

METHODS OF ACTIVATING REGULATORY T CELLS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application Serial No. 62/981,944, filed on February 26, 2020, and U.S. Provisional Patent Application Serial
5 No. 62/975,141, filed on February 11, 2020, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present disclosure relates to the fields of biotechnology and immunology, and more specifically, to methods of stimulating or increasing the proliferation of a T_{reg} cell.

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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
15 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
20 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
25 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 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 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, 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.

Adoptive immunotherapy or cellular therapy requires the culture of immune cells obtained from a subject *in vivo* (and optionally genetic manipulation of the immune cells to express a chimeric antigen receptor or a T-cell receptor) before administration back into the subject. A sufficient number of immune cells is necessary in order to provide a therapeutic effect in the subject. In many examples, immune cells obtained from a subject need to be cultured for three or more weeks before a therapeutically effective number of immune cells can be obtained. In addition, many methods of culturing immune cells obtained from a subject *in vitro* require a layer of feeder cells, which requires subsequent purification or isolation of the immune cells before administration back to the subject.

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SUMMARY

Excellent safety profiles have been shown in patients receiving Treg cells (Esensten et al., *J Allergy Clin Immunol* 142(6): 1710-1718, 2018). Early clinical studies showed encouraging results in using Treg cells to prevent and treat acute and chronic Graft versus Host Diseases (Brunstein et al., *Blood* 117(3): 1061-1070, 2011; Di Ianni et al., *Blood* 117(14): 3921-3928, 2011; Martelli et al., *Blood* 124(4): 638-644, 2014; Theil

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et al., *Cytotherapy* 17(4): 473-486, 2015; Brunstein et al., *Blood* 127(8): 1044-1051, 2016), autoimmune and neurodegenerative diseases (Thonhoff et al., *Neurol Neuroimmunol Neuroinflamm* 5(4): e465, 2018; Dall'Era et al., *Arthritis Rheumatol* 71(3): 431-440, 2019).

5 To use T cell-based adoptive therapies, including Treg cells, ex-vivo cell stimulation step is required for GMP manufacturing setting. Anti-CD3/anti-CD28 antibody-coated magnetic beads (Dynabeads, Thermo Fisher) is most commonly used for cell stimulation and expansion in combination with IL-2 (Highfill et al., *Curr Hematol Malig Rep* 14(4): 269-277, 2019). If using this approach, methods must be developed to
10 ensure T cells are “bead free” at the culture harvest and prior to infusion into patients. In this study, we describe a method of employing 2t2 and 3t15*-28s to effectively substitute IL-2 and anti-CD3/antiCD28-coated magnetic beads, respectively, for T cell stimulation and expansion without using rapamycin. 3t15*-28s is soluble fusion protein complex that can be easily removed from the culture with simple wash steps which eliminates the
15 need for bead removal, saving time and processing cost, and enhancing cell recovery. In addition, we also demonstrated that the inclusion of a separation step using an anti-CD39 antibody in the isolation or the purification of Treg cells before or after cell stimulation and expansion could enrich the CD4⁺ CD25^{hi} CD127^{lo} and CD39^{hi} Tregs, resulting in Treg cells with increased the purity, consistency, and the effector functions.

20 Aging in humans is associated with elevated systemic inflammation (Ferrucci et al., *Blood* 105(6): 2294-2299, 2005; Dinarello, *Am J Clin Nutr* 83(2): 447S-455S, 2006). The process that connects inflammation with aging has been termed inflamm-aging (Franceschi et al., *Ann N Y Acad Sci* 908: 244-254, 2000). The process of aging is connected with major changes that affect the immune system and results in a variety
25 aging-associated pathologies.

 The function of the immune system is to detect and respond to damage to tissues or to the invasion of pathogenic microorganisms. The innate immune cells, the first line of defense against infection, express distinct germ-line encoded pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns
30 (PAMPs) unique to microbes (Janeway, *Cold Spring Harb Symp Quant Biol* 54 Pt 1: 1-13, 1989; Gong et al., *Nat Rev Immunol* 20(2): 95-112, 2020). Danger signals released

by distressed or damaged cells are also recognized by the receptors of damage-associated molecular patterns (DAMPs) (Gong et al., *Nat Rev Immunol* 20(2): 95-112, 2020). Both PAMPs and DAMPs can initiate innate immune responses through the activation of classical PRRs, such as Toll-like receptors (TLRs), and multiple germ-line-encoded receptors, such as NOD-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs) and intracellular DNA sensors (Cao, *Nat Rev Immunol* 16(1): 35-50, 2016). DAMPs can also be sensed by several other receptors. These include receptor for advanced glycation end products (RAGE) (Hudson et al., *Annu Rev Med* 69: 349-364, 2018; Teissier et al., *Biogerontology* 20(3): 279-301, 2019), triggering receptors expressed on myeloid cells (TREMs) (Ford et al., *Curr Opin Immunol* 21(1): 38-46, 2009), several G-protein-coupled receptors (GPCRs) (Heng et al., *Annu Rev Pharmacol Toxicol* 54: 227-249, 2014; Weiss et al., *Trends Immunol* 39(10): 815-829, 2018) and ion channels (Eisenhut et al., *Pflugers Arch* 461(4): 401-421, 2011).

DAMPs-initiated inflammatory responses are referred as sterile inflammation because they are independent of pathogen infection (Chen et al., *Nat Rev Immunol* 10(12): 826-837, 2010). DAMPs can activate both non-immune cells and innate immune cells (Chen et al., *Nat Rev Immunol* 10(12): 826-837, 2010). Activation of these cells leads to the production of cytokines and chemokines, which in turn recruit inflammatory cells and activate adaptive immune responses (Chen et al., *Nat Rev Immunol* 10(12): 826-837, 2010). Some DAMPs are also known to directly activate adaptive immune cells (Lau et al., *J Exp Med* 202(9): 1171-1177, 2005; Qin et al., *J Immunol* 199(1): 72-81, 2017). Although sterile inflammation plays an essential role in tissue repair and regeneration to reestablish the tissue hemostasis after injurious insults, unresolved chronic inflammation due to repeated tissue damage or in response to an overabundance of innate-immune triggers present in tissue is detrimental to the host and may lead to sterile inflammatory diseases, including cancer, metabolic disorders (e.g., diabetes), neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease), and autoimmune diseases (e.g., multiple sclerosis) (Roh et al., *Immune Netw* 18(4): e27, 2018).

Inflammasomes are large, multimeric protein complexes comprising a cytosolic pattern-recognition receptor, the adaptor protein apoptosis-associated Speck-like protein

containing a caspase recruitment domain (ASC), and a caspase-1 (Lamkanfi et al., *Cell* 157(5): 1013-1022, 2014). Their assembly in innate immune cells and other cells is triggered by a variety of stimuli and culminates in the activation of caspase-1 which then cleaves pro-IL-1 β to IL-1 β (Latz et al., *Nat Rev Immunol* 13(6): 397-411, 2013; Walsh et al., *Nat Rev Neurosci* 15(2): 84-97, 2014). To date, diverse inflammasomes have been discovered. Among the various inflammasomes identified, the nucleotide-binding oligomerization domain, leucine-rich repeat-containing receptor (NLR) family pyrin domain-containing 3 (NLRP3) inflammasome is best characterized (Swanson et al., *Nat Rev Immunol* 19(8): 477-489, 2019). The NLRs are recognized as the key sensors of pathogens and danger signals. The NLRP3 inflammasome has a two-step activation mechanism: “priming”, which entails induction of Pro-IL-1 β and NLRP3, and “activation”, wherein a functional inflammasome complex is assembled following uptake of PAMPs or DAMPs. The pathology of various diseases, including Alzheimer’s disease (Heneka et al., *Nature* 493(7434): 674-678, 2013), Parkinson’s disease (Heneka et al., *Nat Rev Neurosci* 19(10): 610-621, 2018), and atherosclerosis (Jin et al., *J Am Heart Assoc* 8(12): e012219, 2019) has been linked to hyperactivation of the NLRP3 inflammasome.

Sterile inflammation can also result from the accumulation of senescent cells. Cellular senescence is defined as an irreversible cell cycle arrest that occurs in responses to cellular stress and prevents transmission of defects to the next generation (Collado et al., *Nat Rev Cancer* 10(1): 51-57, 2010; McHugh et al., *J Cell Biol* 217(1): 65-77, 2018). Cellular senescence plays a major protective role in the process of development, tissue hemostasis, and wound healing (Munoz-Espin et al., *Cell* 155(5): 1104-1118, 2013; Storer et al., *Cell* 155(5): 1119-1130, 2013; Demaria et al., *Dev Cell* 31(6): 722-733, 2014; Yun et al., *Elife* 4, 2015). Cellular senescent is accompanied by a pro-inflammatory phenotype. The phenotype is referred to the senescence-associated secretory phenotype (SASP) (McHugh et al., *J Cell Biol* 217(1): 65-77, 2018). The SASP is characterized by the release of inflammatory cytokines, chemokines, growth factors and proteases. This reinforces cellular senescence through autocrine and paracrine signaling, and recruits and instructs immune cells to clear senescence cells. Thus, cellular senescence and SASP are a vital physiological response that maintains

homeostasis at the cellular level, tissue level and organ level. However, upon persistent damage or during aging, senescent cell clearance is compromised, and dysfunctional cells accumulate. The SASP from these uncleared and accumulated senescent cells is a protracted and chronic source of inflammatory factors that create an inflammatory
 5 microenvironment that results in a diverse range of pathological manifestations (Munoz-Espin et al., *Nat Rev Mol Cell Biol* 15(7): 482-496, 2014; van Deursen, *Nature* 509(7501): 439-446, 2014; McHugh et al., *J Cell Biol* 217(1): 65-77, 2018). In addition, some of the SASP factors prime the inflammasome-containing cells which increases the risk of fueling chronic inflammasome activation to induce sterile inflammation.

10 Regulatory T (Treg) cells are essential mediators of peripheral tolerance and the global immunoregulatory potential in hosts to self and non-self-antigens (Sakaguchi et al., *Cell* 133(5): 775-787, 2008; Sakaguchi et al., *Annu Rev Immunol* 38: 541-566, 2020). Treg cells achieve this immunoregulatory control through multiple suppressive mechanisms. These include IL-2 deprivation, the secretion of inhibitory cytokines (i.e.,
 15 IL-10 and TGF- β) and the acquisition of co-stimulatory molecules from antigen-presenting cells via high-affinity binding to CTLA-4 (Oberle et al., *J Immunol* 179(6): 3578-3587, 2007; Tang et al., *Nat Immunol* 9(3): 239-244, 2008; Zheng et al., *J Immunol* 181(3): 1683-1691, 2008). The adenosine triphosphate (ATP)-adenosine pathway is also utilized by regulatory Treg as a key modulator of innate and adaptive immunity. CD39 is
 20 the dominant ecto-nucleotidase broadly expressed on immune cells (e.g., Tregs), endothelial cells and tumor cell, that hydrolyses ATP and adenosine diphosphate (ADP) to adenosine monophosphate (AMP) (Moesta et al., *Nat Rev Immunol* 20(12): 739-755, 2020). AMP is then hydrolyzed by CD73 to adenosine. Adenosine binds to its receptors A1, A2A, A2B, and A3 displayed on immune cells. A2A and A2B receptors (A2AR and
 25 A2BR) are Gs-coupled receptors that increase intracellular cAMP and PKA levels, playing dominant roles in adenosine-induced immunosuppression in a cAMP-dependent manner. A1 and A3 receptors (A1R and A3R) are Gi/o-coupled receptors that decrease intracellular cAMP favoring cell activation, and therefore are generally viewed as immune-promoting adenosine receptors. In humans, A1R, A2AR and A3R display high
 30 affinity for adenosine whereas A2BR has a significantly lower affinity. A2A and A2BR are expressed on immune cells (Feng et al., *Cancer Cell Int* 20: 110, 2020). Recently, it

