimeric polypeptides, the soluble tissue factor domain and the second target-binding domain directly abut each other. In some embodiments of these single-chain chimeric polypeptides, the single-chain chimeric
 30 polypeptide further includes a linker sequence (e.g., any of the exemplary linkers

described herein) between the soluble tissue factor domain and the second target-binding domain.

In some embodiments of these single-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain is a soluble human IL-2 protein. A non-limiting example of an IL-2 protein that binds specifically to an IL-2 receptor can include a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKH
LQCLEELKPLEEVNLNAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADET
ATIVEFLNRWITFCQSIISTLT (SEQ ID NO: 28).

In some embodiments, an IL-2 protein that binds specifically to an IL-2 receptor can be encoded by a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

GCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCATTACT
GCTGGATTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCAAACCT
CACCAGGATGCTCACATTTAAGTTTACATGCCCAAGAAGGCCACAGAACTGA
AACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAA
TTTAGCTCAAAGCAAAAACCTTTCACTTAAGACCCAGGGACTTAATCAGCAATA
TCAACGTAATAGTTCTGGAATAAAGGGATCTGAAACAACATTCATGTGTGAA
TATGCTGATGAGACAGCAACCATTTGTAGAATTTCTGAACAGATGGATTACCTTT
TGTCAAAGCATCATCTCAACACTAACT (SEQ ID NO: 106).

In some embodiments, an IL-2 protein that binds specifically to an IL-2 receptor can be encoded by a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

GCCCCACCTCCTCCTCCACCAAGAAGACCCAGCTGCAGCTGGAGCATTACT
 GCTGGATTACAGATGATTTTAAACGGCATCAACAACACTACAAGAACCCCAAGC
 TGACTCGTATGCTGACCTTCAAGTTCTACATGCCCCAAGAAGGCCACCGAGCTG
 AAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAGGTGCTGA
 5 ATTTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAATCAGCAACA
 TCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTCATGTGCGAG
 TACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTTGGATCACCTT
 CTGCCAGTCCATCATCTCCACTTTAACC (SEQ ID NO: 107)

In some embodiments of these single-chain chimeric polypeptides, the soluble
 10 tissue factor domain can be any of the exemplary soluble tissue factor domains described
 herein.

In some embodiments, a single-chain chimeric polypeptide can include a
 sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical,
 at least 86% identical, at least 88% identical, at least 90% identical, at least 92%
 15 identical, at least 94% identical, at least 96% identical, at least 98% identical, at least
 99% identical, or 100% identical) to:

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKH
 LQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADET
 ATIVEFLNRWITFCQSIISTLTSGTTNTVAAYNLWKSTNFKTILEWEPKPVNQVYT
 20 VQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG
 EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDV
 FGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRVTNRK
 STDSPVECMGQEKGEFREAPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLT
 RMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIV
 25 LELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT (SEQ ID NO: 108).

In some embodiments, a single-chain chimeric polypeptide is encoded by a
 sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical,
 at least 86% identical, at least 88% identical, at least 90% identical, at least 92%
 identical, at least 94% identical, at least 96% identical, at least 98% identical, at least
 30 99% identical, or 100% identical) to:

GCCCCACCTCCTCCTCCACCAAGAAGACCCAGCTGCAGCTGGAGCATTACT
GCTGGATTACAGATGATTTTAAACGGCATCAACAACACTACAAGAACCCCAAGC
TGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCACCGAGCTG
AAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAGGTGCTGA
5 ATTTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAATCAGCAACA
TCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTCATGTGCGAG
TACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTTGGATCACCTT
CTGCCAGTCCATCATCTCCACTTTAACCAGCGGCACAACCAACACAGTCGCTG
CCTATAACCTCACTTGGAAGAGCACCAACTTCAAAACCATCCTCGAATGGGAA
10 CCCAAACCCGTTAACCAAGTTTACACCGTGAGATCAGCACCAAGTCCGGCG
ACTGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTCACCGAT
GAGATCGTGAAAGATGTGAAACAGACCTACCTCGCCCCGGGTGTTTAGCTACCC
CGCCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACGAGAAC
AGCCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCCACCATCCA
15 AAGCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGGAGGACGAGCG
GACTTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCA
AAGATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGA
CAGCTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAAGGCGAAAA
CTACTGTTTCAGCGTGCAAGCTGTGATCCCCCTCCCGGACCGTGAATAGGAAAA
20 GCACCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGA
GGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCATTAC
TGCTGGATTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCAAAC
TCACCAGGATGCTCACATTAAAGTTTTACATGCCCAAGAAGGCCACAGAACTG
AAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAA
25 ATTTAGCTCAAAGCAAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAAT
ATCAACGTAATAGTTCTGGAATAAAGGGATCTGAAACAACATTCATGTGTGA
ATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGATGGATTACCTT
TTGTCAAAGCATCATCTCAACACTAACT (SEQ ID NO: 109).

In some embodiments, a single-chain chimeric polypeptide can include a
30 sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical,
at least 86% identical, at least 88% identical, at least 90% identical, at least 92%

identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

MKWVTFISLLFLFSSAYSAPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTR
 MLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVL
 5 ELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTSGTTNTVAAYNLTWKSTN
 FKILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLA
 RVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVED
 ERTLVRNNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGEN
 YCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFREAPTSSTKKTQLQLEHLLLDL
 10 QMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSK
 NFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT
 (SEQ ID NO: 110).

In some embodiments, a single-chain chimeric polypeptide is encoded by a
 sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical,
 15 at least 86% identical, at least 88% identical, at least 90% identical, at least 92%
 identical, at least 94% identical, at least 96% identical, at least 98% identical, at least
 99% identical, or 100% identical) to:

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCTCCAGCGCCTACTCC
 GCCCCACCTCCTCCTCCACCAAGAAGACCCAGCTGCAGCTGGAGCATTACT
 20 GCTGGATTACAGATGATTTTAAACGGCATCAACAATAACAAGAACCCCAAGC
 TGAATCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCACCGAGCTG
 AAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCCTCGAGGAGGTGCTGA
 ATTTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAATCAGCAACA
 TCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTCATGTGCGAG
 25 TACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTTGGATCACCTT
 CTGCCAGTCCATCATCTCCACTTTAACCAGCGGCACAACCAACACAGTCGCTG
 CCTATAACCTCACTTGGAAGAGCACCAACTTCAAAACCATCCTCGAATGGGAA
 CCCAAACCCGTTAACCAAGTTTACACCGTGCAGATCAGCACCAAGTCCGGCG
 ACTGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTCACCGAT
 30 GAGATCGTGAAAGATGTGAAACAGACCTACCTCGCCCGGGTGTTTAGCTACCC
 CGCCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACGAGAAC

AGCCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCCACCATCCA
 AAGCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGGAGGACGAGCG
 GACTTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCA
 AAGATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGA
 5 CAGCTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAAGGCGAAAA
 CTACTGTTTCAGCGTGCAAGCTGTGATCCCCCTCCCGGACCGTGAATAGGAAAA
 GCACCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGA
 GGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCATTTAC
 TGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCCAAC
 10 TCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAACTG
 AAACATCTTCAGTGTCTAGAAGAAGAAGTCAAACCTCTGGAGGAAGTGCTAA
 ATTTAGCTCAAAGCAAAAACCTTCACTTAAGACCCAGGGACTTAATCAGCAAT
 ATCAACGTAATAGTTCTGGAAGTAAAGGGATCTGAAACAACATTCATGTGTGA
 ATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGATGGATTACCTT
 15 TTGTCAAAGCATCATCTCAACACTAACT (SEQ ID NO: 111).

Exemplary Embodiments of Single-Chain Chimeric Polypeptides- Type C

In some embodiments of any of the single-chain chimeric polypeptides described
 herein, the first target-binding domain and/or the second target-binding domain can
 20 independently bind specifically to an IL-15 receptor (e.g., a human IL-15 receptor).

In some embodiments of these single-chain chimeric polypeptides, the first target-
 binding domain and the soluble tissue factor domain directly abut each other. In some
 embodiments of these single-chain chimeric polypeptides, the single-chain chimeric
 polypeptide further includes a linker sequence (e.g., any of the exemplary linkers
 25 described herein) between the first target-binding domain and the soluble tissue factor
 domain.

In some embodiments of these single-chain chimeric polypeptides, the soluble
 tissue factor domain and the second target-binding domain directly abut each other. In
 some embodiments of these single-chain chimeric polypeptides, the single-chain chimeric
 30 polypeptide further includes a linker sequence (e.g., any of the exemplary linkers

described herein) between the soluble tissue factor domain and the second target-binding domain.

In some embodiments of these single-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain is a soluble human IL-15 protein.

5 A non-limiting example of an IL-15 protein that binds specifically to an IL-15 receptor can include a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

10 NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCCKVTAMKCFLELQVISLESGD
ASIHDTVENLIILANNSLSSNGNVTESGCKECELEEKNIKEFLQSFVHIVQMFINT
S (SEQ ID NO: 39).

In some embodiments, an IL-15 protein that binds specifically to an IL-15 receptor can be encoded by a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

AACTGGGTGAACGTGATCAGCGATTTAAAGAAGATCGAGGATTTAATCCAGAG
CATGCACATCGACGCCACTCTGTACACTGAGAGCGACGTGCACCCTAGCTGCA
20 AGGTGACTGCCATGAAGTGCTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAG
AGCGGCGATGCCAGCATCCACGACACTGTGGAGAATTTAATCATTTTAGCCAA
CAACTCTTTAAGCAGCAACGGCAACGTGACAGAGAGCGGCTGCAAGGAGTGC
GAGGAGCTGGAGGAGAAGAACATCAAGGAGTTTTTACAGAGCTTCGTGCACA
TCGTGCAGATGTTTCATCAACACTAGC (SEQ ID NO: 112).

25 In some embodiments, an IL-15 protein that binds specifically to an IL-15 receptor can be encoded by a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

30 AACTGGGTGAACGTGATCAGCGATTTAAAGAAGATCGAAGATTTAATTCAGTC
CATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTCTTGTA

GGTGACCGCCATGAAATGTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAGAG
 CGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTTAGCCAATA
 ACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAGGAGTGCGA
 AGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTGTGCACATTG
 5 TCCAGATGTTTCATCAATACCTCC (SEQ ID NO: 113)

In some embodiments of these single-chain chimeric polypeptides, the soluble tissue factor domain can be any of the exemplary soluble tissue factor domains described herein.

In some embodiments, a single-chain chimeric polypeptide can include a
 10 sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLESGD
 15 ASIHDTVENLIILANNSLSSNGNVTESGCKECELEEKNIKEFLQSFVHIVQMFINT
 SSGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTT
 DTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLG
 QPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTYWYKSSSSG
 KKTAKTNTNEFLIDVDKGENYCFVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE
 20 NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLESGD
 ASIHDTVENLIILANNSLSSNGNVTESGCKECELEEKNIKEFLQSFVHIVQMFINT
 S (SEQ ID NO: 114).

In some embodiments, a single-chain chimeric polypeptide is encoded by a
 sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical,
 25 at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

AACTGGGTGAACGTGATCAGCGATTTAAAGAAGATCGAGGATTTAATCCAGAG
 CATGCACATCGACGCCACTCTGTACACTGAGAGCGACGTGCACCCTAGCTGCA
 30 AGGTGACTGCCATGAAGTGCTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAG
 AGCGGCGATGCCAGCATCCACGACACTGTGGAGAATTTAATCATTTTAGCCAA

CAACTCTTTAAGCAGCAACGGCAACGTGACAGAGAGCGGCTGCAAGGAGTGC
GAGGAGCTGGAGGAGAAGAACATCAAGGAGTTTTTACAGAGCTTCGTGCACA
TCGTGCAGATGTTTCATCAACACTAGCAGCGGCACAACCAACACAGTCGCTGCC
TATAACCTCACTTGGAAGAGCACCAACTTCAAAACCATCCTCGAATGGGAACC
5 CAAACCCGTTAACCAAGTTTACACCGTGAGATCAGCACCAAGTCCGGCGAC
TGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTCACCGATGA
GATCGTGAAAGATGTGAAACAGACCTACCTCGCCCGGGTGTTTAGCTACCCCG
CCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACGAGAACAG
CCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCCACCATCCAAA
10 GCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGGAGGACGAGCGGAC
TTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGTTCCGGCAAAG
ATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGACAG
CTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAAGGCGAAAATA
CTGTTTCAGCGTGCAAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAAGCA
15 CCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAGAA
CTGGGTGAACGTCATCAGCGATTAAAGAAGATCGAAGATTTAATTCAGTCCA
TGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTCTTGTAAGG
TGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAGAGCG
GAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTTAGCCAATAACT
20 CTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAGGAGTGCGAAGA
GCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTGTGCACATTGTCC
AGATGTTTCATCAATACCTCC (SEQ ID NO: 115).

In some embodiments, a single-chain chimeric polypeptide can include a
sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical,
25 at least 86% identical, at least 88% identical, at least 90% identical, at least 92%
identical, at least 94% identical, at least 96% identical, at least 98% identical, at least
99% identical, or 100% identical) to:

MKWVTFISLLFLFSSAYSNWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVT
AMKCFLELQVISLESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEK
30 NIKEFLQSFVHIVQMFINTSSGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTV
QISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGE

PLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVF
GKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFQAVIPSRVNRKS
TDSPVECMGQEKGEFRENWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVT
AMKCFLELQVISLESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEK
5 NIKEFLQSFVHIVQMFINTS (SEQ ID NO: 116).

In some embodiments, a single-chain chimeric polypeptide is encoded by a
sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical,
at least 86% identical, at least 88% identical, at least 90% identical, at least 92%
identical, at least 94% identical, at least 96% identical, at least 98% identical, at least
10 99% identical, or 100% identical) to:

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCTCCAGCGCCTACTCC
AACTGGGTGAACGTGATCAGCGATTTAAAGAAGATCGAGGATTTAATCCAGAG
CATGCACATCGACGCCACTCTGTACACTGAGAGCGACGTGCACCCTAGCTGCA
AGGTGACTGCCATGAAGTGCTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAG
15 AGCGGCGATGCCAGCATCCACGACACTGTGGAGAATTTAATCATTTTAGCCAA
CAACTCTTTAAGCAGCAACGGCAACGTGACAGAGAGCGGCTGCAAGGAGTGC
GAGGAGCTGGAGGAGAAGAACATCAAGGAGTTTTTACAGAGCTTCGTGCACA
TCGTGCAGATGTTTCATCAACACTAGCAGCGGCACAACCAACACAGTCGCTGCC
TATAACCTCACTTGGAAGAGCACCAACTTCAAAACCATCCTCGAATGGGAACC
20 CAAACCCGTTAACCAAGTTTACACCGTGCAGATCAGCACCAAGTCCGGCGAC
TGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTCACCGATGA
GATCGTGAAAGATGTGAAACAGACCTACCTCGCCCGGGTGTTTAGCTACCCCG
CCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACGAGAACAG
CCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCCACCATCCAAA
25 GCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGGAGGACGAGCGGAC
TTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGTTCCGGCAAAG
ATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGACAG
CTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAAGGCGAAAATA
CTGTTTCAGCGTGCAAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAAGCA
30 CCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAGAA
CTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATTTAATTCAGTCCA

TGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTCTTGTAAGG
 TGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAGAGCG
 GAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTTAGCCAATAACT
 CTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAGGAGTGCGAAGA
 5 GCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTGTGCACATTGTCC
 AGATGTTTCATCAATACCTCC (SEQ ID NO: 117).

Compositions/Kits

Also provided herein are compositions (e.g., pharmaceutical compositions) that include at least one of any single-chain chimeric polypeptides, any of the cells, or any of the nucleic acids described herein. In some embodiments, the compositions include at least one of any of the single-chain chimeric polypeptides described herein. In some
 10 embodiments, the compositions include any of the immune cells (e.g., any of the immune cells described herein, e.g., any of the immune cells produced using any of the methods described herein).

15 In some embodiments, the pharmaceutical compositions are formulated for different routes of administration (e.g., intravenous, subcutaneous). In some embodiments, the pharmaceutical compositions can include a pharmaceutically acceptable carrier (e.g., phosphate buffered saline).

Single or multiple administrations of pharmaceutical compositions can be given to
 20 a subject in need thereof depending on for example: the dosage and frequency as required and tolerated by the subject. The formulation should provide a sufficient quantity of active agent to effectively treat, prevent or ameliorate conditions, diseases or symptoms.

Also provided herein are kits that include any of the single-chain chimeric polypeptides, compositions, nucleic acids, or cells (e.g., immune cells) described herein.
 25 In some embodiments, the kits can include instructions for performing any of the methods described herein. In some embodiments, the kits can include at least one dose of any of the pharmaceutical compositions described herein.

Nucleic Acids/Vectors

Also provided herein are nucleic acids that encode any of the single-chain
 30 chimeric polypeptides described herein. Also provided herein are vectors that include

any of the nucleic acids encoding any of the single-chain chimeric polypeptides described herein.

Any of the vectors described herein can be an expression vector. For example, an expression vector can include a promoter sequence operably linked to the sequence
5 encoding the single-chain chimeric polypeptide.

Non-limiting examples of vectors include plasmids, transposons, cosmids, and viral vectors (e.g., any adenoviral vectors (e.g., pSV or pCMV vectors), adeno-associated virus (AAV) vectors, lentivirus vectors, and retroviral vectors), and any Gateway®
10 vectors. A vector can, e.g., include sufficient cis-acting elements for expression; other elements for expression can be supplied by the host mammalian cell or in an in vitro expression system. Skilled practitioners will be capable of selecting suitable vectors and mammalian cells for making any of the single-chain chimeric polypeptides described herein.

Cells

15 Also provided herein are cells (e.g., any of the exemplary cells described herein or known in the art) comprising any of the nucleic acids described herein that encode any of the single-chain chimeric polypeptides described herein.

Also provided herein are cells (e.g., any of the exemplary cells described herein or known in the art) that include any of the vectors described herein that encode any of the
20 single-chain chimeric polypeptides described herein.

In some embodiments of any of the methods described herein, the cell can be a eukaryotic cell. As used herein, the term “eukaryotic cell” refers to a cell having a distinct, membrane-bound nucleus. Such cells may include, for example, mammalian (e.g., rodent, non-human primate, or human), insect, fungal, or plant cells. In some
25 embodiments, the eukaryotic cell is a yeast cell, such as *Saccharomyces cerevisiae*. In some embodiments, the eukaryotic cell is a higher eukaryote, such as mammalian, avian, plant, or insect cells. Non-limiting examples of mammalian cells include Chinese hamster ovary cells and human embryonic kidney cells (e.g., HEK293 cells).

Methods of introducing nucleic acids and expression vectors into a cell (e.g., an
30 eukaryotic cell) are known in the art. Non-limiting examples of methods that can be used

to introduce a nucleic acid into a cell include lipofection, transfection, electroporation, microinjection, calcium phosphate transfection, dendrimer-based transfection, cationic polymer transfection, cell squeezing, sonoporation, optical transfection, impalefection, hydrodynamic delivery, magnetofection, viral transduction (e.g., adenoviral and lentiviral
5 transduction), and nanoparticle transfection.

Methods of Producing Single-Chain Chimeric Polypeptides

Also provided herein are methods of producing any of the single-chain chimeric polypeptides described herein that include culturing any of the cells described herein in a culture medium under conditions sufficient to result in the production of the single-chain
10 chimeric polypeptide; and recovering the single-chain chimeric polypeptide from the cell and/or the culture medium.

The recovery of the single-chain chimeric polypeptide from a culture medium or a cell (e.g., a eukaryotic cell) can be performed using techniques well-known in the art (e.g., ammonium sulfate precipitation, polyethylene glycol precipitation, ion-exchange
15 chromatography (anion or cation), chromatography based on hydrophobic interaction, metal-affinity chromatography, ligand-affinity chromatography, and size exclusion chromatography).