has been shown that human CD39^{hi} regulatory T cells exhibits stronger stability, higher Foxp3 expression, and suppressive ability under inflammatory conditions (Gu et al., *Cell Mol Immunol* 14(6): 521-528, 2017). Alternations in Treg cell development, homeostasis or function can predispose these cells to a variety of disease conditions including allergy, autoimmunity, graft rejection, cancer, and response to immunotherapies (Sakaguchi et al., *Annu Rev Immunol* 38: 541-566, 2020). Current research is focused on developing novel therapies to enhance Treg cell functions in vivo through use of cytokines and small molecular weight drugs to support endogenous Treg cell proliferation or activation, ex-vivo manipulated Treg cells in autologous adoptive cell therapy (ACT) to promote immunoregulation in settings of autoimmunity, or antigen-specific Treg cells, including chimeric antigen receptor Treg (CAR-Treg) cells, to strengthen tolerance in allergic inflammation (Ferreira et al., *Nat Rev Drug Discov* 18(10): 749-769, 2019). The present invention is a method that uses Treg cells to deactivate the inflammasome and then subsequently uses immune cells activated by one or more immunotherapeutics to reduce the accumulated senescent cells in an individual to treat inflamm-aging and/or any aging-associated pathologies. Provided is a method of inhibiting inflammasome related diseases and inhibiting senescent related diseases. The Treg cells can be in-vivo enhanced endogenous Treg cells or ex-vivo manipulated Treg cells used in an ACT setting. The immune cells for senescent-cell clearance could be activated by immunotherapeutics in vivo or generated by ex-vivo stimulation and expansion methods to support an ACT administration. The present invention is based on the discovery that single-chain chimeric polypeptides that include a soluble tissue factor domain or multi-chain chimeric polypeptides that include a soluble tissue factor domain, in combination with CD3/CD28-binding agents or IL-2 receptor-activating agents are effective in stimulating or increasing the proliferation of a T_{reg} cell. Based on this discovery provided herein are methods of stimulating or increasing the proliferation of a T_{reg} cell that include culturing a T_{reg} cell in a liquid culture medium over a period of time, where at the beginning of the period of time, the liquid culture medium includes: a CD3/CD28-binding agent; and a single-chain chimeric polypeptide including a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, where the

first target-binding domain and the second target-binding domain bind to a receptor for IL-2.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium further includes an
5 IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the IgG1 antibody construct comprises a heavy chain variable domain comprising CDRs of SEQ ID NOs: 66, 67 or 74, and 68, and a light
10 chain variable domain comprising CDRs of SEQ ID NOs: 69, 70, and 71. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the heavy chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 72, and the light chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 73. In some embodiments of any
15 of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the heavy chain variable domain comprises SEQ ID NO: 72, and the light chain variable domain comprises SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain and the soluble tissue factor domain directly abut each other. In
20 some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the soluble tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain and the
25 second target-binding domain directly abut each other. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain. In some
30 embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain and the second target-binding domain comprise the same amino acid sequence. In some embodiments of any of the

methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain and the second target-binding domain comprise a soluble IL-2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble IL-2 is a human soluble IL-2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain and the second target-binding domain each comprise a sequence that is at least 80% identical to SEQ ID NO: 1. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain and the second target-binding domain each comprise a sequence that is at least 90% identical to SEQ ID NO: 1. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain is a soluble human tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain comprises a sequence of SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain comprises or consists of a sequence from a wildtype soluble human tissue factor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain comprises 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 3. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 3. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence

of SEQ ID NO: 3. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 4. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 4. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 4. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 10 nM to about 500 nM of the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 50 nM to about 150 nM of the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD3/CD28-binding agent is an anti-CD3/anti-CD28 bead. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises the anti-CD3/anti-CD28 bead at a ratio of about 1:1 to about 6:1 beads/cell. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises the anti-CD3/anti-CD28 bead at a ratio of about 3:1 to about 5:1 beads/cell. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD3/CD28-binding agent is an additional single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, wherein: the first target-binding domain of the additional single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the additional single-chain chimeric polypeptide binds to CD28. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium further includes an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the additional single-chain chimeric polypeptide. In some embodiments of any of the

methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the IgG1 antibody construct comprises a heavy chain variable domain comprising CDRs of SEQ ID NOs: 66, 67 or 74, and 68, and a light chain variable domain comprising CDRs of SEQ ID NOs: 69, 70, and 71. In some embodiments of any of the methods of

5 stimulating or increasing the proliferation of a T_{reg} cell described herein, the heavy chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 72, and the light chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or

10 increasing the proliferation of a T_{reg} cell described herein, the heavy chain variable domain comprises SEQ ID NO: 72, and the light chain variable domain comprises SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain of the additional single-chain chimeric polypeptide and the soluble tissue factor domain directly abut each other. In some embodiments of any of the methods of stimulating or increasing

15 the proliferation of a T_{reg} cell described herein, the additional single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the soluble tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain and the second target-binding domain of the additional single-chain

20 chimeric polypeptide directly abut each other. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the additional single-chain chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain. In some

25 embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD3 is human CD3. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain of the additional single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 5. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described

30 herein, the first target-binding domain of the additional single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 5. In some

embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain of the additional single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 5. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described

5 herein, the CD28 is human CD28. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second target-binding domain of the additional single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described

10 herein, the second target-binding domain of the additional single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second target-binding domain of the additional single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 6. In some

15 embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain of the additional single-chain chimeric polypeptide is a soluble human tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain of the additional single-chain chimeric

20 polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain of the additional single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing

25 the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain of the additional single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain of the single-chain chimeric polypeptide comprises or consists of a sequence from a wildtype soluble human

30 tissue factor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain comprises 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain of the additional single-chain chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the additional single-chain chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the additional single-chain chimeric polypeptide comprises a sequence that is at least 80%

identical to SEQ ID NO: 7. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the additional single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 7. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the additional single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 7. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the additional single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 8. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the additional single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 8. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the additional single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 8. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 10 nM to about 1000 nM of the additional single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 50 nM to about 300 nM of the additional single-chain chimeric polypeptide.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD3/CD28 binding agent is a multi-chain chimeric polypeptide comprising: (a) a first chimeric polypeptide comprising: (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and (b) a second chimeric polypeptide comprising: (i) a second domain of a pair of affinity domains; and (ii) a second target-binding domain, wherein the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains; and wherein the first target-binding domain binds to CD3 and the second target-binding domain binds to CD28, or the first target-binding domain binds to CD28 and the second target-binding domain to CD3.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the liquid culture medium further includes an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the multi-chain chimeric polypeptide.

- 5 In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the IgG1 antibody construct comprises a heavy chain variable domain comprising CDRs of SEQ ID NOs: 66, 67 or 74, and 68, and a light chain variable domain comprising CDRs of SEQ ID NOs: 69, 70, and 71. In some embodiments of any of the methods of stimulating or increasing the proliferation of a
- 10 Treg cell described herein, the heavy chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 72, and the light chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the heavy chain variable domain comprises SEQ ID NO: 72, and the light chain
- 15 variable domain comprises SEQ ID NO: 73.

- In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first target-binding domain of the first chimeric polypeptide and the soluble tissue factor domain directly abut each other. In some embodiments of any of the methods of stimulating or increasing the proliferation of
- 20 a Treg cell described herein, the first chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the soluble tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain and the second target-binding domain of the second chimeric polypeptide directly abut each other. In some
- 25 embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain.

- In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the CD3 is human CD3. In some
- 30 embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first target-binding domain of the first chimeric

polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 5. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first target-binding domain of the first chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 5. In
5 some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first target-binding domain of the first chimeric polypeptide comprises a sequence of SEQ ID NO: 5.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain of the first
10 chimeric polypeptide is a soluble human tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble human tissue factor domain of the first chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 2. In some
15 embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble human tissue factor domain of the first chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 2. In some
20 some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble human tissue factor domain of the first chimeric polypeptide comprises a sequence of SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain of the first chimeric polypeptide comprises or consists of a sequence from a wildtype soluble human tissue factor.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain of the first
25 chimeric polypeptide 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
30 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.

5 In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain of the first chimeric polypeptide comprises 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
10 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
15 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 of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain of the first
20 chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 102. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide comprises
25 a sequence that is at least 90% identical to SEQ ID NO: 102. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide comprises a sequence of SEQ ID NO: 102. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide comprises a sequence that is at
30 least 80% identical to SEQ ID NO: 104. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first

chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 104. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide comprises a sequence of SEQ ID NO: 104.

5 In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the CD28 is human CD28. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second target-binding domain of the second chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 6. In
10 some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second target-binding domain of the second chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second target-binding domain of the second chimeric
15 polypeptide comprises a sequence of SEQ ID NO: 6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 106. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric polypeptide
20 comprises a sequence that is at least 90% identical to SEQ ID NO: 106. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric polypeptide comprises a sequence of SEQ ID NO: 106. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric
25 polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 108. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 108. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the
30 second chimeric polypeptide comprises a sequence of SEQ ID NO: 108.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the multi-chain chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the liquid culture medium comprises about 10 nM to about 1000 nM of the multi-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the liquid culture medium comprises about 50 nM to about 300 nM of the multi-chain chimeric polypeptide.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium further comprises an mTOR inhibitor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the mTOR inhibitor is rapamycin. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 10 nM to about 500 nM of the mTOR inhibitor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 50 nM to about 150 nM of the mTOR inhibitor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the liquid culture medium does not comprise an mTOR inhibitor.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the methods further include: periodically adding to the liquid culture medium, after each 36 hours to about 60 hours after the start of the period of time, the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the periodic addition of the single-chain chimeric polypeptide is performed to result in a concentration of about 10 nM to about 500 nM of the single-chain chimeric polypeptide in the liquid culture medium. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the periodic addition of the single-chain chimeric polypeptide is performed to result in a concentration of about 50 nM to about 150 nM of the single-chain chimeric polypeptide in the liquid

culture medium. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the methods further include: periodically adding to the liquid culture medium, after each about 6 days to about 8 days after the start of the period of time, the CD3/CD28-binding agent. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the period of time is about 7 days to about 56 days. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the period of claim is about 15 days to about 25 days. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the method further comprises, before the culturing step, a step of isolating the T_{reg} cell from a sample obtained from a subject. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the method further comprises, after the culturing step, a step of isolating the Treg cell from a sample obtained from a subject.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the isolating comprises the use of fluorescence-assorted cell sorting. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cell is an autologous T_{reg} cell, a haploidentical T_{reg} cell, or an allogeneic T_{reg} cell isolated from peripheral blood or umbilical cord blood. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cell is a CD4⁺CD25⁺Foxp3⁺ cell. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cell is a CD4⁺CD25⁺CD127^{dim} cell. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cells comprises a chimeric antigen receptor.

In another aspect, provided herein are populations of T_{reg} cells generated by any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein. Provided herein are compositions comprising any of the population of T_{reg} cells generated by any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein and a pharmaceutically acceptable carrier. Also provided herein are

methods of treating a subject in need thereof, the methods include administering to the subject a therapeutically effective amount of any of the compositions comprising any of the population of T_{reg} cells generated by any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein and a pharmaceutically acceptable carrier.

- 5 In some embodiments of any of the methods of treating a subject in need thereof, the subject has been identified or diagnosed as having an aging-related disease or an inflammatory disease. In some embodiments of any of the methods of treating a subject in need thereof, the aging-related disease is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma,
- 10 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,
- 15 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, neurodegeneration, stroke, dementia, vascular disease, infection susceptibility, chronic inflammation, and renal dysfunction. In some embodiments of any of the methods of
- 20 treating a subject in need thereof, the inflammatory disease is selected from the group consisting of: rheumatoid arthritis, inflammatory bowel disease, lupus erythematosus, lupus nephritis, diabetic nephropathy, CNS injury, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Crohn's disease, multiple sclerosis, Guillain-Barre syndrome, psoriasis, Grave's disease, ulcerative colitis, nonalcoholic steatohepatitis, and
- 25 mood disorders.

- In another aspect, provided herein are kits that include: (i) a CD3/CD28-binding agent; (ii) a single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, wherein the first target-binding domain and the second target-binding domain bind to a receptor for IL-2;
- 30 and (iii) an mTOR inhibitor. In some embodiments of any of the kits described herein, the kits further include an IgG1 antibody construct that comprises at least one antigen-

binding domain that binds specifically to the soluble tissue factor domain of the single-chain chimeric polypeptide. In some embodiments of any of the kits described herein, the CD3/CD28-binding agent is an anti-CD3/anti-CD28 bead. In some embodiments of any of the kits described herein, the CD3/CD28-binding agent is an additional single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, wherein: the first target-binding domain of the additional single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the additional single-chain chimeric polypeptide binds to CD28. In some embodiments of any of the kits described herein, the kits further include an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the additional single-chain chimeric polypeptide.

In some embodiments of any of the kits described herein, the CD3/CD28-binding agent is a multi-chain chimeric polypeptide comprising: (a) a first chimeric polypeptide comprising: (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and (b) a second chimeric polypeptide comprising: (i) a second domain of a pair of affinity domains; and (ii) a second target-binding domain, wherein: the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains; and wherein the first target-binding domain binds to CD3 and the second target-binding domain binds to CD28, or the first target-binding domain binds to CD28 and the second target-binding domain to CD3. In some embodiments of any of the kits described herein, the liquid culture medium further includes an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the multi-chain chimeric polypeptide.

In another aspect, provided herein are methods of stimulating or increasing the proliferation of a T_{reg} cell, the methods include: culturing a T_{reg} cell in a liquid culture medium over a period of time, wherein at the beginning of the period of time, the liquid culture medium comprises: an IL-2 receptor-activating agent; and a single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, wherein: the first target-binding domain of the additional

single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the additional single-chain chimeric polypeptide binds to CD28. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium further includes an IgG1 antibody construct that

5 comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the IgG1 antibody construct comprises a heavy chain variable domain comprising CDRs of SEQ ID NOs: 66, 67 or 74, and 68, and a light chain variable domain comprising

10 CDRs of SEQ ID NOs: 69, 70, and 71. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the heavy chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 72, and the light chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or

15 increasing the proliferation of a T_{reg} cell described herein, the heavy chain variable domain comprises SEQ ID NO: 72, and the light chain variable domain comprises SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain and the soluble tissue factor domain directly abut each other. In some embodiments of any of the

20 methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the soluble tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain and the second target-binding domain directly

25 abut each other. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD3 is

30 human CD3. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain comprises

a sequence that is at least 80% identical to SEQ ID NO: 5. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain comprises a sequence that is at least 90% identical to SEQ ID NO: 5. In some embodiments of any of the methods of stimulating or

5 increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain comprises a sequence of SEQ ID NO: 5. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD28 is human CD28. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second target-binding domain

10 comprises a sequence that is at least 80% identical to SEQ ID NO: 6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second target-binding domain comprises a sequence that is at least 90% identical to SEQ ID NO: 6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second

15 target-binding domain comprises a sequence of SEQ ID NO: 6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain is a soluble human tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain comprises a sequence that

20 is at least 80% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain

25 comprises a sequence of SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain comprises or consists of a sequence from a wildtype soluble human tissue factor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain does not

30 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain comprises 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 7. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 7. In

some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 7. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 8. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 8. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 8. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 10 nM to about 1000 nM of the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 50 nM to about 300 nM of the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the IL-2 receptor-activating agent is a soluble IL-2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble IL-2 is a human soluble IL-2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the human soluble IL-2 comprises a sequence that is at least 80% identical to SEQ ID NO: 1. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the human soluble IL-2 comprises a sequence that is at least 90% identical to SEQ ID NO: 1. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the human soluble IL-2 comprises a sequence of SEQ ID NO: 1. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 100 IU/mL to about 800 IU/mL of the human soluble IL-2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 400 IU/mL to about 600

IU/mL of the human soluble IL-2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium further comprises an mTOR inhibitor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the mTOR inhibitor is rapamycin. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 10 nM to about 500 nM of the mTOR inhibitor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 50 nM to about 150 nM of the mTOR inhibitor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the liquid culture medium does not comprise an mTOR inhibitor.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the methods further include: periodically adding to the liquid culture medium, after each 36 hours to about 60 hours after the start of the period of time, the IL-2 receptor-activating agent. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the periodic addition of the IL-2 receptor-activating agent is performed to result in a concentration of about 10 nM to about 1000 nM of the IL-2 receptor-activating agent in the liquid culture medium. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the periodic addition of the IL-2 receptor activating agent is performed to result in a concentration of about 50 nM to about 150 nM of the IL-2 receptor-activating agent in the liquid culture medium. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the methods further include: periodically adding to the liquid culture medium, after each about 6 days to about 8 days after the start of the period of time, the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the period of time is about 7 days to about 56 days. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the period of claim is about 15 days to about 25 days. In some embodiments of any of the methods of

stimulating or increasing the proliferation of a T_{reg} cell described herein, the methods further include, before the culturing step, a step of isolating the T_{reg} cell from a sample obtained from a subject. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the method further comprises,
5 after the culturing step, a step of isolating the T_{reg} cell from a sample obtained from a subject.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the isolating comprises the use of fluorescence-assorted cell sorting. In some embodiments of any of the methods of
10 stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cell is an autologous T_{reg} cell, a haploidentical T_{reg} cell, or an allogeneic T_{reg} cell isolated from peripheral blood or umbilical cord blood. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cell is a CD4⁺CD25⁺Foxp3⁺ cell. In some embodiments of any of the methods of stimulating or
15 increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cell is a CD4⁺CD25⁺CD127^{dim} cell. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cells comprises a chimeric antigen receptor.

Provided herein are populations of T_{reg} cells generated by any of the methods of
20 stimulating or increasing the proliferation of a T_{reg} cell described herein. Provided herein are compositions comprising any populations of T_{reg} cells generated by any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein and a pharmaceutically acceptable carrier. Also provided herein are methods of treating a subject in need thereof, the methods include administering to the subject a therapeutically
25 effective amount of any of the compositions comprising any populations of T_{reg} cells generated by any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein and a pharmaceutically acceptable carrier. In some embodiments of any of the methods of treating a subject in need thereof described herein, the subject has been identified or diagnosed as having an aging-related disease or an inflammatory
30 disease. In some embodiments of any of the methods of treating a subject in need thereof described herein, the aging-related disease 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, neurodegeneration, stroke, dementia, vascular disease, infection susceptibility, chronic inflammation, and renal dysfunction. In some embodiments of any of the methods of treating a subject in need thereof described herein, the inflammatory disease is selected from the group consisting of: rheumatoid arthritis, inflammatory bowel disease, lupus erythematosus, lupus nephritis, diabetic nephropathy, CNS injury, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Crohn's disease, multiple sclerosis, Guillain-Barre syndrome, psoriasis, Grave's disease, ulcerative colitis, nonalcoholic steatohepatitis, and mood disorders.

In another aspect, provided herein are kits that include: (i) an interleukin-2 receptor-activating agent; (ii) a single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, wherein: the first target-binding domain binds to CD3 and the second target-binding domain binds to CD28; and (iii) an mTOR inhibitor. In some embodiments of any of the kits described herein, the kits further include an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the single-chain chimeric polypeptide.

In another aspect, provided herein are methods of stimulating or increasing the proliferation of a T_{reg} cell, the methods include: culturing a T_{reg} cell in a liquid culture medium over a period of time, wherein at the beginning of the period of time, the liquid culture medium comprises: a CD3/CD28-binding agent; and a multi-chain chimeric polypeptide comprising: (a) a first chimeric polypeptide comprising: (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of

affinity domains; and (b) a second chimeric polypeptide comprising: (i) a second domain of a pair of affinity domains; and (ii) a second target-binding domain, wherein the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains. In some
5 embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium further includes an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the multi-chain chimeric polypeptide. In some
10 embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the IgG1 antibody construct comprises a heavy chain variable domain comprising CDRs of SEQ ID NOs: 66, 67 or 74, and 68, and a light chain variable domain comprising CDRs of SEQ ID NOs: 69, 70, and 71. In some
15 embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the heavy chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 72, and the light chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described
20 herein, the heavy chain variable domain comprises SEQ ID NO: 72, and the light chain variable domain comprises SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain and the soluble tissue factor domain directly abut each other in the first chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the
25 soluble tissue factor domain in the first chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain and the first domain of the pair of affinity domains directly abut each other in the first chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described
30 herein, the first chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the first domain of the pair of affinity domains in the

first chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second domain of the pair of affinity domains and the second target-binding domain directly abut each other in the second chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, second chimeric polypeptide further comprises a linker sequence between the second domain of the pair of affinity domains and the second target-binding domain in the second chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain and the second target-binding domain bind specifically to the same antigen. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell 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 methods of stimulating or increasing the proliferation of a T_{reg} cell 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 methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, one or both of the first target-binding domain and the second target-binding domain 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 , CD26, 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-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 methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, one or both of the first target-binding domain and the second target-binding domain is a soluble interleukin or cytokine protein. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble interleukin, cytokine, or ligand 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, and FLT3L. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, one or both of the first target-binding domain and the second target-binding domain is a soluble interleukin or cytokine receptor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, 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, or a soluble CD28. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first chimeric polypeptide further comprises one or more additional target-binding domain(s). In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second chimeric polypeptide further comprises one or more additional target-binding domains. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the pair of affinity domains is a sushi domain from an alpha chain of human IL-15 receptor (IL15R α) and a soluble IL-15. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble IL-15 has a D8N or D8A amino acid substitution. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the pair of affinity domains is selected from the group consisting of: barnase and barnstar, a PKA and an AKAP, adapter/docking tag modules based on mutated RNase I fragments, and SNARE modules based on interactions of the proteins syntaxin, synaptotagmin, synaptobrevin, and SNAP25. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described

herein, the soluble tissue factor domain is a soluble human tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain comprises a sequence that is at least 80% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain comprises a sequence that is at least 90% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain comprises a sequence of SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain comprises or consists of a sequence from a wildtype soluble human tissue factor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain comprises 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the multi-chain chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD3/CD28-binding agent is an anti-CD3/anti-CD28 bead. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises the anti-CD3/anti-CD28 bead at a ratio of about 1:1 to about 6:1 beads/cell. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises the anti-CD3/anti-CD28 bead at a ratio of about 3:1 to about 5:1 beads/cell. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD3/CD28-binding agent is a single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, wherein: the first target-binding domain of the single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the single-chain chimeric polypeptide binds to CD28. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium further includes an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the IgG1 antibody construct comprises a heavy chain variable domain comprising CDRs of SEQ ID NOs: 66, 67 or 74, and 68, and a light chain variable domain comprising CDRs of SEQ ID NOs: 69, 70, and 71. In some

embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the heavy chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 72, and the light chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 73. In some embodiments of any

5 of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the heavy chain variable domain comprises SEQ ID NO: 72, and the light chain variable domain comprises SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain of the single-chain chimeric polypeptide and the soluble tissue

10 factor domain directly abut each other. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide further comprises a linker sequence between the first target-binding domain and the soluble tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the

15 soluble tissue factor domain and the second target-binding domain of the single-chain chimeric polypeptide directly abut each other. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain. In some

20 embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD3 is human CD3. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain of the single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 5. In some embodiments of any

25 of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain of the single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 5. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the first target-binding domain of the single-chain chimeric polypeptide comprises

30 a sequence of SEQ ID NO: 5. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the CD28 is human CD28.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second target-binding domain of the single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 6.

6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second target-binding domain of the single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 6.

6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the second target-binding domain of the single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 6.