Methods of culturing cells are well known in the art. Cells can be maintained *in vitro* under conditions that favor proliferation, differentiation and growth. Briefly, cells
20 can be cultured by contacting a cell (e.g., any cell) with a cell culture medium that includes the necessary growth factors and supplements to support cell viability and growth.

Also provided herein are single-chain chimeric polypeptides (e.g., any of the single-chain chimeric polypeptides described herein) produced by any of the methods
25 described herein.

Methods of Stimulating an Immune Cell

Also provided herein are methods of stimulating an immune cell (e.g., any of the exemplary immune cells described herein or known in the art) that include contacting an
30 immune cell with an effective amount of any of the single-chain chimeric polypeptides

described herein or any of the compositions (e.g., pharmaceutical compositions) described herein. In some examples, the immune cell is contacted *in vitro* (e.g., in a suitable liquid culture medium under conditions sufficient to result in stimulation of the immune cell).

5 In some examples, the immune cell has been previously obtained from a subject (e.g., a mammal, e.g., a human). Some embodiments of these methods further include obtaining the immune cell from the subject prior to the contacting step.

In some examples, the immune cell is contacted *in vivo*. In such embodiments, the single-chain chimeric polypeptide is administered to a subject (e.g., a mammal, e.g., a
10 human) in an amount sufficient to result in stimulation of an immune cell in the subject.

In some examples of any of the methods described herein, the immune cell can be an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a memory T cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-
15 infiltrating T cell, a CD8⁺ T cell, a CD4⁺ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, or a natural killer cell, or a combination thereof.

In some examples, the immune cell has previously been genetically-modified to express a chimeric antigen receptor or a recombinant T-cell receptor. In some examples,
20 the immune cell (e.g., any of the immune cells described herein) has previously been genetically-modified to express a co-stimulatory molecule (e.g., CD28).

Some embodiments of these methods can further include, after the contacting step, introducing into the immune cell (e.g., any of the immune cells described herein) a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor.

25 Some embodiments of these methods can further include, after the contacting step, introducing into the immune cell (e.g., any of the immune cells described herein) a nucleic acid encoding a co-stimulatory molecule (e.g., CD28).

Some embodiments of these methods can further include administering a therapeutically effective amount of the immune cell to a subject in need thereof (e.g., any
30 of the exemplary subjects described herein).

In some examples, the subject can be a subject identified or diagnosed as having an age-related disease or condition. Non-limiting examples of age-related diseases or disorders include: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, 5 intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular 10 degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

In some examples, the subject can be a subject that has been identified or diagnosed as having a cancer. Non-limiting examples of cancers include: solid tumor, 15 hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), 20 cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

25 In some examples, the subject can be a subject that has been diagnosed or identified as having an infectious disease. Non-limiting examples of infectious disease include infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, or influenza virus.

30 Activation of an immune cell can be determined using methods known in the art. For example, activation of an immune cell can be determined by detecting the levels of

cytokines and chemokines that are secreted upon activation of an immune cell. Non-limiting examples of cytokines, chemokines, and regulatory molecules that are secreted or upregulated upon activation of an immune cell include: IL-2, IFN- γ , IL-1, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-22, IL-33, leukotriene B₄, CCL5, TNF α , granzymes, perforin, TGF β , STAT3, ROR γ T, FOXP3, STAT6, and GATA3. The detection of these cytokines and chemokines or regulatory molecules can be performed using an immunoassay (e.g., an enzyme-linked immunosorbent assay) or quantitative PCR. For example, activation of an immune cell can result in an increase of about 1% to about 800% (e.g., about 1% to about 750%, about 1% to about 700%, about 1% to about 650%, about 1% to about 600%, about 1% to about 550%, about 1% to about 500%, about 1% to about 450%, about 1% to about 400%, about 1% to about 350%, about 1% to about 300%, about 1% to about 280%, about 1% to about 260%, about 1% to about 240%, about 1% to about 220%, about 1% to about 200%, about 1% to about 180%, about 1% to about 160%, about 1% to about 140%, about 1% to about 120%, about 1% to about 100%, about 1% to about 90%, about 1% to about 80%, about 1% to about 70%, about 1% to about 60%, about 1% to about 50%, about 1% to about 45%, about 1% to about 40%, about 1% to about 35%, about 1% to about 30%, about 1% to about 25%, about 1% to about 20%, about 1% to about 15%, about 1% to about 10%, about 1% to about 5%, about 5% to about 800%, about 5% to about 750%, about 5% to about 700%, about 5% to about 650%, about 5% to about 600%, about 5% to about 550%, about 5% to about 500%, about 5% to about 450%, about 5% to about 400%, about 5% to about 350%, about 5% to about 300%, about 5% to about 280%, about 5% to about 260%, about 5% to about 240%, about 5% to about 220%, about 5% to about 200%, about 5% to about 180%, about 5% to about 160%, about 5% to about 140%, about 5% to about 120%, about 5% to about 100%, about 5% to about 90%, about 5% to about 80%, about 5% to about 70%, about 5% to about 60%, about 5% to about 50%, about 5% to about 45%, about 5% to about 40%, about 5% to about 35%, about 5% to about 30%, about 5% to about 25%, about 5% to about 20%, about 5% to about 15%, about 5% to about 10%, about 10% to about 800%, about 10% to about 750%, about 10% to about 700%, about 10% to about 650%, about 10% to about 600%, about 10% to about 550%, about 10% to about 500%, about 10% to about 450%, about 10% to about 400%,

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Methods of Inducing or Increasing Proliferation of an Immune Cell

Also provided herein are methods of inducing or increasing proliferation of an immune cell (e.g., any of the exemplary immune cells described herein or known in the art) that include contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides described herein or any of the compositions (e.g., pharmaceutical compositions) described herein. In some examples, the immune cell is contacted *in vitro* (e.g., in a suitable liquid culture medium under conditions sufficient to result in stimulation of the immune cell).

In some examples, the immune cell has been previously obtained from a subject (e.g., a mammal, e.g., a human). Some embodiments of these methods further include obtaining the immune cell from the subject prior to the contacting step.

In some examples, the immune cell is contacted *in vivo*. In such embodiments, the single-chain chimeric polypeptide is administered to a subject (e.g., a mammal, e.g., a human) in an amount sufficient to result in stimulation of an immune cell in the subject.

In some examples of any of the methods described herein, the immune cell can be an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a memory T cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T cell, a CD4⁺ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, or a natural killer cell, or a combination thereof.

In some examples, the immune cell has previously been genetically-modified to express a chimeric antigen receptor or a recombinant T-cell receptor. In some examples, the immune cell (e.g., any of the immune cells described herein) has previously been genetically-modified to express a co-stimulatory molecule (e.g., CD28).

Some embodiments of these methods can further include, after the contacting step, introducing into the immune cell (e.g., any of the immune cells described herein) a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor. Some embodiments of these methods can further include, after the contacting step, introducing into the immune cell (e.g., any of the immune cells described herein) a nucleic acid encoding a co-stimulatory molecule (e.g., CD28).

Some embodiments of these methods can further include administering a therapeutically effective amount of the immune cell to a subject in need thereof (e.g., any of the exemplary subjects described herein).

In some examples, the subject can be a subject identified or diagnosed as having an age-related disease or condition. Non-limiting examples of age-related diseases or disorders include: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis,

amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

In some examples, the subject can be a subject that has been identified or diagnosed as having a cancer. Non-limiting examples of cancers include: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

In some examples, the subject can be a subject that has been diagnosed or identified as having an infectious disease. Non-limiting examples of infectious disease include infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, or influenza virus.

Detection of the proliferation of an immune cell can be performed using methods known in the art, e.g., cytometry (e.g., fluorescence-assisted flow cytometry), microscopy, and immunofluorescence microscopy, e.g., by comparing the rate of increase in the concentration of the immune cell in a sample not contacted with a single-chain chimeric polypeptide to the rate of increase in the concentration of the immune cell in a similar sample contacted with any of the single-chain chimeric polypeptides described herein).

In other examples, the proliferation of an immune cell can be indirectly detected by detecting an increase in the level of one or more cytokines or chemokines secreted or regulatory molecules by proliferating immune cells (e.g., one or more of IL-2, IFN- γ , IL-1, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-22, IL-33, leukotriene B4, CCL5, TNF α , granzymes, perforin, TGF β , STAT3, ROR γ T, FOXP3,

STAT6, and GATA3) (e.g., as compared to the level of the one or more cytokines and chemokines in a control not contacted with any of the single-chain chimeric polypeptides described herein).

5 In some embodiments, the methods provided herein can result in an increase (e.g., about 1% to about 800% increase, or any of the subranges of this range described herein) in the rate of increase in the concentration of the immune cell in a sample contacted with any of the single-chain chimeric polypeptides described herein as compared to the rate of increase in a similar control sample not contacted with any of the single-chain chimeric polypeptides described herein.

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Methods of Inducing Differentiation of an Immune Cell

Also provided herein are method of inducing differentiation of an immune cell (e.g., any of the exemplary immune cells described herein or known in the art) into a memory or memory-like immune cell that include contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides described herein or any of the compositions (e.g., pharmaceutical compositions) described herein. In some examples, the immune cell is contacted in vitro (e.g., in a suitable liquid culture medium under conditions sufficient to result in stimulation of the immune cell).

20 In some examples, the immune cell has been previously obtained from a subject (e.g., a mammal, e.g., a human). Some embodiments of these methods further include obtaining the immune cell from the subject prior to the contacting step.

In some examples, the immune cell is contacted in vivo. In such embodiments, the single-chain chimeric polypeptide is administered to a subject (e.g., a mammal, e.g., a human) in an amount sufficient to result in stimulation of an immune cell in the subject.

25 In some examples of any of the methods described herein, the immune cell can be an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8+ T cell, a CD4+ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, or a natural killer cell, or a combination thereof.

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In some examples, the immune cell has previously been genetically-modified to express a chimeric antigen receptor or a recombinant T-cell receptor. In some examples, the immune cell (e.g., any of the immune cells described herein) has previously been genetically-modified to express a co-stimulatory molecule (e.g., CD28).

5 Some embodiments of these methods can further include, after the contacting step, introducing into the immune cell (e.g., any of the immune cells described herein) a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor. Some embodiments of these methods can further include, after the contacting step, introducing into the immune cell (e.g., any of the immune cells described herein) a
10 nucleic acid encoding a co-stimulatory molecule (e.g., CD28).

Some embodiments of these methods can further include administering a therapeutically effective amount of the immune cell to a subject in need thereof (e.g., any of the exemplary subjects described herein).

In some examples, the subject can be a subject identified or diagnosed as having
15 an age-related disease or condition. Non-limiting examples of age-related diseases or disorders include: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic
20 pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple
25 sclerosis, and renal dysfunction.

In some examples, the subject can be a subject that has been identified or diagnosed as having a cancer. Non-limiting examples of cancers include: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma,
30 B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia

(CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

In some examples, the subject can be a subject that has been diagnosed or identified as having an infectious disease. Non-limiting examples of infectious disease include infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, or influenza virus.

In some examples, the immune cell is a NK cell, and the detection of a memory NK cell can include, e.g., the detection of the increased level of one or more of CD25, CD69, CD62L, IL-12, IL-18, IL-33, STAT4, STAT5, Zbtb32, DNAM-1, NKp30, NKp40, NKp46, BIM, Noxa, SOCS1, BNIP3, BNIP3L, IFN- γ , CXCL16, CXCR6, NKG2D, TRAIL, CD49, Ly49D, CD49b, and Ly79H. A description of NK memory cells and methods of detecting the same is described in O'Sullivan et al., *Immunity* 43:634-645, 2015.

In some examples, the immune cell is a T cell, and the detection of memory T cells can include, e.g., the detection of the level of expression of one or more of CD45RO, CCR7, L-selectin (CD62L), CD44, CD45RA, integrin $\alpha\beta$ 7, CD43, CD27, CD28, IL-7R α , CD95, IL-2R β , CXCR3, and LFA-1. In some examples, the immune cell is a B cell and the detection of memory B cells can include, e.g., the detection of the level of expression of CD27. Other types and markers of memory or memory-like immune cells are known in the art.

Methods of Treatment

Also provided herein are methods of treating a subject in need thereof (e.g., any of the exemplary subjects described herein or known in the art) that include administering to the subject a therapeutically effective amount of any of the single-chain chimeric

polypeptides described herein or any of the compositions (e.g., pharmaceutical compositions) described herein.

In some embodiments of these methods, the subject has been identified or diagnosed as having a cancer. Non-limiting examples of cancer include: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma. In some embodiments, these methods can result in a reduction in the number, severity, or frequency of one or more symptoms of the cancer in the subject (e.g., as compared to the number, severity, or frequency of the one or more symptoms of the cancer in the subject prior to treatment). In some embodiments, these methods can result in a reduction (e.g., about 1% reduction to about 99% reduction, about 1% reduction to about 95% reduction, about 1% reduction to about 90% reduction, about 1% reduction to about 85% reduction, about 1% reduction to about 80% reduction, about 1% reduction to about 75% reduction, about 1% reduction to about 70% reduction, about 1% reduction to about 65% reduction, about 1% reduction to about 60% reduction, about 1% reduction to about 55% reduction, about 1% reduction to about 50% reduction, about 1% reduction to about 45% reduction, about 1% reduction to about 40% reduction, about 1% reduction to about 35% reduction, about 1% reduction to about 30% reduction, about 1% reduction to about 25% reduction, about 1% reduction to about 20% reduction, about 1% reduction to about 15% reduction, about 1% reduction to about 10% reduction, about 1% reduction to about 5% reduction, about 5% reduction to about 99% reduction, about 5% reduction to about 95% reduction, about 5% reduction to about 90% reduction, about 5% reduction to about 85% reduction, about 5% reduction to about 80% reduction, about 5% reduction to about 75% reduction, about 5% reduction to about 70% reduction,

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In some examples of these methods, the subject has been identified or diagnosed as having an aging-related disease or condition. Non-limiting examples of aging-related diseases and conditions include Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction. In some examples, these methods can result in a reduction in the number, severity, or frequency of one or more symptoms of the aging-related disease or condition in the subject (e.g., as compared to the number, severity, or frequency of the one or more symptoms of the aging-related disease or condition in the subject prior to treatment). In some examples, the methods can result in a decrease (e.g., about 1% decrease to about 99% decrease, an about 1% decrease to about 95% decrease, about 1% decrease to about 90% decrease, about 1% decrease to about 85% decrease,

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In some examples of these methods, the subject has been diagnosed or identified as having an infectious disease. Non-limiting examples of infectious disease include infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus. In some embodiments, these methods can result in a decrease in the infectious titer (e.g., viral titer) in a subject (e.g., as compared to the infectious titer in the subject prior to treatment). In some embodiments, these methods can result in a reduction in the number, severity, or frequency of one or more symptoms of the infectious disease (e.g., viral infection) in the subject (e.g., as compared to the number, severity, or frequency of the one or more symptoms of the infectious disease in the subject prior to treatment).

The term “subject” refers to any mammal. In some embodiments, the subject or “subject in need of treatment” may be a canine (e.g., a dog), feline (e.g., a cat), equine (e.g., a horse), ovine, bovine, porcine, caprine, primate, e.g., a simian (e.g., a monkey (e.g., marmoset, baboon), or an ape (e.g., a gorilla, chimpanzee, orangutan, or gibbon) or a human; or rodent (e.g., a mouse, a guinea pig, a hamster, or a rat). In some embodiments, the subject or “subject in need of treatment” may be a non-human

mammal, especially mammals that are conventionally used as models for demonstrating therapeutic efficacy in humans (e.g., murine, lapine, porcine, canine or primate animals) may be employed.

5 **Methods of Killing a Cancer Cell, an Infected Cell, or a Senescent Cell**

Also provided herein are methods of killing a cancer cell (e.g., any of the exemplary types of cancer described herein or known in the art), an infected cell (e.g., a cell infected with any of the exemplary viruses described herein or known in the art), or a senescent cell (e.g., a senescent cancer cell, a senescent fibroblast, or a senescent
10 endothelial cell) in a subject in need thereof (e.g., any of the exemplary subjects described herein or known in the art) that include administering to the subject a therapeutically effective amount of any of the single-chain chimeric polypeptides described herein or any of the compositions (e.g., pharmaceutical compositions) described herein.

15 In some embodiments of these methods, the subject has been identified or diagnosed as having a cancer. Non-limiting examples of cancer include: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic
20 lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma,
25 squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

In some examples of these methods, the subject has been identified or diagnosed as having an aging-related disease or condition. Non-limiting examples of aging-related diseases and conditions include Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in
30 pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2

diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular
5 degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

In some examples of these methods, the subject has been diagnosed or identified as having an infectious disease. Non-limiting examples of an infectious disease include
10 infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Senescent Cells

15 Senescence is a form of irreversible growth arrest accompanied by phenotypic changes, resistance to apoptosis and activation of damage-sensing signaling pathways. Cellular senescence was first described in cultured human fibroblast cells that lost their ability to proliferate, reaching permanent arrest after about 50 population doublings (referred to as the Hayflick limit). Senescence is considered a stress response that can be
20 induced by a wide range of intrinsic and extrinsic insults, including oxidative and genotoxic stress, DNA damage, telomere attrition, oncogenic activation, mitochondrial dysfunction, or chemotherapeutic agents.

Senescent cells remain metabolically active and can influence the tissue hemostasis, disease and aging through their secretory phenotype. Senescence is
25 considered as a physiologic process and is important in promoting wound healing, tissue homeostasis, regeneration, and fibrosis regulation. For instance, transient induction of senescent cells is observed during wound healing and contributes to wound resolution. Perhaps one of the most important roles of senescence is its role in tumor suppression. However, the accumulation of senescent cells also drives aging- and aging-related
30 diseases and conditions. The senescent phenotype also can trigger chronic inflammatory responses and consequently augment chronic inflammatory conditions to promote tumor

growth. The connection between senescence and aging was initially based on observations that senescent cells accumulate in aged tissue. The use of transgenic models has enabled the detection of senescent cells systematically in many age-related pathologies. Strategies to selectively eliminate senescent cells has demonstrated that
5 senescent cells can indeed play a causal role in aging and related pathologies.

Senescent cells display important and unique properties which include changes in morphology, chromatin organization, gene expression, and metabolism. There are several biochemical and functional properties associated with cellular senescence, such as (i) increased expression of p16 and p21, inhibitors of cyclin-dependent kinases, (ii)
10 presence of senescence-associated β -galactosidase, a marker of lysosomal activity, (iii) appearance of senescence-associated heterochromatin foci and downregulation of lamin B1 levels, (iv) resistance to apoptosis caused by an increased expression of anti-apoptotic BCL-family protein, and (v) upregulation of CD26 (DPP4), CD36 (Scavenger receptor), forkhead box 4 (FOXO4), and secretory carrier membrane protein 4 (SCAMP4).

15 Senescent cells also express an inflammatory signature, the so-called senescence-associated secretory phenotype (SASP). Through SASP, the senescent cells produce a wide range of inflammatory cytokines (IL-6, IL-8), growth factors (TGF- β), chemokines (CCL-2), and matrix metalloproteinases (MMP-3, MMP-9) that operate in a cell-autonomous manner to reinforce senescence (autocrine effects) and communicate with
20 and modify the microenvironment (paracrine effects). SASP factors can contribute to tumor suppression by triggering senescence surveillance, an immune-mediated clearance of senescent cells. However, chronic inflammation is also a known driver of tumorigenesis, and accumulating evidence indicates that chronic SASP can also boost cancer and aging-related diseases.