6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain of the single-chain chimeric polypeptide is a soluble human tissue factor domain.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain of the single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 2.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain of the single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 2.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble human tissue factor domain of the single-chain chimeric polypeptide comprises or consists of a sequence from a wildtype soluble human tissue factor.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain comprises 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 of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the soluble tissue factor domain of the single-chain chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 7. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 7. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 7. In some embodiments of any of the methods of

stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 8. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 8. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 8. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 10 nM to about 1000 nM of the single-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 50 nM to about 300 nM of the single-chain chimeric polypeptide.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the CD3/CD28 binding agent is an additional multi-chain chimeric polypeptide comprising: (a) a first chimeric polypeptide comprising: (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and (b) a second chimeric polypeptide comprising: (i) a second domain of a pair of affinity domains; and (ii) a second target-binding domain, wherein the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains; and wherein the first target-binding domain binds to CD3 and the second target-binding domain binds to CD28, or the first target-binding domain binds to CD28 and the second target-binding domain to CD3.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the liquid culture medium further includes an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the additional multi-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the IgG1 antibody construct comprises a heavy chain variable domain comprising CDRs of SEQ ID NOs: 66, 67 or 74, and 68,

and a light chain variable domain comprising CDRs of SEQ ID NOs: 69, 70, and 71. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the heavy chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 72, and the light chain variable domain

5 comprises a sequence that is at least 90% identical to SEQ ID NO: 73. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the heavy chain variable domain comprises SEQ ID NO: 72, and the light chain variable domain comprises SEQ ID NO: 73.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first target-binding domain of the first chimeric polypeptide and the soluble tissue factor domain of the additional multi-chain chimeric polypeptide directly abut each other. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide of the additional multi-chain chimeric polypeptide further
15 comprises a linker sequence between the first target-binding domain and the soluble tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain and the second target-binding domain of the second chimeric polypeptide of the additional multi-chain chimeric polypeptide directly abut each other. In some
20 embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric polypeptide of the additional multi-chain chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the CD3 is human CD3. In some
25 embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first target-binding domain of the first chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 5. In some embodiments of any of the methods of stimulating or increasing the proliferation of
30 a Treg cell described herein, the first target-binding domain of the additional single-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO:

5. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first target-binding domain of the additional single-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 5.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain of the first chimeric polypeptide of the additional multi-chain chimeric polypeptide is a soluble human tissue factor domain. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble human tissue factor domain of the first chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble human tissue factor domain of the first chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble human tissue factor domain of the first chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 2. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain of the first chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises or consists of a sequence from a wildtype soluble human tissue factor.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain of the first chimeric polypeptide of the additional multi-chain chimeric polypeptide 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.

5 In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the soluble tissue factor domain of the first chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises 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
10 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
15 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 of any of the methods of stimulating or increasing the
20 proliferation of a Treg cell described herein, the soluble tissue factor domain of the first chimeric polypeptide of the additional multi-chain chimeric polypeptide does not initiate blood coagulation.

 In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide of the
25 additional multi-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 102. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 102. In some embodiments of any
30 of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide of the additional multi-chain chimeric polypeptide

comprises a sequence of SEQ ID NO: 102. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 104. In some embodiments of any
5 of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide of the additional multi-chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 104. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the first chimeric polypeptide of the additional multi-chain
10 chimeric polypeptide comprises a sequence of SEQ ID NO: 104.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the CD28 is human CD28. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second target-binding domain of the second chimeric
15 polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second target-binding domain of the second chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO:
20 6. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second target-binding domain of the second chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 6.

In some embodiments of any of the methods of stimulating or increasing the
25 proliferation of a Treg cell described herein, the second chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence that is at least 80% identical to SEQ ID NO: 106. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a
30 sequence that is at least 90% identical to SEQ ID NO: 106. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described

herein, the second chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 106. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric polypeptide of the additional multi-chain chimeric polypeptide
5 comprises a sequence that is at least 80% identical to SEQ ID NO: 108. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the second chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence that is at least 90% identical to SEQ ID NO: 108. In some embodiments of any of the methods of stimulating or increasing the
10 proliferation of a Treg cell described herein, the second chimeric polypeptide of the additional multi-chain chimeric polypeptide comprises a sequence of SEQ ID NO: 108.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the additional multi-chain chimeric polypeptide does not initiate blood coagulation. In some embodiments of any of the
15 methods of stimulating or increasing the proliferation of a Treg cell described herein, the liquid culture medium comprises about 10 nM to about 1000 nM of the additional multi-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the liquid culture medium comprises about 50 nM to about 300 nM of the additional multi-chain chimeric
20 polypeptide.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium further comprises an mTOR inhibitor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the mTOR inhibitor is
25 rapamycin. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 10 nM to about 500 nM of the mTOR inhibitor. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium comprises about 50 nM to about 150 nM of the mTOR inhibitor.
30 In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the methods further include: periodically adding to the

liquid culture medium, after each 36 hours to about 60 hours after the start of the period of time, the multi-chain chimeric polypeptide. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the periodic addition of the multi-chain chimeric polypeptide is performed to result in a concentration of about 10 nM to about 500 nM of the multi-chain chimeric polypeptide in the liquid culture medium. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the periodic addition of the multi-chain chimeric polypeptide is performed to result in a concentration of about 50 nM to about 150 nM of the multi-chain chimeric polypeptide in the liquid culture medium. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the methods further include: periodically adding to the liquid culture medium, after each about 6 days to about 8 days after the start of the period of time, the CD3/CD28-binding agent. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the period of time is about 7 days to about 56 days. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the period of claim is about 15 days to about 25 days. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the method further comprises, before the culturing step, a step of isolating the T_{reg} cell from a sample obtained from a subject. In some embodiments of any of the methods of stimulating or increasing the proliferation of a Treg cell described herein, the method further comprises, after the culturing step, a step of isolating the Treg cell from a sample obtained from a subject.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the isolating comprises the use of fluorescence-assorted cell sorting. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cell is an autologous T_{reg} cell, a haploidentical T_{reg} cell, or an allogeneic T_{reg} cell isolated from peripheral blood or umbilical cord blood. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cell is a CD4⁺CD25⁺Foxp3⁺ cell. In some embodiments of any of the methods of stimulating or

increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cell is a CD4⁺CD25⁺CD127^{dim} cell. In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the T_{reg} cells comprises a chimeric antigen receptor.

5 Provided herein are populations of T_{reg} cells generated by any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein. Also provided herein are compositions comprising any of the populations of T_{reg} cells generated by any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein and a pharmaceutically acceptable carrier. Provided herein are methods of treating a
10 subject in need thereof, the methods include administering to the subject a therapeutically effective amount of any of the compositions comprising any of the populations of T_{reg} cells generated by any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein and a pharmaceutically acceptable carrier. In some
15 embodiments of any of the methods of treating a subject in need thereof described herein, the subject has been identified or diagnosed as having an aging-related disease or an inflammatory disease. In some embodiments of any of the methods of treating a subject in need thereof described herein, the aging-related disease is selected from the group consisting of: Alzheimer's disease, aneurysm, cystic fibrosis, fibrosis in pancreatitis, glaucoma, hypertension, idiopathic pulmonary fibrosis, inflammatory bowel disease,
20 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
25 degeneration, cachexia, glomerulosclerosis, liver cirrhosis, NAFLD, osteoporosis, amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxia, multiple sclerosis, neurodegeneration, stroke, dementia, vascular disease, infection susceptibility, chronic inflammation, and renal dysfunction. In some embodiments of any of the methods of treating a subject in need thereof described herein, the inflammatory disease
30 is selected from the group consisting of: rheumatoid arthritis, inflammatory bowel disease, lupus erythematosus, lupus nephritis, diabetic nephropathy, CNS injury,

Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Crohn's disease, multiple sclerosis, Guillain-Barre syndrome, psoriasis, Grave's disease, ulcerative colitis, nonalcoholic steatohepatitis, and mood disorders.

In another aspect, provided herein are kits that include: a CD3/CD28-binding agent; a multi-chain chimeric polypeptide comprising: (a) a first chimeric polypeptide comprising: (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; (b) a second chimeric polypeptide comprising: (i) a second domain of a pair of affinity domains; and (ii) a second target-binding domain, wherein the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains; and an mTOR inhibitor. In some embodiments of any of the kits described herein, the kits further include an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the multi-chain chimeric polypeptide. In some embodiments of any of the kits described herein, the CD3/CD28-binding agent is an anti-CD3/anti-CD28 bead. In some embodiments of any of the kits described herein, the CD3/CD28-binding agent is a single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, wherein the first target-binding domain of the additional single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the additional single-chain chimeric polypeptide binds to CD28. In some embodiments of any of the kits described herein, the kits further include an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the single-chain chimeric polypeptide.

In some embodiments of any of the kits described herein, the CD3/CD28-binding agent is an additional multi-chain chimeric polypeptide comprising: (a) a first chimeric polypeptide comprising: (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and (b) a second chimeric polypeptide comprising: (i) a second domain of a pair of affinity domains; and (ii) a second target-binding domain, wherein the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains; and wherein the first target-binding domain binds

to CD3 and the second target-binding domain binds to CD28, or the first target-binding domain binds to CD28 and the second target-binding domain to CD3.

In some embodiments of any of the kits described herein, the liquid culture medium further includes an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the
5 additional multi-chain chimeric polypeptide.

In another aspect, provided herein are methods of isolating a Treg cell from a sample obtained from a subject, wherein the method comprises separating the Treg cell from the sample based on its expression of CD39, thereby isolating the Treg cell.

10 In some embodiments of any of the methods of isolating a Treg cell described herein, the method comprises: mixing the sample with an antibody or ligand capable of binding CD39 under conditions that allow binding of the antibody or ligand to Treg cells expressing CD39; separating the Treg cell bound to the antibody or ligand from other components in the sample, thereby isolating the Treg cell.

15 In some embodiments of any of the methods of isolating a Treg cell described herein, the antibody is a mouse, a humanized, or a human antibody or antigen-binding fragment thereof; and/or the antibody or the ligand is labeled with at least one of biotin, avidin, streptavidin, or a fluorochrome, or is bound to a particle, bead, resin, or solid support. In some embodiments of any of the methods of isolating a Treg cell described
20 herein, separating comprises the use of flow cytometry, fluorescence-activated cell sorting (FACS), centrifugation, or column, plate, particle, or bead-based methods.

In some embodiments of any of the methods of isolating a Treg cell described herein, the method further comprises: mixing the sample with a biotinylated antibody or ligand capable of binding CD39 under conditions that allow binding of the antibody or
25 the ligand to the Treg cell; capturing the Treg cell bound to the biotinylated antibody or the ligand using streptavidin-coated magnetic particles; separating the magnetic particle-bound Treg cell using a magnet; washing the magnet particle-bound Treg cell to remove other components in the sample; and releasing the magnetic particle-bound Treg cell from the magnet into a solution, thereby isolating the Treg cell.

30 In some embodiments of any of the methods of isolating a Treg cell described herein, the Treg cell is an autologous Treg cell, a haploidentical Treg cell, or an

allogeneic Treg cell isolated from a sample comprising fresh or frozen peripheral blood, umbilical cord blood, peripheral blood mononuclear cells, lymphocytes, CD4⁺ T cells or Treg cells. In some embodiments of any of the methods of isolating a Treg cell described herein, the Treg cell is a CD4⁺CD25⁺Foxp3⁺ cell. In some embodiments of any of the methods of isolating a Treg cell described herein, the Treg cell is a CD4⁺CD25⁺CD127^{dim} cell. In some embodiments of any of the methods of isolating a Treg cell described herein, the Treg cells comprises a chimeric antigen receptor. In some embodiments of any of the methods of isolating a Treg cell described herein, the Treg cells are immunosuppressive in vitro and in vivo.

In another aspect, provided herein are populations of isolated Treg cells generated by any one of the methods described herein. In some embodiments of any of the populations of isolated Treg cells described herein, the isolated population of Treg cells are greater than 70% CD39⁺ cells. Also provided are method of expanding any of the populations of isolated Treg cells described herein that include culturing the population of isolated Treg cells under conditions that allow for proliferation of the isolated Treg cells.

Also provided herein are compositions comprising any of the populations of isolated Treg cells described herein and a pharmaceutically acceptable carrier.

Also provided herein are methods of treating a subject in need thereof, comprising administering to the subject a therapeutically effective amount of any of the compositions described herein. In some embodiments of any of the methods of treating a subject in need described herein, the subject has been identified or diagnosed as having an aging-related disease or an inflammatory disease. In some embodiments of any of the methods of treating a subject in need described herein, the aging-related disease 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, neurodegeneration, stroke, dementia, vascular disease, infection susceptibility, chronic inflammation, and renal dysfunction. In some embodiments of any of the methods of treating a subject in need described herein, the inflammatory disease is selected from the group consisting of: rheumatoid arthritis, inflammatory bowel disease, lupus erythematosus, lupus nephritis, diabetic nephropathy, CNS injury, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Crohn's disease, multiple sclerosis, Guillain-Barre syndrome, psoriasis, Grave's disease, ulcerative colitis, nonalcoholic steatohepatitis, and mood disorders.

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 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).

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 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.

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 examples of soluble tissue factor domains are described herein.

5 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
10 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,
15 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.
20 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.

25 A “multi-chain polypeptide” as used herein refers to a polypeptide comprising two or more (e.g., three, four, five, six, seven, eight, nine, or ten) protein chains (e.g., at least a first chimeric polypeptide and a second polypeptide), where the two or more proteins chains associate through non-covalent bonds to form a quaternary structure.

 The term “epitope” means a portion of an antigen that specifically binds to an
30 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
5 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.

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,
10 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.

The term “CD3/CD28-binding agent” means an agent that binds specifically to
15 both CD3 and CD28 on the surface of a mammalian cell (e.g., a T cell, such as a T_{reg} cell) and induces downstream signaling in the mammalian cell. Non-limiting examples of CD3/CD28-binding agents are described herein.

The term “IL-2 receptor activating agent” means an agent that binds specifically to an IL-2 receptor present on a surface of a mammalian cell (e.g., a T cell, such as a T_{reg}
20 cell) and induces downstream signaling in the mammalian cell. Non-limiting examples of IL-2 receptor activating agents are described herein.

The term “IgG1 antibody construct” means a single chain polypeptide or a multi-chain polypeptide that includes at least one antigen-binding domain that binds to a soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains
25 described herein) and at least one IgG1 Fc domain. Non-limiting examples of IgG1 antibody constructs are described herein.

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
30 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.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a diagram showing expansion of Foxp3⁺ T_{reg} cells using either (1) 2t2, CD3/CD28 beads, and rapamycin or (2) recombinant human IL-2, CD3/CD28 beads, and rapamycin.

FIGs. 2A-2I are diagrams showing an increase in Helios⁺, CTLA-4⁺, CD39⁺, CD62L⁺, CD25⁺, CD103⁺, CD69⁺, PD1⁺, and CD49b⁺ markers after stimulation of T_{reg} cells with (1) 2t2, CD3/CD28 beads, and rapamycin or (2) recombinant human IL-2, CD3/CD28 beads, and rapamycin.

FIGs. 3A-3D are diagrams showing glucose metabolism levels upon activation of human T_{reg} cells with (1) 2t2, CD3/CD28 beads, and rapamycin, or (2) recombinant human IL-2, CD3/CD28 beads, and rapamycin. FIG. 3A shows levels of glycolysis of T_{reg} cells. FIG. 3B shows levels of glycolytic capacity of T_{reg} cells. FIG. 3C shows levels of glycolytic reserve of T_{reg} cells. FIG. 3D shows levels of non-glycolytic acidification of T_{reg} cells.

FIG. 4 is a diagram showing the suppression of autologous T responder cells by T_{reg} cells previously stimulated with (1) 2t2, CD3/CD28 beads, and rapamycin, or (2) recombinant human IL-2, CD3/CD28 beads, and rapamycin.

FIG. 5 is a diagram showing the levels of IL-10 secretion by T_{reg} cells in a T responder cell suppression assay, where the T_{reg} cells were previously stimulated with either (1) 2t2 and CD3/CD28 beads in the presence or absence of rapamycin, or (2) recombinant human IL-2 and CD3/CD28 beads in the presence or absence of rapamycin.

FIGs. 6A-6B show exemplary diagrams for a fusion protein generated including anti-CD3scFv/TF/IL15D8N mutant (3t15*) and anti-CD28scFv/IL15R α Su (28s) fusion proteins.

FIG. 7 is a graph showing 3t15*-28s purification elution by immunoaffinity chromatography from an anti-TF antibody affinity column.

FIG. 8 shows size exclusion chromatography analysis of 3t15*-28s.

FIG. 9 shows an SDS gel of 3t15*-28s purified from anti-TF antibody affinity chromatography. Lane 1: 3t15*-28s; Lane 2: SeeBlue Plus 2 prestained standards (numbers on the left side indicate molecular weights in kilodaltons).

FIG. 10A is a graph showing interaction between 3t15*-28s and CD28.

FIG. 10B is a graph showing interaction between 3t15*-28s and CD3.

FIG. 11 is a graph showing the expansion of Foxp3⁺ regulatory T cells in media containing 2t2 and 3t15*-28s.

FIG. 12 is a graph showing the level of proliferating human immune cells following incubation in media containing 2t2 and 3t15*-28s with or without anti-TF antibody.

FIG. 13A shows flow cytometry histograms indicating the purity of CD39⁺ regulatory T cells isolated with different purification schemes.

FIG. 13B shows a flow cytometry histogram indicating the purity of CD39⁺ regulatory T cells isolated using an anti-CD39 Ab reagent.

FIG. 14 shows a bar graph depicting the level of T responder cell proliferation following incubation with regulatory T cells isolated with different methods.

FIG. 15 shows a graph showing T responder cell proliferation (as assessed by levels of IL-10) following incubation with T regulatory cells isolated using different methods.

DETAILED DESCRIPTION

Provided herein are methods of stimulating or increasing the proliferation of a T_{reg} cell (e.g., any of the T_{reg} cells described herein), a population of T_{reg} cells produced using any of the methods described herein, a composition including any of these populations of T_{reg} cells, and methods of treating a subject that include administering any of these populations of T_{reg} cells or compositions.

Tissue Factor

Human tissue factor is a 263 amino-acid transmembrane protein containing three 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 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 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. 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 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 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 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 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. 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 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 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.

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 ~80 Å

above the membrane surface. Upon FVIIa binding to tissue factor, the FVa active site is repositioned $\sim 6\text{\AA}$ closer to the membrane. This modulation may aid in a proper 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
5 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 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
10 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 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*
15 269(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 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
20 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 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
25 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 interact independently with FVIIa, indicating that the mutational effect at the Trp⁴⁵
30 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.

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; 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 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 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 Glu³⁵ of FVIIa would 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.

Multi-Chain Chimeric Polypeptides

Non-limiting examples of cell activating agents are multi-chain chimeric polypeptides that include: (a) a first chimeric polypeptide including: (i) a first target-

binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and (b) a second chimeric polypeptide including: (i) a second domain of a pair of affinity domains; and (ii) a second target-binding domain, where the first chimeric polypeptide and the second chimeric polypeptide associate through the binding
5 of the first domain and the second domain of the pair of affinity domains.

In some embodiments of any of the multi-chain chimeric polypeptides described herein, the first target-binding domain (e.g., any of the first target-binding domains described herein) and the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein) directly abut each other in the first chimeric
10 polypeptide. In some embodiments of any of the multi-chain chimeric polypeptides described herein, the first 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 first target-binding domains described herein) and the soluble tissue factor domain (e.g., any of the exemplary soluble
15 tissue factor domains described herein) in the first chimeric polypeptide.

In some embodiments of any of the multi-chain chimeric polypeptides described herein, the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein) and the first domain of the pair of affinity domains (e.g., any of the exemplary first domains of any of the exemplary pairs of affinity domains
20 described herein) directly abut each other in the first chimeric polypeptide. In some embodiments of any of the multi-chain chimeric polypeptides described herein, the first 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
25 the first domain of the pair of affinity domains (e.g., any of the exemplary first domains of any of the exemplary pairs of affinity domains described herein) in the first chimeric polypeptide.

In some embodiments of any of the multi-chain chimeric polypeptides described herein, the second domain of the pair of affinity domains (e.g., any of the exemplary
30 second domains of any of the exemplary pairs of affinity domains described herein) and the second target-binding domain (e.g., any of the exemplary second target-binding

domains described herein) directly abut each other in the second chimeric polypeptide. In some embodiments of any of the multi-chain chimeric polypeptides described herein, the second 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 domain of the pair of affinity domains (e.g., any of the exemplary second domains of any of the exemplary pairs of affinity domains described herein) and the second target-binding domain (e.g., any of the exemplary second target-binding domains described herein) in the second chimeric polypeptide.

In some embodiments of any of the multi-chain chimeric polypeptides described herein, one or both of 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) 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 , CD26, 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 scMHC I, a ligand for a scMHC II, 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 multi-chain chimeric polypeptides described herein, one or both of 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) is a soluble interleukin or cytokine 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, and SCF.

In some embodiments of any of the multi-chain chimeric polypeptides described herein, one or both of the first target-binding domain and the second target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) is a soluble interleukin or cytokine receptor. Non-limiting examples of soluble interleukin receptors and soluble cytokine receptors include: 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 of any of the multi-chain chimeric polypeptides described herein, one or both of the first target-binding domain and the second target-binding domain is a soluble interleukin or cytokine receptor. In some embodiments of any of the multi-chain chimeric polypeptides described herein, the soluble receptor is a soluble TGF- β receptor II (TGF- β RII) a soluble TGF- β RIII, a soluble receptor for TNF α , a soluble receptor for IL-4, or a soluble receptor for IL-10.

Soluble Tissue Factor Domains

In some embodiments, 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.

Exemplary Soluble Human Tissue Factor Domain (SEQ ID NO: 2)

SGTTNTVAAYNLTKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTD
 TECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQ
 PTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGK
 5 KTAKTNTNEFLIDVDKGENYCFSVQAVIPSRVTNRKSTDSPVECMGQEKGEFRE

Exemplary Nucleic Acid Encoding Soluble Human Tissue Factor Domain (SEQ ID NO: 13)

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAGCACCA
 10 ACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTTTACACC
 GTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCTATACCAC
 CGACACCGAGTGCGATCTCACCGATGAGATCGTGAAAGATGTGAAACAGACC
 TACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGCACTGGTTC
 CGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTACCTCGAGA
 15 CCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGCACAAAGGT
 GAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAACAACACCTTT
 CTCAGCCTCCGGGATGTGTTCGGCAAAGATTTAATCTACACACTGTATTACTGG
 AAGTCCTCTTCTCCGGCAAGAAGACAGCTAAAACCAACACAAACGAGTTTT
 TAATCGACGTGGATAAAGGCGAAAACACTACTGTTTCAGCGTGCAAGCTGTGATC
 20 CCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGTTGAGTGCATGG
 GCCAAGAAAAGGGCGAGTTCCGGGAG

Exemplary Soluble Mouse Tissue Factor Domain (SEQ ID NO: 14)

agipekafnltwistdfktilwqpkptnytytvqisdrsrnwknkcfstt
 25 dtecdltdeivkdvtwayeakvlsvprnrsvhgdgdqlvihgeppftnap
 kflpyrdtnlgqpviqqfeqdgrklrvvkdsltlvrkngtfltlrqvfgk
 dlgyiityrkgsstgkktnitntnefsidveegvsycffvqamifsrktng
 nspgsstvcetqwkstflge

30 Exemplary Soluble Rat Tissue Factor Domain (SEQ ID NO: 15)

Agtppgkafnltwistdfktilwqpkptnytytvqisdrsrnwkykctgt
 tdtecdltdeivkdvnwtyearvlsvpwrnsthgketlfgthgeppftna
 rkflpyrdtkigqpviqkyeqggtklkvtkdsftlvrkngtfltlrqvfg

ndlgyltyrkdsstgrktnththneflidvekgvsycffaqavifsrktn
hkspesitkcteqwksvlge

Exemplary Mutant Soluble Human Tissue Factor Domain (SEQ ID NO: 16)

5 SGTNTVAAYNLTKSTNFATALEWEPKPVNQVYTVQISTKSGDWKSKCFYTT
DTECALTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNL
GQPTIQSFEQVGTKVNVTVEDERTLVARNNTALSLRDVFGKDLIYTLYYWKSSSS
GKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEF
RE

10 **Exemplary Mutant Soluble Human Tissue Factor Domain (SEQ ID NO: 17)**

SGTNTVAAYNLTKSTNFATALEWEPKPVNQVYTVQISTKSGDAKSKCFYTTD
TECALTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLAENSPEFTPYLETNLG
QPTIQSFEQVGTKVNVTVEDERTLVARNNTALSLRDVFGKDLIYTLYYWKSSSSG
KKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFR
15 E

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, at least 99% identical, or 100% identical to SEQ ID NO: 2, 14, 15, 16, or 17. In some embodiments, a soluble tissue factor domain can include a sequence of SEQ ID NO: 2, 14, 15, 16, or 17 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) 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 decrease the activity and/or structural stability of the protein.