25 The secretion profile of senescent cells is context dependent. For instance, the mitochondrial dysfunction-associated senescence (MiDAS), induced by different mitochondrial dysfunction in human fibroblasts, led to the appearance of a SASP that was deficient in IL-1-dependent inflammatory factors. A decrease in the NAD⁺/NADH ratio activated AMPK signaling which induced MiDAS through the activation of p53. As a
30 result, p53 inhibited NF- κ B signaling which is a crucial inducer of pro-inflammatory SASP. In contrast, the cellular senescence caused by persistent DNA damage in human

cells induced an inflammatory SASP, which was dependent on the activation of ataxia-telangiectasia mutated (ATM) kinase but not on that of p53. In particular, the expression and secretion levels of IL-6 and IL-8 were increased. It was also demonstrated that cellular senescence caused by the ectopic expression p16INK4a and p21CIP1 induced the
5 senescent phenotype in human fibroblasts without an inflammatory SASP indicating that the growth arrest itself did not stimulate SASP.

One of the most defining characteristics of senescence is stable growth arrest. This is achieved by two important pathways, the p16/Rb and the p53/p21, both of which are central in tumor suppression. DNA damage results in: (1) high deposition of γ H2Ax
10 (histone coding gene) and 53BP1 (involved in DNA damage response) in chromatin: this leads to activation of a kinase cascade eventually resulting in p53 activation, and (2) activation of p16INK4a and ARF (both encoded by CDKN2A) and P15INK4b (encoded by CDKN2B): p53 induces transcription of cyclin-dependent kinase inhibitor (p21) and along with both p16INK4a and p15INK4b block genes for cell cycle progression (CDK4
15 and CDK6). This eventually leads to hypophosphorylation of Retinoblastoma protein (Rb) and cell cycle arrest at the G1 phase.

Selectively killing senescent cells has been shown to significantly improve the health span of mice in the context of normal aging and ameliorates the consequences of age-related disease or cancer therapy (Ovadya, *J Clin Invest.* 128(4):1247-1254, 2018). In
20 nature, the senescent cells are normally removed by the innate immune cells. Induction of senescence not only prevents the potential proliferation and transformation of damaged/alterd cells, but also favors tissue repair through the production of SASP factors that function as chemoattractants mainly for Natural Killer (NK) cells (such as IL-15 and CCL2) and macrophages (such as CFS-1 and CCL2). These innate immune cells
25 mediate the immunosurveillance mechanism for eliminating stressed cells. Senescent cells usually up-regulate the NK-cell activating receptor NKG2D and DNAM1 ligands, which belong to a family of stress-inducible ligands: an important component of the frontline immune defense against infectious diseases and malignancies. Upon receptor activation, NK cells can then specifically induce the death of senescent cells through their
30 cytolytic machinery. A role for NK cells in the immune surveillance of senescent cells has been pointed out in liver fibrosis (Sagiv, *Oncogene* 32(15): 1971-1977, 2013),

hepatocellular carcinoma (Iannello, *J Exp Med* 210(10): 2057-2069, 2013), multiple myeloma (Soriani, *Blood* 113(15): 3503-3511, 2009), and glioma cells stressed by dysfunction of the mevalonate pathway (Ciaglia, *Int J Cancer* 142(1): 176-190, 2018). Endometrial cells undergo acute cellular senescence and do not differentiate into decidual cells. The differentiated decidual cells secrete IL-15 and thereby recruit uterine NK cells to target and eliminate the undifferentiated senescent cells thus helping to re-model and rejuvenate the endometrium (Brighton, *Elife* 6: e31274, 2017). With a similar mechanism, during liver fibrosis, p53-expressing senescent liver satellite cells skewed the polarization of resident Kupfer macrophages and freshly infiltrated macrophages toward the pro-inflammatory M1 phenotype, which display senolytic activity. F4/80+ macrophages have been shown to play a key role in the clearance of mouse uterine senescent cells to maintain postpartum uterine function.

Senescent cells recruit NK cells by mainly upregulating ligands to NKG2D (expressed on NK cells), chemokines, and other SASP factors. In vivo models of liver fibrosis have shown effective clearance of senescent cells by activated NK cells (Krizhanovsky, *Cell* 134(4): 657-667, 2008). Studies have described various models to study senescence including liver fibrosis (Krizhanovsky, *Cell* 134(4): 657-667, 2008), osteoarthritis (Xu, *J Gerontol A Biol Sci Med Sci* 72(6): 780-785, 2017), and Parkinson's disease (Chinta, *Cell Rep* 22(4): 930-940, 2018). Animal models for studying senescent cells are described in: Krizhanovsky, *Cell* 134(4): 657-667, 2008; Baker, *Nature* 479(7372): 232-236, 2011; Farr, *Nat Med* 23(9): 1072-1079, 2017; Bourgeois, *FEBS Lett* 592(12): 2083-2097, 2018; Xu, *Nat Med* 24(8): 1246-1256, 2018).

Additional Therapeutic Agents

Some embodiments of any of the methods described herein can further include administering to a subject (e.g., any of the subjects described herein) a therapeutically effective amount of one or more additional therapeutic agents. The one or more additional therapeutic agents can be administered to the subject at substantially the same time as a single-chain chimeric polypeptide (e.g., any of the single-chain chimeric polypeptides described herein) or an immune cell (e.g., administered as a single formulation or two or more formulations to the subject). In some embodiments, one or

more additional therapeutic agents can be administered to the subject prior to administration of a single-chain chimeric polypeptide (e.g., any of the single-chain chimeric polypeptides described herein) or an immune cell. In some embodiments, one or more additional therapeutic agents can be administered to the subject after
5 administration of a single-chain chimeric polypeptide (e.g., any of the single-chain chimeric polypeptides described herein) or an immune cell to the subject.

Non-limiting examples of additional therapeutic agents include: anti-cancer drugs, activating receptor agonists, immune checkpoint inhibitors, agents for blocking HLA-specific inhibitory receptors, Glucogen Synthase Kinase (GSK) 3 inhibitors, and
10 antibodies.

Non-limiting examples of anticancer drugs include antimetabolic drugs (e.g., 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, hydroxycarbamide, methotrexate, 6-thioguanine, cladribine, nelarabine, pentostatin, or pemetrexed), plant alkaloids (e.g., vinblastine, vincristine,
15 vindesine, camptothecin, 9-methoxycamptothecin, coronaridine, taxol, nucleosides, diprenylated indole alkaloid, montamine, schischkiniin, protoberberine, berberine, sanguinarine, chelerythrine, chelidonine, liriodenine, clivorine, β -carboline, antofine, tylophorine, cryptolepine, neocryptolepine, corynoline, sampangine, carbazole, crinamine, montanine, ellipticine, paclitaxel, docetaxel, etoposide, teniposide, irinotecan,
20 topotecan, or acridone alkaloids), proteasome inhibitors (e.g., lactacystin, disulfiram, epigallocatechin-3-gallate, marizomib (salinosporamide A), oprozomib (ONX-0912), delanzomib (CEP-18770), epoxomicin, MG132, beta-hydroxy beta-methylbutyrate, bortezomib, carfilzomib, or ixazomib), antitumor antibiotics (e.g., doxorubicin, daunorubicin, epirubicin, mitoxantrone, idarubicin, actinomycin, plicamycin, mitomycin,
25 or bleomycin), histone deacetylase inhibitors (e.g., vorinostat, panobinostat, belinostat, givinostat, abexinostat, depsipeptide, entinostat, phenyl butyrate, valproic acid, trichostatin A, dacinostat, mocetinostat, pracinostat, nicotinamide, cambinol, tenovin 1, tenovin 6, sirtinol, ricolinostat, tefinostat, kevetrin, quisinostat, resminostat, tacedinaline, chidamide, or selisistat), tyrosine kinase inhibitors (e.g., axitinib, dasatinib, encorafenib,
30 erlotinib, imatinib, nilotinib, pazopanib, and sunitinib), and chemotherapeutic agents (e.g., all-trans retinoic acid, azacitidine, azathioprine, doxifluridine, epothilone,

hydroxyurea, imatinib, teniposide, tioguanine, valrubicin, vemurafenib, and lenalidomide). Additional examples of chemotherapeutic agents include alkylating agents, e.g., mechlorethamine, cyclophosphamide, chlorambucil, melphalan, ifosfamide, thiotepa, hexamethylmelamine, busulfan, altretamine, procarbazine, dacarbazine, 5 temozolomide, carmustine, lumustine, streptozocin, carboplatin, cisplatin, and oxaliplatin.

Non-limiting examples of activating receptor agonists include any agonists for activating receptors which activate and enhance the cytotoxicity of NK cells, including anti-CD16 antibodies (e.g., anti-CD16/CD30 bispecific monoclonal antibody (BiMAb)) 10 and Fc-based fusion proteins. Non-limiting examples of checkpoint inhibitors include anti-PD-1 antibodies (e.g., MEDI0680), anti-PD-L1 antibodies (e.g., BCD-135, BGB-A333, CBT-502, CK-301, CS1001, FAZ053, KN035, MDX-1105, MSB2311, SHR-1316, anti-PD-L1/CTLA-4 bispecific antibody KN046, anti-PD-L1/TGFβRII fusion protein M7824, anti-PD-L1/TIM-3 bispecific antibody LY3415244, atezolizumab, or 15 avelumab), anti-TIM3 antibodies (e.g., TSR-022, Sym023, or MBG453) and anti-CTLA-4 antibodies (e.g., AGEN1884, MK-1308, or an anti-CTLA-4/OX40 bispecific antibody ATOR-1015). Non-limiting examples of agents for blocking HLA-specific inhibitory receptors include monalizumab (e.g., an anti-HLA-E NKG2A inhibitory receptor monoclonal antibody). Non-limiting examples of GSK3 inhibitor include tideglusib or 20 CHIR99021. Non-limiting examples of antibodies that can be used as additional therapeutic agents include anti-CD26 antibodies (e.g., YS110), anti-CD36 antibodies, and any other antibody or antibody construct that can bind to and activate an Fc receptor (e.g., CD16) on a NK cell. In some embodiments, an additional therapeutic agent can be insulin or metformin.

25

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

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Example 1. Production of an Exemplary Single-Chain Chimeric Polypeptides

An exemplary single-chain chimeric polypeptide including a first target-binding domain that is an anti-CD3 scFv, a soluble human tissue factor domain, and a second target-binding domain that is an anti-CD28 scFv was generated

- 5 (α CD3scFv/TF/ α CD28scFv) (Figure 1). The nucleic acid and amino acid sequences of this single-chain chimeric polypeptide are shown below.

Nucleic Acid Encoding Exemplary Single-Chain Chimeric Polypeptide

(α CD3scFv/TF/ α CD28scFv) (SEQ ID NO: 4)

- 10 (*Signal peptide*)
 ATGAAGTGGGTGACCTTCATCAGCTTATTATTTTATTCAGCTCCGCCT
 ATTCC
 (*α CD3 light chain variable region*)
 CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGT
 15 GAGAAGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACT
 GGTATCAGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCA
 GCAAGCTGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCAC
 CAGCTACTCTTTAACCATTTCGGCATGGAAGCTGAAGACGCTGCCACCTACT
 ATTGCCAGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCT
 20 CGAAATCAATCGT
 (*Linker*)
 GGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAGC
 (*α CD3 heavy chain variable region*)
 CAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCCGGCGC
 25 CTCCGTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAA
 TGCATTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATAT
 CAACCCTTCCCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCC
 ACTTTAACCCTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTT
 AACCAGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCAC
 30 TACTGTTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGC

(Human tissue factor 219 form)

TCCGGCACCACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGC
 ACCAACTTCAAGACAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTT
 ACACCGTGCAGATCTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTA
 5 CACAACAGACACCGAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAG
 CAAACCTATCTGGCTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCAC
 CGGCTCCGCTGGCGAGCCTCTCTACGAGAATTCCCCCGAATTCACCCCTTATT
 TAGAGACCAATTTAGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCAC
 CAAGGTGAACGTCACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAAC
 10 ACATTTTTATCCCTCCGGGATGTGTTTCGGCAAAGACCTCATCTACACACTGTA
 CTATTGGAAGTCCAGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAAC
 GAGTTTTTAATTGACGTGGACAAAGGCGAGAACTACTGCTTCAGCGTGCAAG
 CCGTGATCCCTTCTCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGA
 GTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

15 *(α CD28 light chain variable region)*

GTCCAGCTGCAGCAGAGCGGACCCGAACCTCGTGAAACCCGGTGCTTCC
 GTGAAAATGTCTTGTAAGGCCAGCGGATACACCTTCACCTCCTATGTGATCCA
 GTGGGTCAAACAGAAAGCCCGGACAAGGTCTCGAGTGGATCGGCAGCATCAA
 CCCTTACAACGACTATACCAAATACAACGAGAAGTTTAAGGGAAAGGCTACT
 20 TTAACCTCCGACAAAAGCTCCATCACAGCCTACATGGAGTTCAGCTCTTTAAC
 ATCCGAGGACAGCGCTCTGTACTATTGCGCCCGGTGGGGCGACGGCAATTAC
 TGGGGACGGGGCACAACACTGACCGTGAGCAGC

(Linker)

GGAGGCGGAGGCTCCGGCGGAGGCGGATCTGGCGGTGGCGGCTCC

25 *(α CD28 light chain variable region)*

GACATCGAGATGACCCAGTCCCCCGCTATCATGTCCGCCTCTTTAGGCGAGC
 GGGTCACAATGACTTGACAGCCTCCTCCAGCGTCTCCTCCTCCTACTTCCAT
 TGGTACCAACAGAAACCCGGAAGCTCCCCTAAACTGTGCATCTACAGCACCA
 GCAATCTCGCCAGCGGCGTGCCCCCTAGGTTTTCCGGAAGCGGAAGCACCAG
 30 CTACTCTTTAACCATCTCCTCCATGGAGGCTGAGGATGCCGCCACCTACTTTT

GTCACCAGTACCACCGGTCCCCACCTTCGGAGGCGGCACCAAACCTGGAGAC
AAAGAGG

Exemplary Single-Chain Chimeric Polypeptide (α CD3scFv/TF/ α CD28scFv) (SEQ

5 ID NO: 3)

(Signal peptide)

MKWVTFISLLFLFSSAYS

(α CD3 light chain variable region)

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDT
10 SKLASGVP AHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEI
NR

(Linker)

GGGSGGGSGGGGS

(α CD3 heavy chain variable region)

15 QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPQGLEWI
GYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDD
HYCLDYWGQGTTLTVSS

(Human tissue factor 219)

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
20 FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYW
KSSSSGKK TAKTNTNEFLIDVDKGENYCF SVQAVIPSR TVNRKSTDSPVECMGQE
KGEFRE

(α CD28 light chain variable region)

25 VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGS
INPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNY
WGRGTTTLTVSS

(Linker)

GGGSGGGSGGGGS

30 *(α CD28 heavy chain variable region)*

DIEMTQSPAIMASASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYSTSNLA
SGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR

A second exemplary single-chain chimeric polypeptide including a first target-binding domain that is an anti-CD28 scFv, a soluble human tissue factor domain, and a
5 second target-binding domain that is an anti-CD3 scFv was generated
(α CD28scFv/TF/ α CD3scFv) (Figure 2). The nucleic acid and amino acid sequences of this single-chain chimeric polypeptide are shown below.

Nucleic Acid Encoding Exemplary Single-Chain Chimeric Polypeptide

10 (α CD28scFv/TF/ α CD3scFv) (SEQ ID NO: 8)

(Signal peptide)

ATGAAATGGGTCACCTTCATCTCTTTACTGTTTTTATTTAGCAGCGCCT
ACAGC

(α CD28 light chain variable region)

15 GTGCAGCTGCAGCAGTCCGGACCCGAAGTGGTCAAGCCCGGTGCCTCC
GTGAAAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCA
ATGGGTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAAT
CCCTACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTC
TGACAAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACT
20 TCTGAGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTG
GGGCCGGGGAAGTACTTTAACAGTGAGCTCC

(Linker)

GGCGGCGGCGGAAGCGGAGGTGGAGGATCTGGCGGTGGAGGCAGC

(α CD28 heavy chain variable region)

25 GACATCGAGATGACACAGTCCCCCGCTATCATGAGCGCCTCTTTAGGA
GAACGTGTGACCATGACTTGTACAGCTTCCTCCAGCGTGAGCAGCTCCTATTT
CCACTGGTACCAGCAGAAACCCGGCTCCTCCCCTAAACTGTGTATCTACTCCA
CAAGCAATTTAGCTAGCGGCGTGCCTCCTCGTTTTAGCGGCTCCGGCAGCACC
TCTTACTCTTTAACCATTAGCTCTATGGAGGCCGAAGATGCCGCCACATACTT
30 TTGCCATCAGTACCACCGGTCCCCTACCTTTGGCGGAGGCACAAAGCTGGAG
ACCAAGCGG

(Human tissue factor 219 form)

AGCGGCACCACCAACACAGTGGCCGCCTACAATCTGACTTGAAATCC
ACCAACTTCAAGACCATCCTCGAGTGGGAGCCCAAGCCCGTTAATCAAGTTT
ATACCGTGCAGATTTCCACCAAGAGCGGCGACTGGAAATCCAAGTGCTTCTA
5 TACCACAGACACCGAGTGCATCTCACCGACGAGATCGTCAAAGACGTGAAG
CAGACATATTTAGCTAGGGTGTCTCTCTACCCCGCTGGAAACGTGGAGAGCA
CCGGATCCGCTGGAGAGCCTTTATACGAGAACTCCCCGAATTCACCCCTAT
CTGGAAACCAATTTAGGCCAGCCACCATCCAGAGCTTCGAACAAGTTGGCA
CAAAGGTGAACGTCACCGTCGAAGATGAGAGGACTTTAGTGCGGAGGAACA
10 ATACATTTTTATCCTTACGTGACGTCTTCGGCAAGGATTTAATCTACACTG
TATTACTGGAAGTCTAGCTCCTCCGGCAAGAAGACCGCCAAGACCAATACCA
ACGAATTTTAAATTGACGTGGACAAGGGCGAGAACTACTGCTTCTCCGTGCA
AGCTGTGATCCCCTCCCGGACAGTGAACCGGAAGTCCACCGACTCCCCCGTG
GAGTGCATGGGCCAAGAGAAGGGAGAGTTTCGTGAG

15 *(α CD3 light chain variable region)*

CAGATCGTGCTGACCCAGTCCCCCGCTATTATGAGCGCTAGCCCCGGT
GAAAAGGTGACTATGACATGCAGCGCCAGCTCTTCCGTGAGCTACATGAACT
GGTATCAGCAGAAGTCCGGCACCAGCCCTAAAAGGTGGATCTACGACACCAG
CAAGCTGGCCAGCGGCGTCCCCGCTCACTTTTCGGGGCTCCGGCTCCGGAACA
20 AGCTACTCTCTGACCATCAGCGGCATGGAAGCCGAGGATGCCGCTACCTATT
ACTGTCAGCAGTGGAGCTCCAACCCCTTCACCTTTGGATCCGGCACCAAGCTC
GAGATTAATCGT

(Linker)

GGAGGCGGAGGTAGCGGAGGAGGCGGATCCGGCGGTGGAGGTAGC

25 *(α CD3 heavy chain variable region)*

CAAGTTCAGCTCCAGCAAAGCGGCGCCGAACTCGCTCGGCCCCGGCGCT
TCCGTGAAGATGTCTTGTAAGGCCTCCGGCTATACCTTCACCCGGTACACAAT
GCACTGGGTCAAGCAACGGCCCCGGTCAAGGTTTAGAGTGGATTGGCTATATC
AACCCCTCCCGGGGCTATACCAACTACAACCAGAAGTTCAAGGACAAAGCCA
30 CCCTCACCACCGACAAGTCCAGCAGCACCGCTTACATGCAGCTGAGCTCTTT

AACATCCGAGGATTCCGCCGTGTACTACTGCGCTCGGTACTACGACGATCATT
 ACTGCCTCGATTACTGGGGCCAAGGTACCACCTTAACAGTCTCCTCC

Exemplary Single-Chain Chimeric Polypeptide (α CD28scFv/TF/ α CD3scFv) (SEQ

5 **ID NO: 7)**

(Signal peptide)

MKWVTFISLLFLFSSAYS

(α CD28 light chain variable region)

VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGS

10 INPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNY
 WGRGTTTLTVSS

(Linker)

GGGGSGGGSGGGGS

(α CD28 heavy chain variable region)

15 DIEMTQSPAISASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYS
 TSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR

(Human tissue factor 219)

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC

FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL

20 ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLYTLYYW
 KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
 KGEFRE

(α CD3 light chain variable region)

QIVLTQSPAISASPGKEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDT

25 SKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEI
 NR

(Linker)

GGGGSGGGSGGGGS

(α CD3 heavy chain variable region)

QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPQGLEWI
 GYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDD
 HYCLDYWGQGTTTLTVSS

5 The nucleic acid encoding α CD3scFv/TF/ α CD28scFv was cloned into a modified retrovirus expression vectors as described previously (Hughes et al., *Hum Gene Ther* 16:457–72, 2005). The expression vector encoding α CD3scFv/TF/ α CD28scFv was transfected into CHO-K1 cells. Expression of the expression vector in CHO-K1 cells allowed for secretion of the soluble α CD3scFv/TF/ α CD28scFv single-chain chimeric
 10 polypeptide (referred to as 3t28), which can be purified by anti-TF antibody affinity and other chromatography methods.