In some examples, the soluble tissue factor domain is not capable of binding to Factor VIIa. In some examples, the soluble tissue factor domain does not convert
5 inactive Factor X into Factor Xa. In some embodiments, any of the single-chain chimeric polypeptides or multi-chain chimeric polypeptides described herein 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
10 mouse tissue factor domain. In some embodiments, the soluble tissue factor domain can 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
15 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
20 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 examples, the soluble tissue factor domain does not include any of: a
25 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
30 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.

5 In some examples, the soluble tissue factor domain includes 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
10 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;
15 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: 16 or SEQ ID NO: 17.

 In some examples, the soluble tissue factor domain can be encoded by a nucleic
20 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% identical, at least 98% identical, at least 99% identical, or 100% identical to SEQ ID NO:
25 13.

 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 amino acids, about 20 amino acids to about 200 amino acids, about 20 amino acids to
30 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
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
5 about 140 amino acids, about 20 amino acids to about 135 amino acids, about 20 amino
acids to about 130 amino acids, about 20 amino acids to about 125 amino acids, about 20
amino acids to about 120 amino acids, about 20 amino acids to about 115 amino acids,
about 20 amino acids to about 110 amino acids, about 20 amino acids to about 105 amino
acids, about 20 amino acids to about 100 amino acids, about 20 amino acids to about 95
10 amino acids, about 20 amino acids to about 90 amino acids, about 20 amino acids to
about 85 amino acids, about 20 amino acids to about 80 amino acids, about 20 amino
acids to about 75 amino acids, about 20 amino acids to about 70 amino acids, about 20
amino acids to about 60 amino acids, about 20 amino acids to about 50 amino acids,
about 20 amino acids to about 40 amino acids, about 20 amino acids to about 30 amino
15 acids, about 30 amino acids to about 220 amino acids, about 30 amino acids to about 215
amino acids, about 30 amino acids to about 210 amino acids, about 30 amino acids to
about 205 amino acids, about 30 amino acids to 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,
20 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 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
25 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 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
30 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,

[illegible]

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[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

amino acids to about 170 amino acids, about 160 amino acids to about 165 amino acids,
about 165 amino acids to about 220 amino acids, about 165 amino acids to about 215
amino acids, about 165 amino acids to about 210 amino acids, about 165 amino acids to
about 205 amino acids, about 165 amino acids to about 200 amino acids, about 165
5 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
amino acids to about 215 amino acids, about 170 amino acids to about 210 amino acids,
10 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,
about 175 amino acids to about 220 amino acids, about 175 amino acids to about 215
15 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
amino acids, about 180 amino acids to about 220 amino acids, about 180 amino acids to
20 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
about 220 amino acids, about 185 amino acids to about 215 amino acids, about 185
25 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
amino acids to about 210 amino acids, about 190 amino acids to about 205 amino acids,
30 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, 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.

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. 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.

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 sequences, e.g., any of the exemplary linker sequences described herein or known in the art).

In some embodiments of any of the first chimeric polypeptides and/or the second 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 first chimeric polypeptides and/or the second 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, 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, 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 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 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 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 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,

[illegible]

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[illegible]

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 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 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: 18). In some embodiments, the linker sequence

can be encoded by a nucleic acid comprising or consisting of:

GGCGGTGGAGGATCCGGAGGAGGTGGCTCCGGCGGCGGAGGATCT (SEQ ID NO: 19). In some embodiments, the linker sequence can comprise or consist of: GGGSGGGS (SEQ ID NO: 20).

5

Target-Binding Domains

In some embodiments of any of the single- or multi-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- or multi-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, about 5 amino acids to about 650 amino acids, about 5 amino acids to about 600 amino acids, about 5 amino acids to about 550 amino acids, about 5 amino acids to about 500 amino acids, about 5 amino acids to about 450 amino acids, about 5 amino acids to about 400 amino acids, about 5 amino acids to about 350 amino acids, about 5 amino acids to about 300 amino acids, about 5 amino acids to about 280 amino acids, about 5 amino acids to about 260 amino acids, about 5 amino acids to about 240 amino acids, about 5 amino acids to about 220 amino acids, about 5 amino acids to about 200 amino acids, about 5 amino acids to about 195 amino acids, about 5 amino acids to about 190 amino acids, about 5 amino acids to about 185 amino acids, about 5 amino acids to about 180 amino acids, about 5 amino acids to about 175 amino acids, about 5 amino

acids to about 170 amino acids, about 5 amino acids to about 165 amino acids, about 5 amino acids to about 160 amino acids, about 5 amino acids to about 155 amino acids, about 5 amino acids to about 150 amino acids, about 5 amino acids to about 145 amino acids, about 5 amino acids to about 140 amino acids, about 5 amino acids to about 135 amino acids, about 5 amino acids to about 130 amino acids, about 5 amino acids to about 125 amino acids, about 5 amino acids to about 120 amino acids, about 5 amino acids to about 115 amino acids, about 5 amino acids to about 110 amino acids, about 5 amino acids to about 105 amino acids, about 5 amino acids to about 100 amino acids, about 5 amino acids to about 95 amino acids, about 5 amino acids to about 90 amino acids, about 5 amino acids to about 85 amino acids, about 5 amino acids to about 80 amino acids, about 5 amino acids to about 75 amino acids, about 5 amino acids to about 70 amino acids, about 5 amino acids to about 65 amino acids, about 5 amino acids to about 60 amino acids, about 5 amino acids to about 55 amino acids, about 5 amino acids to about 50 amino acids, about 5 amino acids to about 45 amino acids, about 5 amino acids to about 40 amino acids, about 5 amino acids to about 35 amino acids, about 5 amino acids to about 30 amino acids, about 5 amino acids to about 25 amino acids, about 5 amino acids to about 20 amino acids, about 5 amino acids to about 15 amino acids, about 5 amino acids to about 10 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).

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 or multi-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
5 single-chain or multi-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 or multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain include the same amino acid sequence.

10 In some embodiments of any of the single-chain or multi-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 or multi-chain chimeric polypeptides described herein, one or both of the first target-binding domain and the
15 second target-binding domain is an antigen-binding domain. In some embodiments of any of the single-chain or multi-chain chimeric polypeptides described herein, the first target-binding domain and the second target-binding domain are each antigen-binding domains. In some embodiments of any of the single-chain or multi-chain chimeric polypeptides described herein, the antigen-binding domain includes or is a scFv or a
20 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 No. 7,723,482), CD3 (see, e.g., those described in U.S. Patent No. 9,226,962), CD33
25 (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-1 (see, e.g., those described in WO 2014/095808), VEGF (see, e.g., those described in
30 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. 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, 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), CD26 (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. 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 PDGF-DD (see, e.g., those described in U.S. Patent No. 9,441,034).

5 The antigen-binding domains present in any of the single-chain or multi-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, 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-
10 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 or multi-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 VNAR domain is a single monomeric variable antibody domain
15 that can be found in cartilaginous fish. Non-limiting aspects of V_HH 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.*
20 *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-
25 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 or multi-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
30 antigen-binding domains in the single-chain or multi-chain chimeric polypeptides described herein are both VNAR domains, or at least one antigen-binding domain is a

VNAR domain. In some embodiments, each of the antigen-binding domains in the single-chain or multi-chain chimeric polypeptides described herein are both scFv domains, or at least one antigen-binding domain is a scFv domain.

In some embodiments, two or more of polypeptides present in the single-chain or multi-chain chimeric polypeptide can assemble (e.g., non-covalently assemble) to form any of the antigen-binding domains described herein, e.g., an antigen-binding fragment of an antibody (e.g., any of the antigen-binding fragments of an antibody described herein), a VHH-scAb, a VHH-Fab, a Dual scFab, a F(ab')₂, a diabody, a crossMab, a DAF (two-in-one), a DAF (four-in-one), a DutaMab, a DT-IgG, a knobs-in-holes common light chain, a knobs-in-holes assembly, a charge pair, a Fab-arm exchange, a SEEDbody, a LUZ-Y, a Fcab, a κλ-body, an orthogonal Fab, a DVD-IgG, a IgG(H)-scFv, a scFv-(H)IgG, IgG(L)-scFv, scFv-(L)IgG, IgG(L,H)-Fv, IgG(H)-V, V(H)-IgG, IgG(L)-V, V(L)-IgG, KIH IgG-scFab, 2scFv-IgG, IgG-2scFv, scFv4-Ig, Zyboby, DVI-IgG, Diabody-CH3, a triple body, a miniantibody, a minibody, a TriBi minibody, scFv-CH3 KIH, Fab-scFv, a F(ab')₂-scFv₂, a scFv-KIH, a Fab-scFv-Fc, a tetravalent HCAb, a scDiabody-Fc, a Diabody-Fc, a tandem scFv-Fc, an Intrabody, a dock and lock, a ImmTAC, an IgG-IgG conjugate, a Cov-X-Body, and a scFv1-PEG-scFv₂. See, e.g., Spiess et al., *Mol. Immunol.* 67:95-106, 2015, incorporated in its entirety herewith, for a description of these elements. Non-limiting examples of an antigen-binding fragment of an antibody include an Fv fragment, a Fab fragment, a F(ab')₂ fragment, and a Fab' fragment. Additional examples of an antigen-binding fragment of an antibody is an antigen-binding fragment of an IgG (e.g., an antigen-binding fragment of IgG1, IgG2, IgG3, or IgG4) (e.g., an antigen-binding fragment of a human or humanized IgG, e.g., human or humanized IgG1, IgG2, IgG3, or IgG4); an antigen-binding fragment of an IgA (e.g., an antigen-binding fragment of IgA1 or IgA2) (e.g., an antigen-binding fragment of a human or humanized IgA, e.g., a human or humanized IgA1 or IgA2); an antigen-binding fragment of an IgD (e.g., an antigen-binding fragment of a human or humanized IgD); an antigen-binding fragment of an IgE (e.g., an antigen-binding fragment of a human or humanized IgE); or an antigen-binding fragment of an IgM (e.g., an antigen-binding fragment of a human or humanized IgM).

An “Fv” fragment includes a non-covalently-linked dimer of one heavy chain variable domain and one light chain variable domain.

A “Fab” fragment includes, the constant domain of the light chain and the first constant domain (CH₁) of the heavy chain, in addition to the heavy and light chain
5 variable domains of the Fv fragment.

A “F(ab')₂” fragment includes two Fab fragments joined, near the hinge region, by disulfide bonds.

A “dual variable domain immunoglobulin” or “DVD-Ig” refers to multivalent and multispecific binding proteins as described, e.g., in DiGiammarino et al., *Methods Mol.*
10 *Biol.* 899:145-156, 2012; Jakob et al., *MABs* 5:358-363, 2013; and U.S. Patent Nos. 7,612,181; 8,258,268; 8,586,714; 8,716,450; 8,722,855; 8,735,546; and 8,822,645, each of which is incorporated by reference in its entirety.

DARTs are described in, e.g., Garber, *Nature Reviews Drug Discovery* 13:799-801, 2014.

15 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.