 An anti-tissue factor antibody affinity column was used to purify the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide. The anti-tissue factor antibody affinity column was connected to a GE Healthcare AKTA Avant system. A flow
 15 rate of 4 mL/min was used for all steps except the elution step, which was 2 mL/min.

 Cell culture harvest including α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide was adjusted to pH 7.4 with 1M Tris base and loaded onto the anti-TF antibody affinity column (described above) which was equilibrated with 5 column volumes of PBS. After sample loading, the column was washed with 5 column volumes
 20 PBS, followed by elution with 6 column volumes 0.1 M acetic acid, pH 2.9. An A280 elution peak was collected and then neutralized to pH 7.5-8.0 by adding 1 M Tris base. The neutralized sample was then buffer exchanged into PBS using Amicon centrifugal filters with a 30 kDa molecular weight cutoff. The data in Figure 2 show that the anti-tissue factor antibody affinity column can bind the α CD3scFv/TF/ α CD28scFv single-
 25 chain chimeric polypeptide, which contains a human soluble tissue factor domain. The buffer-exchanged protein sample was stored at 2-8 °C for further biochemical analysis and biological activity testing.

 After each elution, the anti-tissue factor antibody affinity column was stripped using 6 column volumes of 0.1 M glycine, pH 2.5. The column was then neutralized
 30 using 10 column volumes of PBS, 0.05% NaN₃, and stored at 2-8 °C.

Analytical size exclusion chromatography (SEC) was performed on the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide using a Superdex 200 Increase 10/300 GL gel filtration column (from GE Healthcare) connected to an AKTA Avant system (from GE Healthcare). The column was equilibrated with 2 column volumes of PBS. A flow rate of 0.8 mL/min was used. Two hundred μ L of α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide (1 mg/mL) was injected onto the column using a capillary loop. After injection of the single-chain chimeric polypeptide, 1.25 column volumes of PBS were flowed into the column. The SEC chromatograph is shown in Figure 3. The data show that there are 3 protein peaks, likely representing a monomer and dimer or other different forms of the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide.

To determine the purity and protein molecular weight of the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide, the purified α CD3scFv/TF/ α CD28scFv protein sample from anti-tissue factor antibody affinity column was analyzed by standard sodium dodecyl sulfate polyacrylamide gel (4-12% NuPage Bis-Tris gel) electrophoresis (SDS-PAGE) method under reduced conditions. The gel was stained with InstantBlue for about 30 minutes and destained overnight with purified water. Figure 4 shows the SDS gel of the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide purified using an anti-tissue factor antibody affinity column. The results show that the purified α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide has the expected molecular weight (72 kDa) in reduced SDS gel.

Example 2. Functional Characterization of α CD3scFv/TF/ α CD28scFv Single-Chain Chimeric Polypeptide

ELISA-based methods confirmed the formation of the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide. The α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide was detected using ELISA with one anti-TF monoclonal antibody for capture and a different anti-TF monoclonal antibody for detection (Figure 5). A purified tissue factor protein with a similar concentration was used as a control.

A further in vitro experiment was performed to determine whether the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide is capable of activating human peripheral blood mononuclear cells (PBMCs). Fresh human leukocytes were obtained from the blood bank and peripheral blood mononuclear cells (PBMC) were isolated using density gradient Histopaque (Sigma). The cells were counted and resuspended in 0.2×10^6 /mL in a 96-well flat bottom plate in 0.2 mL of complete media (RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone)). The cells were stimulated with α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide from 0.01 nM to 1000 nM for 3 days at 37 °C, 5% CO₂. After 72 hours, the cells were harvested and surface stained for CD4-488, CD8-PerCP Cy5.5, CD25-BV421, CD69-APCFire750, CD62L-PE Cy7, and CD44-PE (Biolegend) for 30 minutes. After surface staining, the cells were washed (1500 RPM for 5 minutes at room temperature) in FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)). After two washes, the cells were resuspended in 300 μ L of FACS buffer and analyzed by Flow Cytometry (Celesta-BD Bioscience). The data in Figures 6 and 7 show that the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide is able to stimulate both CD8⁺ and CD4⁺ T-cells.

A further experiment was performed, in which PBMCs isolated from blood using Histopaque (Sigma) were counted and resuspended in 0.2×10^6 /mL in a 96-well flat bottom plate in 0.2 mL of complete media (RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone)). The cells were then stimulated with the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide from 0.01 nM to 1000 nM for 3 days at 37 °C, 5% CO₂. After 72 hours, the cells were harvested and surface stained for CD4-488, CD8-PerCP Cy5.5, CD25-BV421, CD69-APCFire750, CD62L-PE Cy7, and CD44-PE (Biolegend) for 30 minutes. After surface staining, the cells were washed (1500 RPM for 5 minutes at room temperature) in FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)). After two washes, the cells were resuspended in 300 μ L of FACS buffer and analyzed by Flow Cytometry (Celesta-BD Bioscience). The data again show that the

α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide was able to stimulate activation of CD4⁺ T cells (Figure 8).

Example 3. Production and characterization of the Exemplary Single-Chain

5 Chimeric Polypeptide IL-2/TF/IL-2

An exemplary single-chain chimeric polypeptide including a first target-binding domain that binds to an IL-2 receptor, a soluble human tissue factor domain, and a second target-binding domain that binds to an IL-2 receptor was generated (IL-2/TF/IL-2) (Figure 9). The nucleic acid and amino acid sequences of this single-chain chimeric polypeptide are shown below.

Nucleic Acid Encoding Exemplary Single-Chain Chimeric Polypeptide (IL-2/TF/IL-2) (SEQ ID NO: 111)

(Signal peptide)

15 ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCTCCAGCGCCT
ACTCC

(First IL-2 fragment)

GCCCCACCTCCTCCTCCACCAAGAAGACCCAGCTGCAGCTGGAGCAT
TTACTGCTGGATTACAGATGATTTTAAACGGCATCAACAACCTACAAGAACC
20 CCAAGCTGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCAC
CGAGCTGAAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAG
GTGCTGAATTTAGCCAGTCCAAGAATTTCCATTAAAGGCCCCGGGATTTAAT
CAGCAACATCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTC
ATGTGCGAGTACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTT
25 GGATCACCTTCTGCCAGTCCATCATCTCCACTTTAACC

(Human tissue factor 219 form)

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAG
CACCAACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTT
TACACCGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCT
30 ATACCACCGACACCGAGTGCATCTACCGATGAGATCGTGAAAGATGTGAA
ACAGACCTACCTCGCCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGC

ACTGGTTCCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTA
 CCTCGAGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGC
 ACAAAGGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAAC
 AACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCAAAGATTTAATCTACACACT
 5 GTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGACAGCTAAAACCAACACA
 AACGAGTTTTTAATCGACGTGGATAAAGGCGAAAACACTACTGTTTCAGCGTGC
 AAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGT
 TGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(Second IL-2 fragment)

10 GCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCAT
 TTACTGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCC
 CAAACTCACCAGGATGCTCACATTTAAGTTTACATGCCCAAGAAGGCCACA
 GAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAG
 TGCTAAATTTAGCTCAAAGCAAAAACCTTCACTTAAGACCCAGGGACTTAAT
 15 CAGCAATATCAACGTAATAGTTCTGGAATAAAGGGATCTGAAACAACATTC
 ATGTGTGAATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGAT
 GGATTACCTTTTGTCAAAGCATCATCTCAACACTAACT

Exemplary Single-Chain Chimeric Polypeptide (IL-2/TF/IL-2) (SEQ ID NO: 110)

20 *(Signal peptide)*

MKWVTFISLLFLFSSAYS

(Human IL-2)

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA
 TELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCE
 25 YADETATIVEFLNRWITFCQSIISTLT

(Human Tissue Factor 219)

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
 FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
 ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYW
 30 KSSSSGKKTAKTNTNEFLIDVDKGENYCFVSQAVIPSRTVNRKSTDSPVECMGQE
 KGEFRE

(Human IL-2)

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA
TELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCE
YADETATIVEFLNRWITFCQSIISTLT

5

The nucleic acid encoding IL-2/TF/IL-2 was cloned into a modified retrovirus expression vector as described previously (Hughes et al., *Hum Gene Ther* 16:457–72, 2005). The expression vector encoding IL-2/TF/IL-2 was transfected into CHO-K1 cells. Expression of the expression vector in CHO-K1 cells allowed for secretion of the soluble
10 IL-2/TF/IL-2 single-chain chimeric polypeptide (referred to as 2t2), which can be purified by anti-TF antibody affinity and other chromatography methods.

IL-2 and IL-2/TF/IL-2 promoted IL-2R β and common γ chain containing 32D β cell proliferation in a similar manner

15 To evaluate the IL-2 activity of IL-2/TF/IL-2, IL-2/TF/IL-2 was compared with recombinant IL-2 for promoting proliferation of 32D β cells that express IL-2R β and common γ chain. IL-2 dependent 32D β cells were washed 5 times with IMDM-10% FBS and seeded to the wells at 2×10^4 cells/well. Serial dilutions of IL-2/TF/IL-2 or IL-2 were added to the cells (Figure 10). Cells were incubated in a CO₂ incubator at 37°C for 3
20 days. Cell proliferation was detected by adding 10 μ l of WST1 to each well on day 3 and incubating for an additional 3 hours in a CO₂ incubator at 37°C. The amount of formazan dye produced was analyzed by measuring the absorbance at 450 nm. As shown in Figure 10, IL-2/TF/IL-2 and IL-2 activated 32D β cells in a similar manner. The EC₅₀ of IL-2/TF/IL-2 and IL-2 was 158.1 pM and 140 pM, respectively.

25

IL-2/TF/IL-2 showed improved ability to promote IL-2R $\alpha\beta\gamma$ containing CTLL-2 cell proliferation as compared to IL-2

To evaluate the IL-2 activity of IL-2/TF/IL-2, IL-2/TF/IL-2 was compared with recombinant IL-2 for promoting proliferation of CTLL-2 cells that express IL-2R α , IL-
30 2R β and common γ chain. IL-2 dependent CTLL-2 cells were washed 5 times with IMDM-10% FBS and seeded to the wells at 2×10^4 cells/well. Serial dilutions of IL-

2/TF/IL-2 or IL-2 were added to the cells (Figure 11). Cells were incubated in a CO₂ incubator at 37°C for 3 days. Cell proliferation was detected by adding 10 µl of WST1 to each well in the day 3 and incubating for an additional 3 hours in a CO₂ incubator at 37°C. The amount of formazan dye produced was analyzed by measuring the absorbance at 450 nm. As shown in Figure 3, IL-2/TF/IL-2 promoted CTLL-2 cell proliferation 4-5-fold stronger than IL-2. The EC₅₀ of IL-2/TF/IL-2 was 123.2 pM and IL-2 was 548.2 pM.

IL-2/TF/IL-2 suppressed the increase of the high fat-induced hyperglycemia in ApoE^{-/-} mice

Six-week-old female ApoE^{-/-} mice (Jackson Lab) were fed with standard chow diet or high diet fat containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Harlan Laboratories) and maintained in the standard conditions. At week 7, mice fed with high fat diet were randomly assigned into the control group and treatment group. Mice then received either IL-2/TF/IL-2 (treatment group) or PBS (chow diet group and control group) per subcutaneous injection at a dosage of 3 mg/kg. Three days post dosing, the mice were fasted overnight, and blood samples were collected through retro-orbital venous plexus puncture. Overnight fasting glucose levels were measured using a OneTouch Glucometer. As shown in Figure 12, the results showed that IL-2/TF/IL-2 injection effectively suppresses the increase of glucose levels in ApoE^{-/-} mice.

IL-2/TF/IL-2 significantly upregulate the ratio of CD4⁺CD25⁺FoxP3⁺ T regulatory (Treg) cells in blood lymphocytes

Six-week-old female ApoE^{-/-} mice (Jackson Lab) were fed with standard chow diet or high diet fat containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Harlan Laboratories) and maintained in the standard conditions. At week 7, mice fed with the high fat diet were randomly assigned into control group and treatment group. Mice then received either IL-2/TF/IL-2 (treatment group) or PBS (chow diet group and control group) per subcutaneous injection at a dosage of 3mg/kg. Three days after the dosing, overnight fasting blood samples were

collected through retro-orbital venous plexus puncture and incubated with ACK lysing buffer (Thermo Fisher Scientific) at 37°C for 5 minutes. Samples were then resuspended in FACS buffer (1 X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)) and surface stained with FITC-anti-CD4 and APC-anti-CD25 antibodies (BioLegend) for 30 minutes. Surface-stained samples were further fixed and premetallized with Fix/Perm buffer (BioLegend) and intracellular stained with PE-anti-Foxp3 antibody (BioLegend). After staining, cells were washed twice with FACS buffer followed by centrifugation at 1500 RPM for 5 minutes at room temperature. The cells were analyzed by flow cytometry (Celesta-BD Bioscience). As shown in Figure 13, IL-2/TF/IL-2 treatment significantly increased Treg populations in blood lymphocytes (3.5%±0.32) compared to the untreated groups (0.4%±0.16 for chow diet group and 0.46%±0.09 for high fat diet group).

Purification elution chromatograph of IL-2/TF/IL-2 from anti-TF antibody affinity column

IL-2/TF/IL-2 harvested from cell culture was loaded onto the anti-TF antibody affinity column equilibrated with 5 column volumes of PBS. After sample loading, the column was washed with 5 column volumes of PBS, followed by elution with 6 column volumes of 0.1M acetic acid, pH 2.9. A280 elution peak was collected and then neutralized to pH 7.5-8.0 with 1M Tris base. The neutralized sample was then buffer exchanged into PBS using Amicon centrifugal filters with a 30 kDa molecular weight cutoff. As shown in Figure 14, the anti-TF antibody affinity column bound to IL-2/TF/IL-2 which contains TF as a fusion domain. The buffer-exchanged protein sample was stored at 2-8 °C for further biochemical analyses and biological activity tests. After each elution, the anti-TF antibody affinity column was stripped using 6 column volumes of 0.1M glycine, pH 2.5. The column was then neutralized using 5 column volumes of PBS, and 7 column volumes of 20% ethanol for storage. The anti-TF antibody affinity column was connected to a GE Healthcare AKTA Avant system. The flow rate was 4 mL/min for all steps except for the elution step, which was 2 mL/min.

30

Analytical size exclusion chromatography (SEC) analysis of IL-2/TF/IL-2

To analyze IL-2/TF/IL-2 using analytical size exclusion chromatography (SEC), a Superdex 200 Increase 10/300 GL gel filtration column (from GE Healthcare) was connected to an AKTA Avant system (from GE Healthcare). The column was
5 equilibrated with 2 column volumes of PBS. The flow rate was 0.7 mL/min. A sample containing IL-2/TF/IL-2 in PBS was injected into the Superdex 200 column using a capillary loop, and analyzed by SEC. The SEC chromatograph of the sample is shown in Figure 15. The SEC results indicated two protein peaks for IL-2/TF/IL-2.

10 *Reduced SDS-PAGE of IL-2/TF/IL-2*

To determine the purity and molecular weight of the protein, IL-2/TF/IL-2 protein sample purified with anti-TF antibody affinity column was analyzed by sodium dodecyl sulfate polyacrylamide gel (4-12% NuPage Bis-Tris gel) electrophoresis (SDS-PAGE) method under reduced condition. After electrophoresis, the gel was stained with
15 InstantBlue for about 30 min, followed by destaining overnight in purified water.

To verify that the IL-2/TF/IL-2 protein undergoes glycosylation after translation in CHO cells, a deglycosylation experiment was conducted using the Protein Deglycosylation Mix II kit from New England Biolabs according to the manufacturer's instructions. Figures 16A and 16B show the reduced SDS-PAGE analysis of the sample
20 in non-deglycosylated (lane 1 in red outline) and deglycosylated (lane 2 in yellow outline) state. The results show that the IL-2/TF/IL-2 protein is glycosylated when expressed in CHO cells. After deglycosylation, the purified sample ran with expected molecular weights (56 kDa) in reduced SDS gel. Lane M was loaded with 10 µL of SeeBlue Plus2 Prestained Standard.

25

In vivo characterization of IL-2/TF/IL-2

IL-2/TF/IL-2 was subcutaneously injected into C57BL/6 mice at various doses to determine the immunostimulatory activity of IL-2/TF/IL-2 in vivo. Mice were subcutaneously treated with control solution (PBS) or IL-2/TF/IL-2 at 0.1, 0.4, 2 and 10
30 mg/kg. The treated mice were euthanized day 3 post treatment. The mouse spleens were collected and weighed day 3 post treatment. Single splenocyte suspensions were

prepared, and the prepared splenocytes were stained for CD4⁺ T cells, CD8⁺ T cells and NK cells (with fluorochrome-conjugated anti-CD4, -CD8, and -NK1.1 antibodies), and analyzed by flow cytometry. The results showed that IL-2/TF/IL-2 was effective at expanding splenocytes based on spleen weight (Figure 17A) especially at 0.1-10 mg/kg. The percentage of CD8⁺ T cells were higher compared to control-treated mice (Figure 17B) at 2 and 10 mg/kg. The percentage of NK cells were higher compared to control-treated mice (Figure 17B) at all doses tested.

It has been known that IL-2 upregulates CD25 expression by immunocytes. We therefore accessed CD25 expression of CD4⁺ T cells, CD8⁺ T cells and NK cells in the IL-2/TF/IL-2 treated mice. C57BL/6 mice were subcutaneously treated with IL-2/TF/IL-2 as described in the paragraph above. The splenocytes were stained with fluorochrome-conjugated anti-CD4, -CD8, CD25 and NK1.1 monoclonal antibodies. The CD25 expression (MFI) of splenocyte subsets was analyzed by flow cytometry. As shown in Figure 18, at the doses and time point (day 3) tested, IL-2/TF/IL-2 significantly upregulated CD25 expression by CD4⁺ T cells but not CD8⁺ T cells or NK cells.

The pharmacokinetics of IL-2/TF/IL-2 in C57BL/6 mice was also investigated. IL-2/TF/IL-2 was subcutaneously injected into C57BL/6 mice at 1 mg/kg. The mouse blood was drawn from tail vein at various time points as shown in Figure 19 and the serum was prepared. IL-2/TF/IL-2 concentrations were determined with ELISA (Capture: anti-tissue factor antibody; Detection: biotinylated anti-human IL-2 antibody followed by SA-HRP and ABTS substrate). The half-life of IL-2/TF/IL-2 was 1.83 hours calculated with PK Solutions 2.0 (Summit Research Services).

IL-2/TF/IL-2 attenuated the formation of high fat-induced atherosclerotic plaques in ApoE^{-/-} mice

Six-week-old female ApoE^{-/-} mice (The Jackson Laboratory) were fed with standard chow diet or high diet fat (21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch) (TD88137, Harlan Laboratories) and maintained in the standard conditions. At week 7, mice fed with high fat diet (HFD) were randomly assigned into control group and treatment group. Mice were then administrated either IL-2/TF/IL-2 (treatment group) or PBS (chow diet group and control group) subcutaneously at a

dosage of 3mg/kg weekly for 4 weeks. At week 12, all mice were euthanized by isoflurane. Aortas were collected, opened longitudinally and stained with Sudan IV solution (0.5%) using *en face* method. The percentage of plaque area (red color as shown in Figure 20A) relative to total aorta area was then quantified with Image J software.

- 5 Figure 20A shows a representative view of atherosclerotic plaques from each group. Figure 20B shows the results of quantitative analysis of atherosclerotic plaques of each group. The percentage of plaque areas in control group (HF Diet) was much higher than the treatment group (HFD+IL-2/TF/IL-2), being 10.28% vs 4.68 %.

10 *IL-2/TF/IL-2 suppresses the progression of type 2 diabetes*

Male BKS.Cg-Dock7^m +/+ Lepr^{db}/J (db/db (Jackson Lab)) mice were fed with standard chow diet and received drinking water ad libitum. At the age of six weeks, mice were randomly assigned into control group and treatment group. The treatment group received IL-2/TF/IL-2 by subcutaneous injection at 3 mg/kg bi-weekly, while control

15 group received vehicle (PBS) only. Overnight fasting glucose levels were measure weekly using a OneTouch Glucometer. The results showed that IL-2/TF/IL-2 effectively suppressed the increase of glucose levels in BKS.Cg-Dock7^m +/+ Lepr^{db}/J mice.

20 *IL-2/TF/IL-2 significantly upregulates the ratio of CD4⁺CD25⁺FoxP3⁺ T regulatory cells in blood lymphocytes after the first injection*

Male BKS.Cg-Dock7^m +/+ Lepr^{db}/J (db/db) (The Jackson Laboratory) mice were fed with standard chow diet and received drinking water ad libitum. At the age of six weeks, mice were randomly assigned into control group and treatment group. The treatment group received IL-2/TF/IL-2 by subcutaneous injection at 3 mg/kg bi-weekly,

25 while the control group received vehicle (PBS) only. Four days after the first drug injection, overnight fasting blood samples were collected and incubated with ACK lysing buffer (Thermo Fisher Scientific) at 37°C for 5 minutes. Samples were then resuspended in FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)) and surface stained with FITC-anti-CD4 and APC-anti-CD25

30 antibodies (BioLegend) for 30 minutes. Surface-stained samples were further fixed and premetallized with Fix/Perm buffer (BioLegend) and intracellular stained with PE-anti-

Foxp3 antibody (BioLegend). After staining, cells were washed twice with FACs buffer and were analyzed by flow cytometry (Celesta-BD Bioscience). The percentage of CD4⁺CD25⁺FoxP3⁺ Tregs in blood lymphocytes were measured. As shown in Figure 22, the results showed that IL-2/TF/IL-2 significantly upregulated the ratio of Tregs in blood lymphocytes. * p<0.05

Example 4. Production and characterization of the Exemplary Single-Chain Chimeric Polypeptide IL-15/TF/IL-15

A second exemplary single-chain chimeric polypeptide including a first target-binding domain that binds to an IL-15 receptor, a soluble human tissue factor domain, and a second target-binding domain that binds to an IL-15 receptor was generated (IL-15/TF/IL-15 or IL-15/TF/IL-15) (Figure 23). The nucleic acid and amino acid sequences of this single-chain chimeric polypeptide are shown below.

15 Nucleic Acid Encoding Exemplary Single-Chain Chimeric Polypeptide (IL-15/TF/IL-15) (SEQ ID NO: 117)

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCTCCAGCGCCT
ACTCC

20 *(First IL-15 fragment)*

AACTGGGTGAACGTGATCAGCGATTTAAAGAAGATCGAGGATTTAATC
CAGAGCATGCACATCGACGCCACTCTGTACACTGAGAGCGACGTGCACCCTA
GCTGCAAGGTGACTGCCATGAAGTGCTTTTTACTGGAGCTGCAAGTTATCTCT
TTAGAGAGCGGCGATGCCAGCATCCACGACACTGTGGAGAATTTAATCATTT
25 TAGCCAACAACCTCTTTAAGCAGCAACGGCAACGTGACAGAGAGCGGCTGCA
AGGAGTGCGAGGAGCTGGAGGAGAAGAACATCAAGGAGTTTTTACAGAGCT
TCGTGCACATCGTGCAGATGTTTCATCAACACTAGC

(Human tissue factor 219 form)

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAG
30 CACCAACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTT
TACACCGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCT

ATACCACCGACACCGAGTGCATCTCACCGATGAGATCGTGAAAGATGTGAA
 ACAGACCTACCTCGCCCGGGTGTCTTAGCTACCCCGCCGGCAATGTGGAGAGC
 ACTGGTTCCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTA
 CCTCGAGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGC
 5 ACAAAGGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAAC
 AACACCTTTCTCAGCCTCCGGGATGTGTTCGGCAAAGATTTAATCTACACACT
 GTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGACAGCTAAAACCAACACA
 AACGAGTTTTTAATCGACGTGGATAAAGGCGAAAACACTACTGTTTCAGCGTGC
 AAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGT
 10 TGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(Second IL-15 fragment)

AACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATTTAATT
 CAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTC
 TTGTAAGGTGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTTATCTCTT
 15 TAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTT
 AGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAG
 GAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTG
 TGCACATTGTCCAGATGTTTCATCAATACCTCC

20 Exemplary Single-Chain Chimeric Polypeptide (IL-15/TF/IL-15) (SEQ ID NO: 116)

(Signal peptide)

MKWVTFISLLFLFSSAYS

(Human IL-15)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLLELQVIS
 25 LESGDASIHDVTENLIILANNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQ
 MFINTS

(Human Tissue Factor 219)

SGTNTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
 FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
 30 ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLYTLYYW

KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
KGEFRE

(Human IL-15)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCCKVTAMKCFLELQVIS
5 LESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQ
MFINTS

The nucleic acid encoding IL-15/TF/IL-15 was cloned into a modified retrovirus expression vector as described previously (Hughes et al., *Hum Gene Ther* 16:457–72,
10 2005). The expression vector encoding IL-15/TF/IL-15 was transfected into CHO-K1 cells. Expression of the expression vector in CHO-K1 cells allowed for secretion of the soluble IL-15/TF/IL-15 single-chain chimeric polypeptide (referred to as 15t15), which can be purified by anti-TF antibody affinity and other chromatography methods.

IL-15/TF/IL-15 promotes IL-2R β and common γ chain containing 32D β cell proliferation

15 IL-15 activity of IL-15/TF/IL-15 was compared with recombinant IL-15 in IL2R β and common γ chain expressed 32D β cells. IL-15 dependent 32D β cells were washed five times with IMDM-10% FBS and seeded to the wells at 2×10^4 cells/well. Serial dilutions of IL-15/TF/IL-15 or IL-15 were added to the cells (Figure 24). Cells were incubated in a CO₂ incubator at 37°C for 3 days. Cell proliferation was detected by
20 adding 10 μ l of WST1 to each well in the day 3 and incubating for an additional 3 hours in a CO₂ incubator at 37°C. The amount of formazan dye produced was analyzed by measuring the absorbance at 450 nm. As shown in Figure 24, IL-15/TF/IL-15 promoted 32D β cell proliferation less efficiently as compared to IL-15. The EC₅₀ of IL-15/TF/IL-15 and IL-15 was 161.4 pM and 1.6 pM, respectively.

25

Purification elution chromatograph of IL-15/TF/IL-15 from anti-TF antibody affinity column

IL-15/TF/IL-15 harvested from cell culture was loaded onto the anti-TF antibody affinity column equilibrated with 5 column volumes of PBS. After sample loading, the
30 column was washed with 5 column volumes of PBS, followed by elution with 6 column volumes of 0.1M acetic acid, pH 2.9. A280 elution peak was collected and then

neutralized to pH 7.5-8.0 with 1M Tris base. The neutralized sample was then buffer exchanged into PBS using Amicon centrifugal filters with a 30 kDa molecular weight cutoff. As shown in Figure 25, the anti-TF antibody affinity column bound to IL-15/TF/IL-15 which contains TF as a fusion domain. The buffer-exchanged protein sample
5 was stored at 2-8 °C for further biochemical analyses and biological activity tests. After each elution, the anti-TF antibody affinity column was stripped using 6 column volumes of 0.1M glycine, pH 2.5. The column was then neutralized using 5 column volumes of PBS, and 7 column volumes of 20% ethanol for storage. The anti-TF antibody affinity column was connected to a GE Healthcare AKTA Avant system. The flow rate was 4
10 mL/min for all steps except for the elution step, which was 2 mL/min.

Reduced SDS-PAGE of IL-15/TF/IL-15

To determine the purity and molecular weight of the protein, IL-15/TF/IL-15 (15t15) protein sample purified with anti-TF antibody affinity column was analyzed by
15 sodium dodecyl sulfate polyacrylamide gel (4-12% NuPage Bis-Tris gel) electrophoresis (SDS-PAGE) method under reduced condition. After electrophoresis, the gel was stained with InstantBlue for about 30 min, followed by destaining overnight in purified water.

To verify that the IL-15/TF/IL-15 protein undergoes glycosylation after translation in CHO cells, a deglycosylation experiment was conducted using the Protein
20 Deglycosylation Mix II kit from New England Biolabs and the manufacturer's instructions. Figures 26A and 26B show the reduced SDS-PAGE analysis of the sample in non-deglycosylated (lane 1 in red outline) and deglycosylated (lane 2 in yellow outline) state. The results showed that the IL-15/TF/IL-15 protein is glycosylated when expressed in CHO cells. After deglycosylation, the purified sample ran with expected
25 molecular weights (50 kDa) in reduced SDS gel. Lane M was loaded with 10 µL of SeeBlue Plus2 Prestained Standard.

Example 5: Stimulation of NK cells *in vivo* by IL-2/TF/IL-2 (2t2)

A set of experiments was performed to determine the effect of the 2t2 construct
30 on immune stimulation in C57BL/6 mice. In these experiments, C57BL/6 mice were subcutaneously treated with control solution (PBS) or 2t2 at 0.1, 0.4, 2, and 10 mg/kg.

Treated mice were euthanized 3 days post-treatment. Spleen weight was measured and single splenocyte suspensions were prepared. Splenocytes suspensions were stained with conjugated anti-CD4, anti-CD8, and anti-NK1.1 (NK) antibodies. The percentage of CD4⁺ T cells, CD8⁺ T cells, and NK cells, and CD25 expression on lymphocyte subsets were analyzed by flow cytometry. Figure 27A shows that 2t2 was effective at expanding splenocytes based on spleen weight especially at a dose level of 0.1-10 mg/kg. Following treatment, the percentage of CD8⁺ T cells were higher in 2t2-treated mice compared to control-treated mice at 2 and 10 mg/kg (Figure 27B). The percentage of NK cells were also higher in 2t2-treated mice compared to control-treated mice at all doses of 2t2 tested (Figure 27B). Additionally, 2t2 significantly upregulated CD25 expression by CD4⁺ T cells, but not CD8⁺ T cells and NK cells following treatment at 0.4 to 10 mg/kg (Figure 27C).

A set of experiments was performed to determine the effect of the 2t2 construct on immune stimulation in ApoE^{-/-} mice fed with a Western diet. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories). After 8-weeks of the Western diet, the mice were injected subcutaneously with 2t2 at 3 mg/kg. Three days post treatment, mice were fasted for 16 hours and then blood samples were collected through retro-orbital venous plexus puncture. The blood was mixed with 10 µL 0.5 M EDTA, and 20 µL blood was taken for lymphocyte subsets analysis. The red blood cells were lysed with ACK (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4) and the lymphocytes were stained with anti-mouse CD8a and anti-mouse NK1.1 antibodies for 30 minutes at 4 °C in FACS staining buffer (1% BSA in PBS). The cells were washed once and analyzed with a BD FACS Celesta. For Treg staining, ACK treated blood lymphocytes were stained with anti-mouse CD4 and anti-mouse CD25 antibodies for 30 minutes at 4 °C in FACS staining buffer. The cells were washed once and resuspended in fixation/permeabilization working solution and incubated at room temperature for 60 minutes. The cells were washed once and resuspended in permeabilization buffer. The samples were centrifuged at 300-400 x g for 5 minutes at room temperature and the supernatant was then discarded. The cell pellet was resuspended in residual volume and

the volume adjusted to about 100 μ L with 1 x permeabilization buffer. Anti-Foxp3 antibody was added to the cells, and the cells were incubated for 30 minutes at room temperature. Permeabilization buffer (200 μ L) was added to the cells, and the cells were centrifuged at 300-400 x g for 5 minutes at room temperature. The cells were resuspended in flow cytometry staining buffer and analyzed on a flow cytometer. Figures 28B-28C show that treatment with 2t2 increased the percentage of NK cells and CD8⁺ T cells in ApoE^{-/-} mice fed with Western diet. Figure 28A shows that treatment with 2t2 also increased the percentage of Treg cells.

10 **Example 6: Induction of proliferation of immune cells *in vivo***

A set of experiments was performed to determine the effect of the 2t2 construct on immune cell stimulation in C57BL/6 mice. In these experiments, C57BL/6 mice were subcutaneously treated with control solution (PBS) or 2t2 at 0.1, 0.4, 2, and 10 mg/kg. Treated mice were euthanized 3 days post-treatment. Spleen weight was measured and single splenocyte suspensions were prepared. The splenocyte suspensions were stained with conjugated anti-CD4, anti-CD8, and anti-NK1.1 (NK) antibodies. The percentage of CD4⁺ T cells, CD8⁺ T cells, and NK cells were analyzed by flow cytometry. Figure 29A shows that 2t2 treatment was effective at expanding splenocytes based on spleen weight especially at 0.1-10 mg/kg. The percentage of CD8⁺ T cells was higher compared to control-treated mice at 2 and 10 mg/kg (Figure 29B). The percentage of NK cells was higher compared to control-treated mice at all doses of 2t2 tested (Figure 29B). These results demonstrate that 2t2 treatment was able to induce proliferation of CD8⁺ T cells and NK cells in C57BL/6 mice.

A set of experiments was performed to determine the effect of the 2t2 construct on immune stimulation in ApoE^{-/-} mice fed with a Western diet. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories). After 8-week of the Western diet, the mice were injected subcutaneously with 2t2 at 3 mg/kg. Three days post-treatment, the mice were fasted for 16 hours and then blood samples were collected through retro-orbital venous plexus puncture. The blood was mixed with 10 μ L 0.5 M EDTA and 20 μ L blood

was taken for lymphocyte subsets analysis. The red blood cells were lysed with ACK (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4) and the lymphocytes were stained with anti-mouse CD8a and anti-mouse NK1.1 antibodies for 30 minutes at 4 °C in FACS staining buffer (1% BSA in PBS). The cells were washed once and resuspended in

5 Fixation Buffer (BioLegend Cat# 420801) for 20 minutes at room temperature. The cells were centrifuged at 350 x g for 5 minutes, the fixed cells were resuspended in Intracellular Staining Permeabilization Wash Buffer (BioLegend Cat# 421002) and then centrifuged at 350 x g for 5 minutes. The cells were then stained with anti-Ki67 antibody for 20 minutes at RT. The cells were washed twice with Intracellular Staining

10 Permeabilization Wash Buffer and centrifuged at 350 x g for 5 minutes. The cells were then resuspended in FACS staining buffer. Lymphocyte subsets were analyzed with a BD FACS Celesta. Figure 30A and 30B shows treatment of ApoE^{-/-} mice with 2t2 also induced proliferation (Ki67-positive staining) in NK and CD8⁺ T cells.

15 **Example 7: Treatment of Diabetes**

A set of experiments was performed to investigate amelioration of Western diet-induced hyperglycemia in ApoE^{-/-} mice by 2t2. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch

20 (TD88137, Envigo Laboratories). After 8-weeks of the Western diet, the mice were injected subcutaneously with 2t2 at 3 mg/kg. Three days post-treatment, the mice were fasted for 16 hours and then blood samples were collected through retro-orbital venous plexus puncture. Blood glucose was detected with a glucose meter (OneTouch UltraMini) and GenUltimated test strips using a drop of fresh blood. As shown in Figure

25 31A, 2t2 treatment significantly reduced hyperglycemia induced by the Western diet (p<0.04). The plasma insulin and resistin levels were analyzed with Mouse Rat Metabolic Array by Eve Technologies. HOMA-IR was calculated using the following formula: homeostatic model assessment-insulin resistance = Glucose (mg/dL) * Insulin (mU/mL)/405. As shown in Figure 31B, 2t2 treatment reduced insulin resistance

30 compared to the untreated group. 2t2 (p<0.02) reduced resistin levels significantly

compared to the untreated group as shown in Figure 31C, which may relate to the reduced insulin resistance induced by 2t2 (Figure 31B).

Example 8. Upregulation of CD44 memory T cells

5 C57BL/6 mice were subcutaneously treated with 2t2. The treated mice were euthanized and the single splenocyte suspensions were prepared 4 days (TGFRt15-TGFRs) or 3 days (2t2) following the treatment. The prepared splenocytes were stained with fluorochrome-conjugated anti-CD4, anti-CD8 and anti-CD44 antibodies and the percentages of CD44^{high} T cells in CD4⁺ T cells or CD8⁺ T cells were analyzed by flow
10 cytometry. The results show that 2t2 upregulated expression of the memory marker CD44 on CD4⁺ and CD8⁺ T cells (Figure 32). These findings indicate that 2t2 was able to induce mouse T cells to differentiate into memory T cells.

Example 9. Tissue factor coagulation assays following treatment with single-chain 15 or multi-chain chimeric polypeptides

A set of experiments was performed to assess blood coagulation following treatment with single-chain or multi-chain chimeric polypeptides. To initiate the blood coagulation cascade pathway, tissue factor (TF) binds to Factor VIIa (FVIIa) to form a TF/FVIIa complex. The TF/FVIIa complex then binds Factor X (FX) and converts FX to
20 FXa.

An assay to measure blood coagulation involves measuring activation of Factor X (FX). Briefly, TF/VIIa activates blood coagulation Factor X (FX) to Factor Xa (FXa) in the presence of calcium and phospholipids. TF₂₄₃, which contains the transmembrane domain of TF, has much higher activity in activating FX to FXa than TF₂₁₉, which does
25 not contain the transmembrane domain. TF/VIIa dependent activation of FX is determined by measuring FXa activity using an FXa-specific chromogenic substrate S-2765 (Diapharma, West Chester, OH). The color change of S-2765 can be monitored spectrophotometrically and is proportional to the proteolytic activity of FXa.