20 Soluble Interleukin or Cytokine Protein

In some embodiments of any of the single-chain or multi-chain chimeric polypeptides described herein, one or both of the first target-binding domain and the second target-binding domain can be a soluble interleukin protein or soluble cytokine protein. In some embodiments, the soluble interleukin or soluble cytokine protein is
25 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, and FLT3L. Non-limiting examples of soluble IL-2, IL-3, IL-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: 75)

30 aptssstkkk qlqlchllld lqmilnginn yknpkltrml tfkfympkka
telkhlqcle eelkpleevl nlaqsknfhl rprdlisnin vivlelkgse

ttfmceyade tativeflnr witfcqsiis tlt

Human Soluble IL-3 (SEQ ID NO: 76)

apmtqttplkt swvnscnmid eiithlkqpp lplldfnnln gedqdilmen
5 nlrpnleaf nravkslqna saiesilknl lpclplataa ptrhpihikd
gdwnefrrkl tfylktlena qaqqttlsia if

Human Soluble IL-7 (SEQ ID NO: 77)

dcdiegkdgkqyesv lmvsidqlld smkeigsncl nnefnffkrh icdankegmf
10 lfraarklrq flkmnstgdf dlhlkvseg ttillnctgq vkgrkpaalg
eaqptkslee nkslkeqkkl ndlcflkrll geiktcwnki lmgtkch

Human Soluble IL-8 (SEQ ID NO: 78)

egavlprsak elrcqikty skpfhpkfik elrviesgph canteiivkl
15 sdgrelcldp kenwvqrvve kflkraens

Human Soluble IL-10 (SEQ ID NO: 79)

spgggtqsensc thfpgnlpnm lrdlrdafrs vktffqmkdq ldnlllkesl
ledfkgyigc qalsemiqfy leevmpqaen qdpdikahvn slgenlktlr
20 lrlrrchrfl pcenkskave qvknafnklq ekgiyakamse fdifinyiea
ymtmkirn

Human Soluble IL-15 (SEQ ID NO: 80)

nwvnvisdlkki edliqsmhid atlytesdvh psckvtamkc fllelqvisl
25 esgdasihdt venliilann slssngnvte sgckeceele eknikeflqs
fvhivqmfin ts

Human Soluble IL-15 D8N Mutant (SEQ ID NO: 99)

NWVNVISNLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLESGDASI
30 HDTVENLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

Human Soluble IL-15 D8N Mutant (SEQ ID NO: 100)

AACTGGGTGAACGTCATCAGCAATTTAAAGAAGATCGAAGATTTAATTCAGTC
 CATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTCTTGTA
 GGTGACCGCCATGAAATGTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAGAG
 5 CGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTTAGCCAATA
 ACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAGGAGTGCGA
 AGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTGTGCACATTG
 TCCAGATGTTTCATCAATACCTCC

10 Human Soluble IL-17 (SEQ ID NO: 81)

gitiprn pgcpnsedkn fprrvmvnlh ihrrntntnp krssdyynrs
 tspwnlhrne dperypsviw eakcrhlgi nadgnvdyhm nsvpiqqeil
 vlrrepphpc nsfrlekilv svgtctvtpi vhhva

15 Human Soluble IL-18 (SEQ ID NO: 82)

yfgklesklsvirn lndqvlfidq gnrplfedmt dsdcrdnapr tifiismykd
 sqprgmavti svkcekistl scenkiisfk emnppdnkd tksdiiffqr
 svpghdnkmq fesssyegyf lacekerdlf klilkkedel gdrsimftvq ned

20 Human Soluble PDGF-DD (SEQ ID NO: 83)

rdtsatpqsasi kalrnanlrr desnhltdly rrdetiqvkg ngyvqsprfp
 nsyprnlllt wrhlsqentr iqlvfdnqfg leeaendicr ydfvevedis
 etstiirgrw cghkevppri ksrtngikit fksddyfvak pgfkiiysll
 edfqpaaase tnwesvtssi sgvsynspsv tdptliadal dkkiaefdtv
 25 edllkyfnpe swqedlenmy ldtpyrgrs yhdrkskvd1 drlnddakry
 sctprnysvn ireelklanv vffprcllvq rcggncgcgt vnwrscctns
 gktvkkyhev lqfepghikr rgraktmalv diqldhherc dcicssrppr

30

Human Soluble SCF (SEQ ID NO: 84)

egicrnrvtnnvkdv tkivanlpkd ymitlkyvpg mdvlpshcwi semvvqlsds
 ltdlldkfsn iseglsnysi idklvnivdd lvecvkenss kdlkksfksp
 eprlftpeef frifnrsida fkdfvvaset sdcvvsstls pekdsrvsvt
 5 kpfmlppvaa sslrndssss nrkaknppgd sslhwaamal palfsliigf
 afgalywkkr qpsltraven iqineednei smlqekeref gev

Human Soluble FLT3L (SEQ ID NO: 85)

tqdcsfqhspissd favkirelsd yllqdypvtv asnlqdealc gglwrlvlaq
 10 rwmerlktva gskmqgller vnteihfvtk cafqpppscl rfvqtnisrl
 lqetseqlva lkpwithrqnf srclelqcqp dsstlpppws prpleatapt
 apqppllllll llpvglllla aawclhwqrt rrrtprpgeq vppvpspqdl
 llveh

15 Non-limiting examples of soluble MICA, MICB, ULBP1, ULBP2, ULBP3,
 ULBP4, ULBP5, and ULBP6 are provided below.

Human Soluble MICA (SEQ ID NO: 86)

ephsrlry nltvlswdgs vqsgfltevh ldgqpflrce rpkcrakpqq
 20 qwaedvlgkn twdretrdlt gngkdrlrmtl ahikdqkegl hslqeirvce
 ihednstrss qhfyydgelf lsqnletkew tmpqssraqt lamnvrnflk
 edamktkthy hamhadclqe lrrylksgvv lrrtvppmvn vtrseasegn
 itvtcrasgf ypwnitlswr qdgvslshdt qqwgdvlpdg ngtyqtwvat
 ricqgeeqrft tcymehsgnh sthpvpqsgkv lvlqshwqtf hvsavaaaai
 25 fviiiifyvrc ckkktsaaeg pelvslqvld qhpvgtsdhr datqlgfgqpl
 msdlgstgst ega

Human Soluble MICB (SEQ ID NO: 87)

aephsrlry nlmvlsqdes vqsgflaegh ldgqpflryd rpkrrakpqq
 30 qwaedvlgak twdtetedlt engqdlrrtl thikdqkqgl hslqeirvce

ihedsstrgs rhfyydgelf lsqnletqes tvpqssraqt lamnvtnfwk
 edamkthky ramqadclqk lqrylksgva irrtpmmvn vtcsevsegn
 itvtcrassf yprnitltwr qdgvslshnt qqwgdvlpdg ngtyqtwvat
 rirqgeeqrf tcymehsgnh gthpvpsgkv lvlqsqrtdf pyvsaampcf
 5 viiiilcvpc ckkktsaaeg pelvslqvld qhpvgtdghr
 daaqlgfgpl msatgstgst ega

Human Soluble ULBP1 (SEQ ID NO: 88)

wvdthclcydfiit pksrpepqcw evqglvderp flhydcvnhk akafaslgkk
 10 vnvtktweeq tetlrdvddf lkgqllldiqv enlipieplt lqarmscehe
 ahghgrgswq flfngqkfl fdsmnrkwt lhpqakmte kweknrdvtm
 ffqkislqdc kmwleeflmy weqmdptkp pslapg

Human Soluble ULBP2 (SEQ ID NO: 89)

15 gradphslcyditvi pkfrpgprwc avqgqvdekt flhydcgnkt vtpvsplgkk
 lnvttawkaq npvlrevvdi lteqlrdiql enytpkeplt lqarmsceqk
 aeghssgswq fsfdgqifll fdsekrmwtt vhpqarkmke kwendkvvam
 sfhyfsmgdc igwledflmg mdstlepszag aplams

20 Human Soluble ULBP3 (SEQ ID NO: 90)

dahslwynfti ihlprhgqqw cevqsqvddk nflsydcgsd kvlsmgglee
 qlyatdawgk qlemlrevgq rlrleladte ledftpsgpl tlqvrmscec
 eadgyirgsw qfsfdgrkfl lfdsnnrkwt vvahagarmk ekwekdsglt
 tffkmvsmrd ckswlrdflm hrkkrlpta pptmapg

25

Human Soluble ULBP4 (SEQ ID NO: 91)

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

gladp hslcyditvi pkfrpgprwc avqqgvdekt flhydcgskt
vtpvsplgkk lnvttawkaq npvlrevvdi lteqlldiql enyipkeplt
lqarmsceqk aeghsgswq lsfdgqifll fdsenrmwtt vhpqarkmke
kwendkdmtn sfhyismgdc tgwledflmg mdstlepszag apptmssg

```

rrddp hslcyditvi pkfrpgprwc avqqgvdekt flhydcgnkt
vtpvsplgkk lnavtmawkaq npvlrevvdi lteqlldiql enytpkeplt
10 lqarmsceqk aeghssgswq fsidgqtfll fdsekrmwtt vhpqarkmke
kwendkdvam sfhyismgdc igwledflmg mdstlepszag aplanmssg

```

15 Soluble Receptor

93

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

Pairs of Affinity Domains

10 In some embodiments, a multi-chain chimeric polypeptide includes: 1) a first chimeric polypeptide that includes a first domain of a pair of affinity domains, and 2) a second chimeric polypeptide that includes a second domain of a pair of affinity domains such that the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity
 15 domains. In some embodiments, the pair of affinity domains is a sushi domain from an alpha chain of human IL-15 receptor (IL15R α) and a soluble IL-15. A sushi domain, also known as a short consensus repeat or type 1 glycoprotein motif, is a common motif in protein-protein interaction. Sushi domains have been identified on a number of protein-binding molecules, including complement components C1r, C1s, factor H, and C2m, as
 20 well as the nonimmunologic molecules factor XIII and β 2-glycoprotein. A typical Sushi domain has approximately 60 amino acid residues and contains four cysteines (Ranganathan, *Pac. Symp Biocomput.* 2000:155-67). The first cysteine can form a disulfide bond with the third cysteine, and the second cysteine can form a disulfide bridge with the fourth cysteine. In some embodiments in which one member of the pair of
 25 affinity domains is a soluble IL-15, the soluble IL15 has a D8N or D8A amino acid substitution. In some embodiments in which one member of the pair of affinity domains is an alpha chain of human IL-15 receptor (IL15R α), the human IL15R α is a mature full-length IL15R α . In some embodiments, the pair of affinity domains is barnase and barnstar. In some embodiments, the pair of affinity domains is a PKA and an AKAP. In
 30 some embodiments, the pair of affinity domains is an adapter/docking tag module based on mutated RNase I fragments (Rossi, *Proc Natl Acad Sci USA.* 103:6841-6846, 2006;

Sharkey et al., *Cancer Res.* 68:5282-5290, 2008; Rossi et al., *Trends Pharmacol Sci.* 33:474-481, 2012) or SNARE modules based on interactions of the proteins syntaxin, synaptotagmin, synaptobrevin, and SNAP25 (Deyev et al., *Nat Biotechnol.* 1486-1492, 2003).

5 In some embodiments, a first chimeric polypeptide of a multi-chain chimeric polypeptide includes a first domain of a pair of affinity domains and a second chimeric polypeptide of the multi-chain chimeric polypeptide includes a second domain of a pair of affinity domains, wherein the first domain of the pair of affinity domains and the second domain of the pair of affinity domains bind to each other with a dissociation
10 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 first domain of the pair of affinity domains and the second domain of the pair of affinity domains bind to each other with a K_D of 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}
15 M, about 1×10^{-7} M to about 1×10^{-9} M, about 1×10^{-8} M to about 1×10^{-10} M, about 1×10^{-9} M to about 1×10^{-11} M, about 1×10^{-10} M to about 1×10^{-12} M, about 1×10^{-11} M to about 1×10^{-13} M, about 1×10^{-4} M to about 1×10^{-5} M, about 1×10^{-5} M to about 1×10^{-6} M, about 1×10^{-6} M to about 1×10^{-7} M, about 1×10^{-7} M to about 1×10^{-8} M, about 1×10^{-8} M to about 1×10^{-9} M, about 1×10^{-9} M to about 1×10^{-10} M, about 1×10^{-10} M to
20 about 1×10^{-11} M, about 1×10^{-11} M to about 1×10^{-12} M, or about 1×10^{-12} M to about 1×10^{-13} M (inclusive). Any of a variety of different methods known in the art can be used to determine the K_D value of the binding of the first domain of the pair of affinity domains and the second domain of the pair of affinity domains (e.g., an electrophoretic mobility shift assay, a filter binding assay, surface plasmon resonance, and a biomolecular
25 binding kinetics assay, etc.).

In some embodiments, a first chimeric polypeptide of a multi-chain chimeric polypeptide includes a first domain of a pair of affinity domains and a second chimeric polypeptide of the multi-chain chimeric polypeptide includes a second domain of a pair of affinity domains, wherein the first domain of the pair of affinity domains, the second
30 domain of the pair of affinity domains, or both is about 10 to 100 amino acids in length. For example, a first domain of a pair of affinity domains, a second domain of a pair of

affinity domains, or both 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 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 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 first domain of a pair of affinity domains, a second domain of a pair of affinity domains, or both 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 first and/or second domains of a pair of affinity domains 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 first and/or second domains of a pair of affinity domains remains intact. For example, a sushi domain from an alpha chain of human IL-15 receptor (IL15R α) can include one or more additional amino acids at the N-terminus and/or the C-terminus, while still retaining the ability to bind to a soluble IL-15. Additionally or alternatively, a

soluble IL-15 can include one or more additional amino acids at the N-terminus and/or the C-terminus, while still retaining the ability to bind to a sushi domain from an alpha chain of human IL-15 receptor (IL15R α).