In these experiments, FX activation with a multi-chain chimeric polypeptide
30 (18t15-12s, mouse (m)21t15, 21t15-TGFRs, and 21t15-7s) was compared with a positive control (Innovin) or TF₂₁₉. TF₂₁₉ (or TF₂₁₉-containing multi-chain chimeric

polypeptides)/FVIIa complexes were mixed at an equal molar concentration (0.1 nM each) in a volume of 50 μ L in round bottom wells of a 96-well ELISA plate, after which 10 μ L of 180 nM FX was added. After 15 minutes of incubation at 37 °C, during which time FX was converted to FXa, 8 μ L of 0.5 M EDTA (which chelates calcium and thus terminates FX activation by TF/VIIa) was added to each well to stop FX activation. Next, 10 μ L of 3.2 mM S-2765 substrate was added to the reaction mixture. Immediately, the plate absorbance was measured at 405 nm and was recorded as the absorbance at time 0. The plate was then incubated for 10-20 minutes at 37 °C. The color change was monitored by reading absorbance at 405 nm following the incubation. Results of FX activation as measured by FXa activity using chromogenic substrate S-2765 are shown in Figure 33. In this experiment, Innovin, which is a commercial prothrombin reagent containing lipidated recombinant human TF₂₄₃, was used as a positive control for FX activation. Innovin was reconstituted with purified water to about 10 nM of TF₂₄₃. Next, 0.1 nM TF/VIIa complex was made by mixing an equal volume of 0.2nM of FVIIa with 0.2 nM of Innovin. Innovin demonstrated very potent FX activation activity, while TF₂₁₉ and TF₂₁₉-containing multi-chain chimeric polypeptides had very low FX activation activity, confirming that TF₂₁₉ is not active in a TF/FVIIa complex for activating natural substrate FX *in vivo*.

20 **Example 10: Induction of Treg cells by 2t2**

The peripheral blood mononuclear cells (PBMC) of a healthy donor (Donor 163) were isolated from 5 mL of whole blood buffy coats by Ficoll Paque Plus (GE17144003). The PBMC were then lysed with ACK to remove red blood cells. Cells were washed with IMDM-10% FBS and counted. 1.8×10^6 cells (100 μ L/tube) were seeded to the flow tubes and incubated with 50 μ L of descending 2t2 or IL2 (15000, 1500, 150, 15, 1.5, 0.15, or 0 pM) and 50 μ L of pre-staining antibodies (anti-CD8-BV605 and anti-CD127-AF647). Cells were incubated for 30 min at 37 °C in water bath. 200 μ L of pre-warmed BD Phosflow Fix Buffer I (Cat# 557870, Becton Dickinson Biosciences) was added for 10 min at 37° C in water bath to stop the stimulation. Cells (4.5×10^5 cells/100 μ L) were transferred to a V-shape 96-well plate and were spun down followed by permeabilization with 100 μ L of -20 °C pre-cooled BD Phosflow Perm Buffer III (Cat# BD Biosciences)

for 30 min on ice. The cells were then extensively washed x2 with 200 μ L of FACS buffer and stained with a panel of fluorescent antibodies (anti-CD25-PE, CD4-PerCP-Cy5.5, CD56-BV421, CD45RA-PE-Cy7 and pSTAT5a-AF488) to distinguish between different lymphocyte subpopulations and evaluate the pSTAT5a status. Cells were spun
5 down and resuspended in 200 μ L of FACS buffer for FACSCelesta analysis. As shown in Figure C1A, 6 pM of 2t2 was sufficient to induce the phosphorylation of Stat5a in CD4⁺CD25^{hi} T_{reg} cells while 43.11 pM of IL-2 was required to induce phosphorylation of Stat5a in the same population of lymphocytes. In contrast, 2t2 was less active (Figure 34B) or equally active (Figure 34C) as compared to IL2 in inducing phosphorylation of
10 Stat5a in CD4⁺CD25⁻T_{con} and CD8⁺T_{con} cells. These results suggest that 2t2 is superior as compared to IL2 in activating T_{reg} in human PBMC, and that 2t2 demonstrates increased T_{reg} selectivity compared to IL-2 in human blood lymphocyte pStat5a responses.

15

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the
20 following claims.

A. Exemplary Embodiments

Embodiment A1. A single-chain chimeric polypeptide comprising:

- (i) a first target-binding domain;
- 5 (ii) a soluble tissue factor domain; and
- (iii) a second target-binding domain.

Embodiment A2. The single-chain chimeric polypeptide of embodiment A1, wherein the first target-binding domain and the soluble tissue factor domain directly abut
10 each other.

Embodiment A3. The single-chain chimeric polypeptide of embodiment A1, wherein the single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the soluble tissue factor domain.
15

Embodiment A4. The single-chain chimeric polypeptide of any one of embodiments A1-A3, wherein the soluble tissue factor domain and the second target-binding domain directly abut each other.

20 Embodiment A5. The single-chain chimeric polypeptide of any one of embodiments A1-A3, wherein the single-chain chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain.

25 Embodiment A6. The single-chain chimeric polypeptide of embodiment A1, wherein the first target-binding domain and the second target-binding domain directly abut each other.

Embodiment A7. The single-chain chimeric polypeptide of embodiment A1,
30 wherein the single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the second target-binding domain.

Embodiment A8. The single-chain chimeric polypeptide of embodiment A6 or A7, wherein the second target-binding domain and the soluble tissue factor domain directly abut each other.

5 Embodiment A9. The single-chain chimeric polypeptide of embodiment A6 or A7, wherein the single-chain chimeric polypeptide further comprises a linker sequence between the second target-binding domain and the soluble tissue factor domain.

 Embodiment A10. The single-chain chimeric polypeptide of any one of
10 embodiments A1-A9, wherein the first target-binding domain and the second target-binding domain bind specifically to the same antigen.

 Embodiment A11. The single-chain chimeric polypeptide of embodiment A10,
 wherein the first target-binding domain and the second target-binding domain bind
15 specifically to the same epitope.

 Embodiment A12. The single-chain chimeric polypeptide of embodiment A11,
 wherein the first target-binding domain and the second target-binding domain comprise
 the same amino acid sequence.

20

 Embodiment A13. The single-chain chimeric polypeptide of any one of
embodiments A1-A9, wherein the first target-binding domain and the second target-binding domain bind specifically to different antigens.

25 Embodiment A14. The single-chain chimeric polypeptide of any one of
embodiments A1-A13, wherein one or both of the first target-binding domain and the
second target-binding domain is an antigen-binding domain.

 Embodiment A15. The single-chain chimeric polypeptide of embodiment A14,
30 wherein the first target-binding domain and the second target-binding domain are each an
antigen-binding domain.

Embodiment A16. The single-chain chimeric polypeptide of embodiment A13, wherein antigen-binding domain comprises a scFv or a single domain antibody.

Embodiment A17. The single-chain chimeric polypeptide of any one of
 5 embodiments A1-A16, wherein one or both of the first target-binding domain and the
 second target-binding domain bind to a target selected from the group consisting of:
 CD16a, CD28, CD3, CD33, CD20, CD19, CD22, CD123, IL-1R, IL-1, VEGF, IL-6R,
 IL-4, IL-10, PDL-1, TIGIT, PD-1, TIM3, CTLA4, MICA, MICB, IL-6, IL-8, TNF α ,
 CD26a, CD36, ULBP2, CD30, CD200, IGF-1R, MUC4AC, MUC5AC, Trop-2, CMET,
 10 EGFR, HER1, HER2, HER3, PSMA, CEA, B7H3, EPCAM, BCMA, P-cadherin,
 CEACAM5, a UL16-binding protein, HLA-DR, DLL4, TYRO3, AXL, MER, CD122,
 CD155, PDGF-DD, a ligand of TGF- β receptor II (TGF- β RII), a ligand of TGF- β RIII, a
 ligand of DNAM-1, a ligand of NKp46, a ligand of NKp44, a ligand of NKG2D, a ligand
 of NKp30, a ligand for a scMHCI, a ligand for a scMHCII, a ligand for a scTCR, a
 15 receptor for IL-1, a receptor for IL-2, a receptor for IL-3, a receptor for IL-7, a receptor
 for IL-8, a receptor for IL-10, a receptor for IL-12, a receptor for IL-15, a receptor for IL-
 17, a receptor for IL-18, a receptor for IL-21, a receptor for PDGF-DD, a receptor for
 stem cell factor (SCF), a receptor for stem cell-like tyrosine kinase 3 ligand (FLT3L), a
 receptor for MICA, a receptor for MICB, a receptor for a ULP16-binding protein, a
 20 receptor for CD155, a receptor for CD122, and a receptor for CD28.

Embodiment A18. The single-chain chimeric polypeptide of any one of
 25 embodiments A1-A16, wherein one or both of the first target-binding domain and the
 second target-binding domain is a soluble interleukin, a soluble cytokine protein, or a
 soluble cell surface protein.

Embodiment A19. The single-chain chimeric polypeptide of embodiment A18,
 wherein the soluble interleukin, soluble cytokine protein, or soluble cell surface protein is
 selected from the group consisting of: IL-1, IL-2, IL-3, IL-7, IL-8, IL-10, IL-12, IL-15,
 30 IL-17, IL-18, IL-21, PDGF-DD, SCF, FLT3L, MICA, MICB, and a ULP16-binding
 protein.

Embodiment A20. The single-chain chimeric polypeptide of any one of embodiments A1-A16, wherein one or both of the first target-binding domain and the second target-binding domain is a soluble interleukin receptor, a soluble cytokine receptor, or a soluble cell surface receptor.

5

Embodiment A21. The single-chain chimeric polypeptide of embodiment A20, wherein the soluble interleukin receptor, soluble cytokine receptor, or soluble cell surface receptor is a soluble TGF- β receptor II (TGF- β RII), a soluble TGF- β RIII, a soluble NKG2D, a soluble NK30, a soluble NKp44, a soluble NKp46, a soluble DNAM1, a scMHCI, a scMHCII, a scTCR, a soluble CD155, or a soluble CD28.

10

Embodiment A22. The single-chain chimeric polypeptide of any one of embodiments A1-A21, wherein the soluble tissue factor domain is a soluble human tissue factor domain.

15

Embodiment A23. The single-chain chimeric polypeptide of embodiment A22, wherein the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 9.

20

Embodiment A24. The single-chain chimeric polypeptide of embodiment A23, wherein the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 9.

25

Embodiment A25. The single-chain chimeric polypeptide of embodiment A24, wherein the soluble human tissue factor domain comprises a sequence that is at least 95% identical to SEQ ID NO: 9.

30

Embodiment A26. The single-chain chimeric polypeptide of any one of embodiments A22-A25, wherein the soluble human tissue factor domain does not comprise one or more of:

a lysine at an amino acid position that corresponds to amino acid position 20 of mature wildtype human tissue factor protein;

an isoleucine at an amino acid position that corresponds to amino acid position 22 of mature wildtype human tissue factor protein;

5 a tryptophan at an amino acid position that corresponds to amino acid position 45 of mature wildtype human tissue factor protein;

an aspartic acid at an amino acid position that corresponds to amino acid position 58 of mature wildtype human tissue factor protein;

10 a tyrosine at an amino acid position that corresponds to amino acid position 94 of mature wildtype human tissue factor protein;

an arginine at an amino acid position that corresponds to amino acid position 135 of mature wildtype human tissue factor protein; and

a phenylalanine at an amino acid position that corresponds to amino acid position 140 of mature wildtype human tissue factor protein.

15

Embodiment A27. The single-chain chimeric polypeptide of embodiment A26, wherein the soluble human tissue factor domain does not comprise any of:

a lysine at an amino acid position that corresponds to amino acid position 20 of mature wildtype human tissue factor protein;

20 an isoleucine at an amino acid position that corresponds to amino acid position 22 of mature wildtype human tissue factor protein;

a tryptophan at an amino acid position that corresponds to amino acid position 45 of mature wildtype human tissue factor protein;

25 an aspartic acid at an amino acid position that corresponds to amino acid position 58 of mature wildtype human tissue factor protein;

a tyrosine at an amino acid position that corresponds to amino acid position 94 of mature wildtype human tissue factor protein;

an arginine at an amino acid position that corresponds to amino acid position 135 of mature wildtype human tissue factor protein; and

30 a phenylalanine at an amino acid position that corresponds to amino acid position 140 of mature wildtype human tissue factor protein.

Embodiment A28. The single-chain chimeric polypeptide of any one of embodiments A1-A27, wherein the soluble tissue factor domain is not capable of binding Factor VIIa.

5 Embodiment A29. The single-chain chimeric polypeptide of any one of embodiments A1-A28, wherein the soluble tissue factor domain does not convert inactive Factor X into Factor Xa.

10 Embodiment A30. The single-chain chimeric polypeptide of any one of embodiments A1-A29, wherein the single-chain chimeric polypeptide does not blood stimulate coagulation in a mammal.

15 Embodiment A31. The single-chain chimeric polypeptide of any one of embodiments A1-A30, wherein the single-chain chimeric polypeptide further comprises one or more additional target-binding domains at its N- and/or C-terminus.

20 Embodiment A32. The single-chain chimeric polypeptide of embodiment A31, wherein the single-chain chimeric polypeptide comprises one or more additional target-binding domains at its N-terminus.

 Embodiment A33. The single-chain chimeric polypeptide of embodiment A32, wherein one or more additional target-binding domains directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

25 Embodiment A34. The single-chain chimeric polypeptide of embodiment A33, wherein the single-chain chimeric polypeptide further comprises a linker sequence between one of the at least one additional target-binding domains and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

Embodiment A35. The single-chain chimeric polypeptide of embodiment A31, wherein the single-chain chimeric polypeptide comprises one or more additional target-binding domains at its C-terminus.

5 Embodiment A36. The single-chain chimeric polypeptide of embodiment A35, wherein one of the one or more additional target-binding domains directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

10 Embodiment A37. The single-chain chimeric polypeptide of embodiment A35, wherein the single-chain chimeric polypeptide further comprises a linker sequence between one of the at least one additional target-binding domains and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

15 Embodiment A38. The single-chain chimeric polypeptide of embodiment A31, wherein the single-chain chimeric polypeptide comprises one or more additional target binding domains at its N-terminus and the C-terminus.

20 Embodiment A39. The single-chain chimeric polypeptide of embodiment A38, wherein one of the one or more additional antigen binding domains at the N-terminus directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

25 Embodiment A40. The single-chain chimeric polypeptide of embodiment A38, wherein the single-chain chimeric polypeptide further comprises a linker sequence between one of the one or more additional antigen-binding domains at the N-terminus and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

30 Embodiment A41. The single-chain chimeric polypeptide of embodiment A38, wherein one of the one or more additional antigen binding domains at the C-terminus

directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

Embodiment A42. The single-chain chimeric polypeptide of embodiment A38,
5 wherein the single-chain chimeric polypeptide further comprises a linker sequence between one of the one or more additional antigen-binding domains at the C-terminus and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

10 Embodiment A43. The single-chain chimeric polypeptide of any one of embodiments A31-A42, wherein two or more of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains bind specifically to the same antigen.

15 Embodiment A44. The single-chain chimeric polypeptide of embodiment A43, wherein two or more of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains bind specifically to the same epitope.

20 Embodiment A45. The single-chain chimeric polypeptide of embodiment A44, wherein two or more of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains comprise the same amino acid sequence.

25 Embodiment A46. The single-chain chimeric polypeptide of embodiment A43, wherein the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains each bind specifically to the same antigen.

Embodiment A47. The single-chain chimeric polypeptide of embodiment A46,
30 wherein the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains each bind specifically to the same epitope.

Embodiment A48. The single-chain chimeric polypeptide of embodiment A47, wherein the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains each comprise the same amino acid sequence.

5 Embodiment A49. The single-chain chimeric polypeptide of any one of embodiments A31-A42, wherein the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains bind specifically to different antigens.

10 Embodiment A50. The single-chain chimeric polypeptide of any one of embodiments A31-A49, wherein one or more of the first target-binding domain, the second target-binding domain, and the one or more target-binding domains is an antigen-binding domain.

15 Embodiment A51. The single-chain chimeric polypeptide of embodiment A50, wherein the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains are each an antigen-binding domain.

 Embodiment A52. The single-chain chimeric polypeptide of embodiment A51,
20 wherein antigen-binding domain comprises a scFv or a single domain antibody.

 Embodiment A53. The single-chain chimeric polypeptide of any one of embodiments A31-A52, wherein one or more of the first target-binding domain, the second target-binding domain, and the one or more target-binding domains bind
25 specifically to a target selected from the group consisting of: CD16a, CD28, CD3, CD33, CD20, CD19, CD22, CD123, IL-1R, IL-1, VEGF, IL-6R, IL-4, IL-10, PDL-1, TIGIT, PD-1, TIM3, CTLA4, MICA, MICB, IL-6, IL-8, TNF α , CD26a, CD36, ULBP2, CD30, CD200, IGF-1R, MUC4AC, MUC5AC, Trop-2, CMET, EGFR, HER1, HER2, HER3, PSMA, CEA, B7H3, EPCAM, BCMA, P-cadherin, CEACAM5, a UL16-binding protein,
30 HLA-DR, DLL4, TYRO3, AXL, MER, CD122, CD155, PDGF-DD, a ligand of TGF- β receptor II (TGF- β RII), a ligand of TGF- β RIII, a ligand of DNAM-1, a ligand of NKp46,

a ligand of NKp44, a ligand of NKG2D, a ligand of NKp30, a ligand for a scMHCI, a ligand for a scMHCII, a ligand for a scTCR, a receptor for IL-1, a receptor for IL-2, a receptor for IL-3, a receptor for IL-7, a receptor for IL-8, a receptor for IL-10, a receptor for IL-12, a receptor for IL-15, a receptor for IL-17, a receptor for IL-18, a receptor for IL-21, a receptor for PDGF-DD, a receptor for stem cell factor (SCF), a receptor for stem cell-like tyrosine kinase 3 ligand (FLT3L), a receptor for MICA, a receptor for MICB, a receptor for a ULP16-binding protein, a receptor for CD155, a receptor for CD122, and a receptor for CD28.

10 Embodiment A54. The single-chain chimeric polypeptide of any one of embodiments A31-A52, wherein one or more of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains is a soluble interleukin, a soluble cytokine protein, or a soluble cell surface protein.

15 Embodiment A55. The single-chain chimeric polypeptide of embodiment A54, wherein the soluble interleukin, soluble cytokine protein, or soluble cell surface protein is selected from the group consisting of: IL-1, IL-2, IL-3, IL-7, IL-8, IL-10, IL-12, IL-15, IL-17, IL-18, IL-21, PDGF-DD, SCF, FLT3L, MICA, MICB, and a ULP16-binding protein.

20 Embodiment A56. The single-chain chimeric polypeptide of any one of embodiments A31-A52, wherein one or more of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains is a soluble interleukin receptor, a soluble cytokine receptor, or a soluble cell surface receptor.

25 Embodiment A57. The single-chain chimeric polypeptide of embodiment A56, wherein the soluble receptor is a soluble TGF- β receptor II (TGF- β RII), a soluble TGF- β RIII, a soluble NKG2D, a soluble NK30, a soluble NKp44, a soluble NKp46, a soluble DNAM1, a scMHCI, a scMHCII, a scTCR, a soluble CD155, a soluble CD122, a soluble CD3, or a soluble CD28.

30

Embodiment A58. The single-chain chimeric polypeptide of any one of embodiments A1-A57, wherein the single-chain chimeric polypeptide further comprises a signal sequence at its N-terminal end.

5 Embodiment A59. The single-chain chimeric polypeptide of any one of embodiments A1-A58, wherein the single-chain chimeric polypeptide further comprises a peptide tag positioned at the N-terminal end or the C-terminal end of the single-chain chimeric polypeptide.

10 Embodiment A60. A composition comprising any of the single-chain chimeric polypeptides of embodiments A1-A59.

Embodiment A61. The composition of embodiment A60, wherein the composition is a pharmaceutical composition.

15

Embodiment A62. A kit comprising at least one dose of the composition of embodiment A60 or A61.

Embodiment A63. A method of stimulating an immune cell, the method
20 comprising:

contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides of embodiments A1-A59 or the composition of embodiment A60 or A61.

25 Embodiment A64. The method of embodiment A63, wherein the immune cell is contacted in vitro.

Embodiment A65. The method of embodiment A64, wherein the immune cell was previously obtained from a subject.

30

Embodiment A66. The method of embodiment A65, wherein the method further comprises obtaining the immune cell from the subject prior to the contacting step.

Embodiment A67. The method of embodiment A63, wherein the immune cell is
5 contacted in vivo.

Embodiment A68. The method of any one of embodiments A63-A67, wherein the immune cell is selected from the group consisting of: an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid
10 progenitor cell, a Treg cell, a memory T cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T cell, a CD4⁺ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, and a natural killer cell.

Embodiment A69. The method of any one of embodiments A63-A68, wherein the
15 immune cell has previously been genetically modified to express a chimeric antigen receptor or a recombinant T-cell receptor.

Embodiment A70. The method of any one of embodiments A63-A68, wherein the
20 method further comprises, after the contacting step, introducing into the immune cell a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor.

Embodiment A71. The method of any one of embodiments A63-A70, wherein the
method further comprises administering the immune cell to a subject in need thereof.

Embodiment A72. The method of embodiment A71, wherein the subject has been
25 identified or diagnosed as having an age-related disease or condition.

Embodiment A73. The method of embodiment A72, wherein the age-related
30 disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic

pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

10 Embodiment A74. The method of embodiment A71, wherein the subject has been identified or diagnosed as having a cancer.

Embodiment A75. The method of embodiment A74, wherein the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

25 Embodiment A76. The method of embodiment A71, wherein the subject has been diagnosed or identified as having an infectious disease.

Embodiment A77. The method of embodiment A76, wherein the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus,

coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Embodiment A78. A method of inducing or increasing proliferation of an
5 immune cell, the method comprising:
contacting an immune cell with an effective amount of any of the single-chain
chimeric polypeptides of embodiments A1-A59 or the composition of embodiment A60
or A61.

10 Embodiment A79. The method of embodiment A88, wherein the immune cell is
contacted in vitro.

Embodiment A80. The method of embodiment A79, wherein the immune cell was
previously obtained from a subject.

15 Embodiment A81. The method of embodiment A80, wherein the method further
comprises obtaining the immune cell from the subject prior to the contacting step.

Embodiment A82. The method of embodiment A78, wherein the immune cell is
20 contacted in vivo.

Embodiment A83. The method of any one of embodiments A78-A82, wherein the
immune cell is selected from the group consisting of: an immature thymocyte, a
peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid
25 progenitor cell, a Treg cell, a memory T cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2
cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T
cell, a CD4⁺ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a
dendritic cell, a basophil, an eosinophil, and a natural killer cell.

Embodiment A84. The method of any one of embodiments A78-A83, wherein the immune cell has previously been genetically modified to express a chimeric antigen receptor or a recombinant T-cell receptor.

5 Embodiment A85. The method of any one of embodiments A78-A83, wherein the method further comprises, after the contacting step, introducing into the immune cell a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor.

 Embodiment A86. The method of any one of embodiments A78-A85, wherein the
10 method further comprises administering the immune cell to a subject in need thereof.

 Embodiment A87. The method of embodiment A86, wherein the subject has been identified or diagnosed as having an age-related disease or condition.

15 Embodiment A88. The method of embodiment A87, wherein the age-related disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy,
20 lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis,
25 Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

 Embodiment A89. The method of embodiment A86, wherein the subject has been identified or diagnosed as having a cancer.

30 Embodiment A90. The method of embodiment A89, wherein the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma,

osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

Embodiment A91. The method of embodiment A86, wherein the subject has been diagnosed or identified as having an infectious disease.

Embodiment A92. The method of embodiment A86, wherein the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Embodiment A93. A method of inducing differentiation of an immune cell into a memory or memory-like immune cell, the method comprising:

contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides of embodiments A1-A59 or the composition of embodiment A60 or A61.

Embodiment A94. The method of embodiment A93, wherein the immune cell is contacted in vitro.

Embodiment A95. The method of embodiment A94, wherein the immune cell was previously obtained from a subject.

Embodiment A96. The method of embodiment A95, wherein the method further comprises obtaining the immune cell from the subject prior to the contacting step.

Embodiment A97. The method of embodiment A93, wherein the immune cell is
5 contacted in vivo.

Embodiment A98. The method of any one of embodiments A93-A97, wherein the immune cell is selected from the group consisting of: an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a
10 Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T cell, a CD4⁺ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, and a natural killer cell.

Embodiment A99. The method of any one of embodiments A93-A98, wherein the
15 immune cell has previously been genetically modified to express a chimeric antigen receptor or a recombinant T-cell receptor.

Embodiment A100. The method of any one of embodiments A93-A98, wherein
20 the method further comprises, after the contacting step, introducing into the immune cell a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor.

Embodiment A101. The method of any one of embodiments A93-A100, wherein
25 the method further comprises administering the immune cell to a subject in need thereof.

Embodiment A102. The method of embodiment A101, wherein the subject has been identified or diagnosed as having an age-related disease or condition.

Embodiment A103. The method of embodiment A102, wherein the age-related
30 disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic

pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

10 Embodiment A104. The method of embodiment A101, wherein the subject has been identified or diagnosed as having a cancer.

Embodiment A105. The method of embodiment A104, wherein the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

25 Embodiment A106. The method of embodiment A101, wherein the subject has been diagnosed or identified as having an infectious disease.

Embodiment A107. The method of embodiment A106, wherein the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus,

coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Embodiment A108. A method of killing a cancer cell, an infected cell, or a
5 senescent cell in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of any of the single-chain chimeric polypeptides of embodiments A1-A59 or the composition of embodiment A60 or A61

Embodiment A109. The method of embodiment A108, wherein the subject has
10 been identified or diagnosed as having a cancer.

Embodiment A110. The method of embodiment A109, wherein the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing
15 sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma,
20 gastic and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

Embodiment A111. The method of embodiment A108, wherein the subject has
25 been identified or diagnosed as having an aging-related disease or condition.

Embodiment A112. The method of embodiment A111, wherein the aging-related disease or condition is selected from the group consisting of: Alzheimer's disease,
30 aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration,

macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease,
5 age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

Embodiment A113. A method of treating a subject in need thereof, the method
10 comprising administering to the subject a therapeutically effective amount of any of the single-chain chimeric polypeptides of embodiments A1-A59 or the composition of embodiment A60 or A61

Embodiment A114. The method of embodiment A113, wherein the subject has
15 been identified or diagnosed as having a cancer.

Embodiment A115. The method of embodiment A114, wherein the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing
20 sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma,
25 gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

Embodiment A116. The method of embodiment A113, wherein the subject has
30 been identified or diagnosed as having an aging-related disease or condition.

Embodiment A117. The method of embodiment A116, wherein the aging-related disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

Embodiment A118. The method of embodiment A113, wherein the subject has been diagnosed or identified as having an infectious disease.

Embodiment A119. The method of embodiment A118, wherein the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Embodiment A120. Nucleic acid encoding any of the single-chain chimeric polypeptides of any one of embodiments A1-A59.

Embodiment A121. A vector comprising the nucleic acid of embodiment A120.

Embodiment A122. The vector of embodiment A121, wherein the vector is an expression vector.

Embodiment A123. A cell comprising the nucleic acid of embodiment A120 or the vector of embodiment A121 or A122.

Embodiment A124. A method of producing a single-chain chimeric polypeptide, the method comprising:

culturing the cell of embodiment A123 in a culture medium under conditions sufficient to result in the production of the single-chain chimeric polypeptide; and

5 recovering the single-chain chimeric polypeptide from the cell and/or the culture medium.

Embodiment A125. A single-chain chimeric polypeptide produced by the method of embodiment A124.

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Embodiment A126. The single-chain chimeric polypeptide of embodiment A26, wherein the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 96.

15

Embodiment A127. The single-chain chimeric polypeptide of embodiment A126, wherein the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 96.

20

Embodiment A128. The single-chain chimeric polypeptide of embodiment A127, wherein the soluble human tissue factor domain comprises a sequence that is at least 95% identical to SEQ ID NO: 96.

25

Embodiment A129. The single-chain chimeric polypeptide of embodiment A128, wherein the soluble human tissue factor domain comprises a sequence that is 100% identical to SEQ ID NO: 96.

30

Embodiment A130. The single-chain chimeric polypeptide of embodiment A26, wherein the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 97.

Embodiment A131. The single-chain chimeric polypeptide of embodiment A130, wherein the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 97.

5 Embodiment A132. The single-chain chimeric polypeptide of embodiment A131, wherein the soluble human tissue factor domain comprises a sequence that is at least 95% identical to SEQ ID NO: 97.

 Embodiment A133. The single-chain chimeric polypeptide of embodiment A132,
10 wherein the soluble human tissue factor domain comprises a sequence that is 100% identical to SEQ ID NO: 97.

B. Exemplary Embodiments

15 Embodiment B1. A single-chain chimeric polypeptide comprising:

- (i) a first target-binding domain;
- (ii) a soluble tissue factor domain; and
- (iii) a second target-binding domain,

wherein:

20 the first target-binding domain and the second target-binding domain each specifically bind to an IL-2 receptor; or

 the first target-binding domain and the second target-binding domain each specifically bind to an IL-15 receptor.

25 Embodiment B2. The single-chain chimeric polypeptide of embodiment B1, wherein the first target-binding domain and the soluble tissue factor domain directly abut each other.

 Embodiment B3. The single-chain chimeric polypeptide of embodiment B1,
30 wherein the single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the soluble tissue factor domain.

Embodiment B4. The single-chain chimeric polypeptide of any one of embodiments B1-B3, wherein the soluble tissue factor domain and the second target-binding domain directly abut each other.

5 Embodiment B5. The single-chain chimeric polypeptide of any one of embodiments B1-B3, wherein the single-chain chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain.

10 Embodiment B6. The single-chain chimeric polypeptide of embodiment B1, wherein the first target-binding domain and the second target-binding domain directly abut each other.

 Embodiment B7. The single-chain chimeric polypeptide of embodiment B1,
15 wherein the single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the second target-binding domain.

 Embodiment B8. The single-chain chimeric polypeptide of embodiment B6 or B7,
 wherein the second target-binding domain and the soluble tissue factor domain directly
20 abut each other.

 Embodiment B9. The single-chain chimeric polypeptide of embodiment B6 or B7,
 wherein the single-chain chimeric polypeptide further comprises a linker sequence
 between the second target-binding domain and the soluble tissue factor domain.

25 Embodiment B10. The single-chain chimeric polypeptide of any one of embodiments B1-B9, wherein both the first target-binding domain and the second target-binding domain is a soluble interleukin protein.

Embodiment B11. The single-chain chimeric polypeptide of embodiment B10, wherein the first target-binding domain and the second target-binding domain is a soluble IL-2 protein.

5 Embodiment B12. The single-chain chimeric polypeptide of embodiment B11, wherein the soluble IL-2 protein is a soluble human IL-2 protein.

Embodiment B13. The single-chain chimeric polypeptide of embodiment B12, wherein the soluble human IL-2 protein comprises SEQ ID NO: 28.

10

Embodiment B14. The single-chain chimeric polypeptide of embodiment B10, wherein the first target-binding domain and the second target-binding domain is a soluble IL-15 protein.

15 Embodiment B15. The single-chain chimeric polypeptide of embodiment B14, wherein the soluble IL-15 protein is a soluble human IL-15 protein.

Embodiment B16. The single-chain chimeric polypeptide of embodiment B15, wherein the soluble human IL-15 protein comprises SEQ ID NO: 39.

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Embodiment B17. The single-chain chimeric polypeptide of any one of embodiments B1-B16, wherein the soluble tissue factor domain is a soluble human tissue factor domain.

25 Embodiment B18. The single-chain chimeric polypeptide of embodiment B17, wherein the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 9.

Embodiment B19. The single-chain chimeric polypeptide of embodiment B18,
30 wherein the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 9.

Embodiment B20. The single-chain chimeric polypeptide of embodiment B19, wherein the soluble human tissue factor domain comprises a sequence that is at least 95% identical to SEQ ID NO: 9.

- 5 Embodiment B21. The single-chain chimeric polypeptide of any one of embodiments B17-B20, wherein the soluble human tissue factor domain does not comprise one or more of:
- a lysine at an amino acid position that corresponds to amino acid position 20 of mature wildtype human tissue factor protein;
 - 10 an isoleucine at an amino acid position that corresponds to amino acid position 22 of mature wildtype human tissue factor protein;
 - a tryptophan at an amino acid position that corresponds to amino acid position 45 of mature wildtype human tissue factor protein;
 - an aspartic acid at an amino acid position that corresponds to amino acid position
 - 15 58 of mature wildtype human tissue factor protein;
 - a tyrosine at an amino acid position that corresponds to amino acid position 94 of mature wildtype human tissue factor protein;
 - an arginine at an amino acid position that corresponds to amino acid position 135 of mature wildtype human tissue factor protein; and
 - 20 a phenylalanine at an amino acid position that corresponds to amino acid position 140 of mature wildtype human tissue factor protein.

Embodiment B22. The single-chain chimeric polypeptide of embodiment B21, wherein the soluble human tissue factor domain does not comprise any of:

- 25 a lysine at an amino acid position that corresponds to amino acid position 20 of mature wildtype human tissue factor protein;- an isoleucine at an amino acid position that corresponds to amino acid position 22 of mature wildtype human tissue factor protein;
- a tryptophan at an amino acid position that corresponds to amino acid position 45
- 30 of mature wildtype human tissue factor protein;

an aspartic acid at an amino acid position that corresponds to amino acid position 58 of mature wildtype human tissue factor protein;

a tyrosine at an amino acid position that corresponds to amino acid position 94 of mature wildtype human tissue factor protein;

5 an arginine at an amino acid position that corresponds to amino acid position 135 of mature wildtype human tissue factor protein; and

a phenylalanine at an amino acid position that corresponds to amino acid position 140 of mature wildtype human tissue factor protein.

10 Embodiment B23. The single-chain chimeric polypeptide of any one of embodiments B1-B22, wherein the soluble tissue factor domain is not capable of binding Factor VIIa.

Embodiment B24. The single-chain chimeric polypeptide of any one of
15 embodiments B1-B23, wherein the soluble tissue factor domain does not convert inactive Factor X into Factor Xa.

Embodiment B25. The single-chain chimeric polypeptide of any one of
embodiments B1-B24, wherein the single-chain chimeric polypeptide does not blood
20 stimulate coagulation in a mammal.

Embodiment B26. The single-chain chimeric polypeptide of any one of
embodiments B1-B25, wherein the single-chain chimeric polypeptide further comprises
one or more additional target-binding domains at its N- and/or C-terminus.

25 Embodiment B27. The single-chain chimeric polypeptide of embodiment B26,
wherein the single-chain chimeric polypeptide comprises one or more additional target-
binding domains at its N-terminus.

Embodiment B28. The single-chain chimeric polypeptide of embodiment B27, wherein one or more additional target-binding domains directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

5 Embodiment B29. The single-chain chimeric polypeptide of embodiment B28, wherein the single-chain chimeric polypeptide further comprises a linker sequence between one of the at least one additional target-binding domains and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

10 Embodiment B30. The single-chain chimeric polypeptide of embodiment B26, wherein the single-chain chimeric polypeptide comprises one or more additional target-binding domains at its C-terminus.

 Embodiment B31. The single-chain chimeric polypeptide of embodiment B30,
15 wherein one of the one or more additional target-binding domains directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

 Embodiment B32. The single-chain chimeric polypeptide of embodiment B30,
20 wherein the single-chain chimeric polypeptide further comprises a linker sequence between one of the at least one additional target-binding domains and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

 Embodiment B33. The single-chain chimeric polypeptide of embodiment B26,
25 wherein the single-chain chimeric polypeptide comprises one or more additional target binding domains at its N-terminus and the C-terminus.

 Embodiment B34. The single-chain chimeric polypeptide of embodiment B33,
 wherein one of the one or more additional antigen binding domains at the N-terminus
30 directly abuts the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

Embodiment B35. The single-chain chimeric polypeptide of embodiment B33, wherein the single-chain chimeric polypeptide further comprises a linker sequence between one of the one or more additional antigen-binding domains at the N-terminus and the first target-binding domain, the second target-binding domain, or the soluble
5 tissue factor domain.

Embodiment B36. The single-chain chimeric polypeptide of embodiment B33, wherein one of the one or more additional antigen binding domains at the C-terminus directly abuts the first target-binding domain, the second target-binding domain, or the
10 soluble tissue factor domain.

Embodiment B37. The single-chain chimeric polypeptide of embodiment B33, wherein the single-chain chimeric polypeptide further comprises a linker sequence between one of the one or more additional antigen-binding domains at the C-terminus
15 and the first target-binding domain, the second target-binding domain, or the soluble tissue factor domain.

Embodiment B38. The single-chain chimeric polypeptide of any one of embodiments B26-B37, wherein each of the first target-binding domain, the second
20 target-binding domain, and the one or more additional target-binding domains bind specifically to an IL-2 receptor or an IL-15 receptor.

Embodiment B39. The single-chain chimeric polypeptide of embodiment B38, wherein each of the first target-binding domain, the second target-binding domain, and
25 the one or more additional target-binding domains comprise the same amino acid sequence.

Embodiment B40. The single-chain chimeric polypeptide of any one of embodiments B26-B37, wherein the one or more additional target-binding domains is an
30 antigen-binding domain.

Embodiment B41. The single-chain chimeric polypeptide of embodiment B40, wherein the antigen-binding domain comprises a scFv or a single domain antibody.

Embodiment B42. The single-chain chimeric polypeptide of any one of
 5 embodiments B26-B37, B40, and B41, wherein the one or more additional target-binding
 domains bind specifically to a target selected from the group consisting of: CD16a,
 CD28, CD3, CD33, CD20, CD19, CD22, CD123, IL-1R, IL-1, VEGF, IL-6R, IL-4, IL-
 10 10, PDL-1, TIGIT, PD-1, TIM3, CTLA4, MICA, MICB, IL-6, IL-8, TNF α , CD26a,
 CD36, ULBP2, CD30, CD200, IGF-1R, MUC4AC, MUC5AC, Trop-2, CMET, EGFR,
 10 HER1, HER2, HER3, PSMA, CEA, B7H3, EPCAM, BCMA, P-cadherin, CEACAM5, a
 UL16-binding protein, HLA-DR, DLL4, TYRO3, AXL, MER, CD122, CD155, PDGF-
 DD, a ligand of TGF- β receptor II (TGF- β RII), a ligand of TGF- β RIII, a ligand of
 DNAM-1, a ligand of NKp46, a ligand of NKp44, a ligand of NKG2D, a ligand of
 NKp30, a ligand for a scMHCI, a ligand for a scMHCII, a ligand for a scTCR, a receptor
 15 for IL-1, a receptor for IL-2, a receptor for IL-3, a receptor for IL-7, a receptor for IL-8, a
 receptor for IL-10, a receptor for IL-12, a receptor for IL-15, a receptor for IL-17, a
 receptor for IL-18, a receptor for IL-21, a receptor for PDGF-DD, a receptor for stem cell
 factor (SCF), a receptor for stem cell-like tyrosine kinase 3 ligand (FLT3L), a receptor
 for MICA, a receptor for MICB, a receptor for a ULP16-binding protein, a receptor for
 20 CD155, a receptor for CD122, and a receptor for CD28.

Embodiment B43. The single-chain chimeric polypeptide of any one of
 embodiments B6-B37, B40, and B41, wherein the one or more additional target-binding
 domains is a soluble interleukin or cytokine protein.

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Embodiment B44. The single-chain chimeric polypeptide of embodiment B43,
 wherein the soluble interleukin or cytokine protein is selected from the group consisting
 of: IL-1, IL-2, IL-3, IL-7, IL-8, IL-10, IL-12, IL-15, IL-17, IL-18, IL-21, PDGF-DD, and
 SCF.

30

Embodiment B45. The single-chain chimeric polypeptide of any one of embodiments B6-B37, B40, and B41, wherein the one or more additional target-binding domains is a soluble interleukin or cytokine receptor.

5 Embodiment B46. The single-chain chimeric polypeptide of embodiment B45, wherein the soluble receptor is a soluble TGF- β receptor II (TGF- β RII) and a soluble TGF- β RIII.

 Embodiment B47. The single-chain chimeric polypeptide of any one of
10 embodiments B1-B46, wherein the single-chain chimeric polypeptide further comprises a signal sequence at its N-terminal end.

 Embodiment B48. The single-chain chimeric polypeptide of any one of
 embodiments B1-B47, wherein the single-chain chimeric polypeptide further comprises a
15 peptide tag positioned at the N-terminal end or the C-terminal end of the single-chain chimeric polypeptide.

 Embodiment B49. A composition comprising any of the single-chain chimeric polypeptides of embodiments B1-B48.
20

 Embodiment B50. The composition of embodiment B49, wherein the composition is a pharmaceutical composition.

 Embodiment B51. A kit comprising at least one dose of the composition of
25 embodiment B49 or B50.

 Embodiment B52. A method of stimulating an immune cell, the method comprising:
 contacting an immune cell with an effective amount of any of the single-chain
30 chimeric polypeptides of embodiments B1-B48 or the composition of embodiment B49 or B50.

Embodiment B53. The method of embodiment B52, wherein the immune cell is contacted in vitro.

5 Embodiment B54. The method of embodiment B53, wherein the immune cell was previously obtained from a subject.

Embodiment B55. The method of embodiment B54, wherein the method further comprises obtaining the immune cell from the subject prior to the contacting step.

10

Embodiment B56. The method of embodiment B52, wherein the immune cell is contacted in vivo.

Embodiment B57. The method of any one of embodiments B52-B56, wherein the
15 immune cell is selected from the group consisting of: an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a memory T cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T cell, a CD4⁺ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a
20 dendritic cell, a basophil, an eosinophil, and a natural killer cell.

Embodiment B58. The method of any one of embodiments B52-B57, wherein the immune cell has previously been genetically modified to express a chimeric antigen receptor or a recombinant T-cell receptor.

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Embodiment B59. The method of any one of embodiments B52-B57, wherein the method further comprises, after the contacting step, introducing into the immune cell a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor.

30 Embodiment B60. The method of any one of embodiments B52-B59, wherein the method further comprises administering the immune cell to a subject in need thereof.

Embodiment B61. The method of embodiment B60, wherein the subject has been identified or diagnosed as having an age-related disease or condition.

Embodiment B62. The method of embodiment B61, wherein the age-related
5 disease or condition is selected from the group consisting of: Alzheimer's disease,
aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic
pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration,
macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy,
lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney
10 transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia,
wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease,
age-associated loss of lung tissue elasticity, macular degeneration, cachexia,
glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis,
Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

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Embodiment B63. The method of embodiment B60, wherein the subject has been identified or diagnosed as having a cancer.

Embodiment B64. The method of embodiment B63, wherein the cancer is
20 selected from the group consisting of: solid tumor, hematological tumor, sarcoma,
osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing
sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell
non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL),
acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic
25 leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma,
retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma,
gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer,
colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and
neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular
30 carcinoma.

Embodiment B65. The method of embodiment B60, wherein the subject has been diagnosed or identified as having an infectious disease.

Embodiment B66. The method of embodiment B65, wherein the infectious
5 disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Embodiment B67. A method of inducing or increasing proliferation of an
10 immune cell, the method comprising:
contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides of embodiments B1-B48 or the composition of embodiment B49 or B50.

Embodiment B68. The method of embodiment B67, wherein the immune cell is
15 contacted in vitro.

Embodiment B69. The method of embodiment B68, wherein the immune cell was previously obtained from a subject.

20

Embodiment B70. The method of embodiment B60, wherein the method further comprises obtaining the immune cell from the subject prior to the contacting step.

Embodiment B71. The method of embodiment B67, wherein the immune cell is
25 contacted in vivo.

Embodiment B72. The method of any one of embodiments B67-B71, wherein the immune cell is selected from the group consisting of: an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a memory T cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2
30 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T

cell, a CD4⁺ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, and a natural killer cell.

Embodiment B73. The method of any one of embodiments B67-B72, wherein the
5 immune cell has previously been genetically modified to express a chimeric antigen receptor or a recombinant T-cell receptor.

Embodiment B74. The method of any one of embodiments B67-B72, wherein the
method further comprises, after the contacting step, introducing into the immune cell a
10 nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor.

Embodiment B75. The method of any one of embodiments B67-B74, wherein the
method further comprises administering the immune cell to a subject in need thereof.

15 Embodiment B76. The method of embodiment B75, wherein the subject has been identified or diagnosed as having an age-related disease or condition.

Embodiment B77. The method of embodiment B76, wherein the age-related
disease or condition is selected from the group consisting of: Alzheimer's disease,
20 aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia,
25 wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

30 Embodiment B78. The method of embodiment B75, wherein the subject has been identified or diagnosed as having a cancer.

Embodiment B79. The method of embodiment B78, wherein the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell
5 non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer,
10 colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

Embodiment B80. The method of embodiment B75, wherein the subject has been
15 diagnosed or identified as having an infectious disease.

Embodiment B81. The method of embodiment B75, wherein the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus,
20 hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Embodiment B82. A method of inducing differentiation of an immune cell into a memory or memory-like immune cell, the method comprising:
contacting an immune cell with an effective amount of any of the single-chain
25 chimeric polypeptides of embodiments B1-B48 or the composition of embodiment B49 or B50.

Embodiment B83. The method of embodiment B82, wherein the immune cell is contacted in vitro.

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Embodiment B84. The method of embodiment B83, wherein the immune cell was previously obtained from a subject.

Embodiment B85. The method of embodiment B84, wherein the method further
5 comprises obtaining the immune cell from the subject prior to the contacting step.

Embodiment B86. The method of embodiment B82, wherein the immune cell is contacted in vivo.

10 Embodiment B87. The method of any one of embodiments B82-B86, wherein the immune cell is selected from the group consisting of: an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T cell, a CD4⁺ T cell,
15 a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, and a natural killer cell.

Embodiment B88. The method of any one of embodiments B82-B87, wherein the immune cell has previously been genetically modified to express a chimeric antigen
20 receptor or a recombinant T-cell receptor.

Embodiment B89. The method of any one of embodiments B82-B87, wherein the method further comprises, after the contacting step, introducing into the immune cell a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor.
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Embodiment B90. The method of any one of embodiments B82-B89, wherein the method further comprises administering the immune cell to a subject in need thereof.

Embodiment B91. The method of embodiment B90, wherein the subject has been
30 identified or diagnosed as having an age-related disease or condition.

Embodiment B92. The method of embodiment B91, wherein the age-related disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

Embodiment B93. The method of embodiment B90, wherein the subject has been identified or diagnosed as having a cancer.

Embodiment B94. The method of embodiment B93, wherein the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

Embodiment B95. The method of embodiment B90, wherein the subject has been diagnosed or identified as having an infectious disease.

Embodiment B96. The method of embodiment B95, wherein the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

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Embodiment B97. A method of killing a cancer cell, an infected cell, or a senescent cell in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of any of the single-chain chimeric polypeptides of embodiments B1-B48 or the composition of embodiment B49 or B50.

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Embodiment B98. The method of embodiment B97, wherein the subject has been identified or diagnosed as having a cancer.

Embodiment B99. The method of embodiment B98, wherein the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

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Embodiment B100. The method of embodiment B97, wherein the subject has been identified or diagnosed as having an aging-related disease or condition.

Embodiment B101. The method of embodiment B100, wherein the aging-related disease or condition is selected from the group consisting of: Alzheimer's disease,

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aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney
5 transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

10

Embodiment B102. A method of treating a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of any of the single-chain chimeric polypeptides of embodiments B1-B48 or the composition of
embodiment B49 or B50.

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Embodiment B103. The method of embodiment B102, wherein the subject has been identified or diagnosed as having a cancer.

Embodiment B104. The method of embodiment B103, wherein the cancer is
20 selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic
25 leukemia (ALL), myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular
30 carcinoma.

Embodiment B105. The method of embodiment B102, wherein the subject has been identified or diagnosed as having an aging-related disease or condition.

Embodiment B106. The method of embodiment B105, wherein the aging-related
5 disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney
10 transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

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Embodiment B107. The method of embodiment B102, wherein the subject has been diagnosed or identified as having an infectious disease.

Embodiment B108. The method of embodiment B107, wherein the infectious
20 disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

Embodiment B109. A nucleic acid encoding any of the single-chain chimeric
25 polypeptides of any one of embodiments B1-B48.

Embodiment B110. A vector comprising the nucleic acid of embodiment B109.

Embodiment B111. The vector of embodiment B110, wherein the vector is an
30 expression vector.

Embodiment B112. A cell comprising the nucleic acid of embodiment B109 or the vector of embodiment B110 or B111.

Embodiment B113. A method of producing a single-chain chimeric polypeptide,
5 the method comprising:
culturing the cell of embodiment B112 in a culture medium under conditions
sufficient to result in the production of the single-chain chimeric polypeptide; and
recovering the single-chain chimeric polypeptide from the cell and/or the culture
medium.

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Embodiment B114. A single-chain chimeric polypeptide produced by the method of embodiment B113.

Embodiment B115. The single-chain chimeric polypeptide of embodiment B21,
15 wherein the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 96.

Embodiment B116. The single-chain chimeric polypeptide of embodiment B115,
wherein the soluble human tissue factor domain comprises a sequence that is at least 90%
20 identical to SEQ ID NO: 96.

Embodiment B117. The single-chain chimeric polypeptide of embodiment B116,
wherein the soluble human tissue factor domain comprises a sequence that is at least 95%
identical to SEQ ID NO: 96.

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Embodiment B118. The single-chain chimeric polypeptide of embodiment B117,
wherein the soluble human tissue factor domain comprises a sequence that is 100%
identical to SEQ ID NO: 96.

Embodiment B119. The single-chain chimeric polypeptide of embodiment B21, wherein the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 97.

5 Embodiment B120. The single-chain chimeric polypeptide of embodiment B119, wherein the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 97.

10 Embodiment B121. The single-chain chimeric polypeptide of embodiment B120, wherein the soluble human tissue factor domain comprises a sequence that is at least 95% identical to SEQ ID NO: 97.

15 Embodiment B122. The single-chain chimeric polypeptide of embodiment B121, wherein the soluble human tissue factor domain comprises a sequence that is 100% identical to SEQ ID NO: 97.

WHAT IS CLAIMED IS:

1. A single-chain chimeric polypeptide comprising:
 - (i) a first target-binding domain;
 - (ii) a soluble tissue factor domain; and
 - (iii) a second target-binding domain.
2. The single-chain chimeric polypeptide of claim 1, wherein the first target-binding domain and the soluble tissue factor domain directly abut each other.
3. The single-chain chimeric polypeptide of claim 1, wherein the single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the soluble tissue factor domain.
4. The single-chain chimeric polypeptide of any one of claims 1-3, wherein the soluble tissue factor domain and the second target-binding domain directly abut each other.
5. The single-chain chimeric polypeptide of any one of claims 1-3, wherein the single-chain chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain.
6. The single-chain chimeric polypeptide of any one of claims 1-5, wherein the first target-binding domain and the second target-binding domain bind specifically to the same antigen.
7. The single-chain chimeric polypeptide of claim 6, wherein the first target-binding domain and the second target-binding domain bind specifically to the same epitope.

8. The single-chain chimeric polypeptide of claim 7, wherein the first target-binding domain and the second target-binding domain comprise the same amino acid sequence.

9. The single-chain chimeric polypeptide of any one of claims 1-5, wherein the first target-binding domain and the second target-binding domain bind specifically to different antigens.

10. The single-chain chimeric polypeptide of any one of claims 1-9, wherein one or both of the first target-binding domain and the second target-binding domain is an antigen-binding domain.

11. The single-chain chimeric polypeptide of any one of claims 1-10, wherein one or both of the first target-binding domain and the second target-binding domain bind to a target selected from the group consisting of: CD16a, CD28, CD3, CD33, CD20, CD19, CD22, CD123, IL-1R, IL-1, VEGF, IL-6R, IL-4, IL-10, PDL-1, TIGIT, PD-1, TIM3, CTLA4, MICA, MICB, IL-6, IL-8, TNF α , CD26a, CD36, ULBP2, CD30, CD200, IGF-1R, MUC4AC, MUC5AC, Trop-2, CMET, EGFR, HER1, HER2, HER3, PSMA, CEA, B7H3, EPCAM, BCMA, P-cadherin, CEACAM5, a UL16-binding protein, HLA-DR, DLL4, TYRO3, AXL, MER, CD122, CD155, PDGFDD, a ligand of TGF- β receptor II (TGF- β RII), a ligand of TGF- β RIII, a ligand of DNAM1, a ligand of NKp46, a ligand of NKp44, a ligand of NKG2D, a ligand of NKp30, a ligand for a scMHCI, a ligand for a scMHCII, a ligand for a scTCR, a receptor for IL-1, a receptor for IL-2, a receptor for IL-3, a receptor for IL-7, a receptor for IL-8, a receptor for IL-10, a receptor for IL-12, a receptor for IL-15, a receptor for IL-17, a receptor for IL-18, a receptor for IL-21, a receptor for PDGF-DD, a receptor for stem cell factor (SCF), a receptor for stem cell-like tyrosine kinase 3 ligand (FLT3L), a receptor for MICA, a receptor for MICB, a receptor for a ULP16-binding protein, a receptor for CD155, a receptor for CD122, and a receptor for CD28.

12. The single-chain chimeric polypeptide of any one of claims 1-10, wherein one or both of the first target-binding domain and the second target-binding domain is a soluble interleukin, a soluble cytokine protein, or a soluble cell surface protein.

13. The single-chain chimeric polypeptide of claim 12, wherein the soluble interleukin, soluble cytokine protein, or soluble cell surface protein is selected from the group consisting of: IL-1, IL-2, IL-3, IL-7, IL-8, IL-10, IL-12, IL-15, IL-17, IL-18, IL-21, PDGF-D, SCF, FLT3L, MICA, MICB, and a ULP16-binding protein.

14. The single-chain chimeric polypeptide of any one of claims 1-10, wherein one or both of the first target-binding domain and the second target-binding domain is a soluble interleukin receptor, a soluble cytokine receptor, or a soluble cell surface receptor.

15. The single-chain chimeric polypeptide of claim 14, wherein the soluble interleukin receptor, the soluble cytokine receptor, or the soluble cell surface receptor is a soluble TGF- β receptor II (TGF- β RII), a soluble TGF- β RIII, a soluble NKG2D, a soluble NKp30, a soluble NKp44, a soluble NKp46, a soluble DNAM-1, a scMHCI, a scMHCII, a scTCR, a soluble CD155, or a soluble CD28.

16. The single-chain chimeric polypeptide of any one of claims 1-15, wherein the soluble tissue factor domain is a soluble human tissue factor domain.

17. The single-chain chimeric polypeptide of claim 16, wherein the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 9.

18. The single-chain chimeric polypeptide of any one of claims 1-17, wherein the single-chain chimeric polypeptide further comprises one or more additional target-binding domains at its N- and/or C-terminus.

19. A composition comprising any of the single-chain chimeric polypeptides of claims 1-18.

20. The composition of claim 19, wherein the composition is a pharmaceutical composition.

21. A kit comprising at least one dose of the composition of claim 19 or 20.

22. A method of stimulating an immune cell, the method comprising:
contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides of claims 1-18 or the composition of claim 19 or 20.

23. A method of inducing or increasing proliferation of an immune cell, the method comprising:
contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides of claims 1-18 or the composition of claim 19 or 20.

24. A method of inducing differentiation of an immune cell into a memory or memory-like immune cell, the method comprising:
contacting an immune cell with an effective amount of any of the single-chain chimeric polypeptides of claims 1-18 or the composition of claim 19 or 20.

25. The method of any one of claims 22-24, wherein the immune cell is contacted in vitro.

26. The method of any one of claims 22-24, wherein the immune cell is contacted in vivo.

27. The method of any one of claims 22-26, wherein the immune cell is selected from the group consisting of: an immature thymocyte, a peripheral blood lymphocyte, a naïve T cell, a pluripotent Th cell precursor, a lymphoid progenitor cell, a Treg cell, a

Th17 cell, a Th22 cell, a Th9 cell, a Th2 cell, a Th1 cell, a Th3 cell, $\gamma\delta$ T cell, an $\alpha\beta$ T cell, a tumor-infiltrating T cell, a CD8⁺ T cell, a CD4⁺ T cell, a natural killer T cell, a mast cell, a macrophage, a neutrophil, a dendritic cell, a basophil, an eosinophil, and a natural killer cell.

28. The method of any one of claims 22-27, wherein the immune cell has previously been genetically modified to express a chimeric antigen receptor or a recombinant T-cell receptor.

29. The method of any one of claims 22-28, wherein the method further comprises administering the immune cell to a subject in need thereof.

30. A method of killing a cancer cell, an infected cell, or a senescent cell in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of any of the single-chain chimeric polypeptides of claims 1-18 or the composition of claim 19 or 20.

31. A method of treating a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of any of the single-chain chimeric polypeptides of claims 1-18 or the composition of claim 19 or 20.

32. The method of claim 30 or 31, wherein the subject has been identified or diagnosed as having a cancer, an aging-related disease or condition, or an infectious disease.

33. The method of claim 32, wherein the cancer is selected from the group consisting of: solid tumor, hematological tumor, sarcoma, osteosarcoma, glioblastoma, neuroblastoma, melanoma, rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, B-cell neoplasms, multiple myeloma, B-cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL),

myelodysplastic syndromes (MDS), cutaneous T-cell lymphoma, retinoblastoma, stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, squamous cell head and neck carcinoma, endometrial cancer, cervical cancer, liver cancer, and hepatocellular carcinoma.

34. The method of claim 32, wherein the aging-related disease or condition is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease, intervertebral disc degeneration, macular degeneration, osteoarthritis, type 2 diabetes mellitus, adipose atrophy, lipodystrophy, atherosclerosis, cataracts, COPD, idiopathic pulmonary fibrosis, kidney transplant failure, liver fibrosis, loss of bone mass, myocardial infarction, sarcopenia, wound healing, alopecia, cardiomyocyte hypertrophy, osteoarthritis, Parkinson's disease, age-associated loss of lung tissue elasticity, macular degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, and renal dysfunction.

35. The method of claim 32, wherein the infectious disease is infection with human immunodeficiency virus, cytomegalovirus, adenovirus, coronavirus, rhinovirus, rotavirus, smallpox, herpes simplex virus, hepatitis B virus, hepatitis A virus, and hepatitis C virus, papillomavirus, and influenza virus.

36. Nucleic acid encoding any of the single-chain chimeric polypeptides of any one of claims 1-18.

37. A vector comprising the nucleic acid of claim 36.

38. A cell comprising the nucleic acid of claim 36 or the vector of claim 37.

39. A method of producing a single-chain chimeric polypeptide, the method comprising:

culturing the cell of claim 38 in a culture medium under conditions sufficient to result in the production of the single-chain chimeric polypeptide; and

recovering the single-chain chimeric polypeptide from the cell and/or the culture medium.

40. A single-chain chimeric polypeptide produced by the method of claim 39.

41. The single-chain chimeric polypeptide of claim 16, wherein the human soluble tissue factor domain does not initiate blood coagulation.

42. The single-chain chimeric polypeptide of any one of claims 1-18, wherein the soluble tissue factor domain comprises or consists of a sequence from a wildtype soluble human tissue factor.