A non-limiting example of a sushi domain from an alpha chain of IL-15 receptor alpha (IL15R α) 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
ITCPPPMSEVHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAH
WTPPSLKCIR (SEQ ID NO: 94). In some embodiments, a sushi domain from an alpha
chain of IL15R α can be encoded by a nucleic acid including
ATTACATGCCCCCTCCCATGAGCGTGGAGCACGCCGACATCTGGGTGAAGAG
CTATAGCCTCTACAGCCGGGAGAGGTATATCTGTAACAGCGGCTTCAAGAGGA
AGGCCGGCACCAGCAGCCTCACCGAGTGCGTGCTGAATAAGGCTACCAACGT
GGCTCACTGGACAACACCCTCTTTAAAGTGCATCCGG (SEQ ID NO: 95).

In some embodiments, a soluble IL-15 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
NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGD
ASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINT
S (SEQ ID NO: 96). In some embodiments, a soluble IL-15 can be encoded by a nucleic acid including the sequence of
AACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATTTAATTCAGTC
CATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTCTTGTA
GGTGACCGCCATGAAATGTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAGAG
CGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTTAGCCAATA
ACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAGGAGTGCGA
AGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTGTGCACATTG
TCCAGATGTTTCATCAATACCTCC (SEQ ID NO: 97).

Single Chain Chimeric Polypeptides

A single-chain chimeric polypeptide includes: a first target-binding domain (e.g., any of the target-binding domains described herein or known in the art), a soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein or known in the art), and a second target-binding domain (e.g., any of the 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 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).

5 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
10 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).

15 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
20 950 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, about 50 amino acids to about 550 amino acids, about 50 amino acids to about 500 amino
25 acids, about 50 amino acids to about 480 amino acids, about 50 amino acids to about 460 amino acids, about 50 amino acids to about 440 amino acids, about 50 amino acids to about 420 amino acids, about 50 amino acids to about 400 amino acids, about 50 amino acids to about 380 amino acids, about 50 amino acids to about 360 amino acids, about 50 amino acids to about 340 amino acids, about 50 amino acids to about 320 amino acids,
30 about 50 amino acids to about 300 amino acids, about 50 amino acids to about 280 amino acids, about 50 amino acids to about 260 amino acids, about 50 amino acids to about 240

[illegible]

amino acids to about 400 amino acids, about 150 amino acids to about 380 amino acids,
about 150 amino acids to about 360 amino acids, about 150 amino acids to about 340
amino acids, about 150 amino acids to about 320 amino acids, about 150 amino acids to
about 300 amino acids, about 150 amino acids to about 280 amino acids, about 150
5 amino acids to about 260 amino acids, about 150 amino acids to about 240 amino acids,
about 150 amino acids to about 220 amino acids, about 150 amino acids to about 200
amino acids, about 200 amino acids to about 3000 amino acids, about 200 amino acids to
about 2500 amino acids, about 200 amino acids to about 2000 amino acids, about 200
amino acids to about 1500 amino acids, about 200 amino acids to about 1000 amino
10 acids, about 200 amino acids to about 950 amino acids, about 200 amino acids to about
900 amino acids, about 200 amino acids to about 850 amino acids, about 200 amino acids
to about 800 amino acids, about 200 amino acids to about 750 amino acids, about 200
amino acids to about 700 amino acids, about 200 amino acids to about 650 amino acids,
about 200 amino acids to about 600 amino acids, about 200 amino acids to about 550
15 amino acids, about 200 amino acids to about 500 amino acids, about 200 amino acids to
about 480 amino acids, about 200 amino acids to about 460 amino acids, about 200
amino acids to about 440 amino acids, about 200 amino acids to about 420 amino acids,
about 200 amino acids to about 400 amino acids, about 200 amino acids to about 380
amino acids, about 200 amino acids to about 360 amino acids, about 200 amino acids to
20 about 340 amino acids, about 200 amino acids to about 320 amino acids, about 200
amino acids to about 300 amino acids, about 200 amino acids to about 280 amino acids,
about 200 amino acids to about 260 amino acids, about 200 amino acids to about 240
amino acids, about 200 amino acids to about 220 amino acids, about 220 amino acids to
about 3000 amino acids, about 220 amino acids to about 2500 amino acids, about 220
25 amino acids to about 2000 amino acids, about 220 amino acids to about 1500 amino
acids, about 220 amino acids to about 1000 amino acids, about 220 amino acids to about
950 amino acids, about 220 amino acids to about 900 amino acids, about 220 amino acids
to about 850 amino acids, about 220 amino acids to about 800 amino acids, about 220
amino acids to about 750 amino acids, about 220 amino acids to about 700 amino acids,
30 about 220 amino acids to about 650 amino acids, about 220 amino acids to about 600
amino acids, about 220 amino acids to about 550 amino acids, about 220 amino acids to

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

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.

10 ***Exemplary IL-2 Receptor Activating Agents that are Single-Chain Chimeric Polypeptides***

In some embodiments, a single-chain chimeric polypeptide can be an IL-2 receptor activating agent. In such embodiments, one or both of the first target-binding domain and/or the second target-binding domain bind to a receptor for IL-2 (e.g., a human receptor for IL-2).

In some embodiments of any of the single-chain chimeric polypeptides that are an IL-2 receptor activating agent, the first target-binding domain and/or the second target-binding domain can be an agonistic antigen-binding domain that binds specifically to an IL-2 receptor (e.g., a human IL-2 receptor) (see, e.g., those described in Gaulton et al., *Clin. Immunol. Immunopathol.* 36(1):18-29, 1985)).

In some embodiments of any of the single-chain chimeric polypeptides that are an IL-2 receptor activating agent, one or both of the first target-binding domain and the second target-binding domain can be a soluble interleukin 2 (IL-2) protein. An exemplary sequence of soluble IL-2 is provided below.

25 **Human Soluble IL-2 (SEQ ID NO: 1)**

```
aptssstkkt qlqlchllld lqmilnginn yknpkltrml tfkfympkka
telkhlqcle eelkpleevl nlaqsknfhl rprdlisnin vivlelkgse
ttfmceyade tativeflnr witfcgsiis tlt
```

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 SEQ ID NO: 1.

In some embodiments, a soluble human IL-2 protein 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
GCTGGATTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCAAAC
CACCAGGATGCTCACATTTAAGTTTACATGCCCAAGAAGGCCACAGAACTGA
AACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAA
TTTAGCTCAAAGCAAAAACCTTTCCTTAAGACCCAGGGACTTAATCAGCAATA
TCAACGTAATAGTTCTGGAATAAAGGGATCTGAAACAACATTCATGTGTGAA
TATGCTGATGAGACAGCAACCATTTGTAGAATTTCTGAACAGATGGATTACCTTT
TGTCAAAGCATCATCTCAACACTAACT (SEQ ID NO: 31).
```

In some embodiments, a soluble human IL-2 protein 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
GCTGGATTACAGATGATTTTAAACGGCATCAACAACCTACAAGAACCCCAAGC
TGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCACCGAGCTG
AAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAGGTGCTGA
ATTTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAATCAGCAACA
TCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTCATGTGCGAG
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TACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTTGGATCACCTT
CTGCCAGTCCATCATCTCCACTTTAACC (SEQ ID NO: 32)

In some embodiments, a single-chain chimeric polypeptide that is an IL-2
receptor activating agent can include a sequence that is at least 80% identical (e.g., at
5 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:

APTSSSTKKTQLQLEHLLLDLQMLNGINNYKNPKLTRMLTFKFYMPKKATELKH
LQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADET
10 ATIVEFLNRWITFCQSIISTLTSGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYT
VQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG
EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDV
FGKDLIYTYWYKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRK
STDSPVECMGQEKGEFREAPTSSSTKKTQLQLEHLLLDLQMLNGINNYKNPKLT
15 RMLTFKFYMPKKATELKH
LQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIV
LELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT (SEQ ID NO: 3).

In some embodiments, a single-chain chimeric polypeptide that is an IL-2
receptor activating agent 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,
20 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
TGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCACCGAGCTG
25 AAGCATTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAGGTGCTGA
ATTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAATCAGCAACA
TCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTCATGTGCGAG
TACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTTGGATCACCTT
CTGCCAGTCCATCATCTCCACTTTAACCAGCGGCACAACCAACACAGTCGCTG
30 CCTATAACCTCACTTGAAGAGCACCAACTTCAAAACCATCCTCGAATGGGAA
CCCAAACCCGTTAACCAAGTTTACACCGTGCAGATCAGCACCAAGTCCGGCG

ACTGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTCACCGAT
 GAGATCGTGAAAGATGTGAAACAGACCTACCTCGCCCGGGTGTTCAGCTACCC
 CGCCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACGAGAAC
 AGCCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCCACCATCCA
 5 AAGCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGGAGGACGAGCG
 GACTTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCA
 AAGATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGA
 CAGCTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAAGGCGAAAA
 CTACTGTTTCAGCGTGCAAGCTGTGATCCCCCTCCCGGACCGTGAATAGGAAAA
 10 GCACCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGA
 GGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCATTAC
 TGCTGGATTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCAAAC
 TCACCAGGATGCTCACATTAAAGTTTTACATGCCCAAGAAGGCCACAGAACTG
 AAACATCTTCAGTGTCTAGAAGAAGAAGTCAAACCTCTGGAGGAAGTGCTAA
 15 ATTTAGCTCAAAGCAAAAACCTTTCCTTAAGACCCAGGGACTTAATCAGCAAT
 ATCAACGTAATAGTTCTGGAAGTAAAGGGATCTGAAACAACATTCATGTGTGA
 ATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGATGGATTACCTT
 TTGTCAAAGCATCATCTCAACACTAACT (SEQ ID NO: 11).

In some embodiments, a single-chain chimeric polypeptide that is an IL-2
 20 receptor activating agent 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:
 MKWVTFISLLFLFSSAYSAPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTR
 25 MLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVL
 ELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTSGTTNTVAAYNLTWKSTN
 FKILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLA
 RVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVED
 ERTLVRNNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGEN
 30 YCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFREAPTSSSTKKTQLQLEHLLLDL
 QMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSK

NFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT
(SEQ ID NO: 4).

In some embodiments, a single-chain chimeric polypeptide that is an IL-2 receptor activating agent 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:

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCTCCAGCGCCTACTCC
GCCCCACCTCCTCCTCCACCAAGAAGACCCAGCTGCAGCTGGAGCATTACTC
10 GCTGGATTTACAGATGATTTTAAACGGCATCAACAACACTACAAGAACCCCAAGC
TGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCACCGAGCTG
AAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAGGTGCTGA
ATTTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAATCAGCAACA
TCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTCATGTGCGAG
15 TACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTTGGATCACCTT
CTGCCAGTCCATCATCTCCACTTTAACCAGCGGCACAACCAACACAGTCGCTG
CCTATAACCTCACTTGGAAGAGCACCAACTTCAAAACCATCCTCGAATGGGAA
CCCAAACCCGTTAACCAAGTTTACACCGTGCAGATCAGCACCAAGTCCGGCG
ACTGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTCACCGAT
20 GAGATCGTGAAAGATGTGAAACAGACCTACCTCGCCCCGGGTGTTTAGCTACCC
CGCCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACGAGAAC
AGCCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCCACCATCCA
AAGCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGGAGGACGAGCG
GACTTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCA
25 AAGATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGA
CAGCTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAAGGCGAAAA
CTACTGTTTCAGCGTGCAAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAA
GCACCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGA
GGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCATTTAC
30 TGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCAAAC
TCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAACTG

AAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAA
 ATTTAGCTCAAAGCAAAAACCTTTCACCTTAAGACCCAGGGACTTAATCAGCAAT
 ATCAACGTAATAGTTCTGGAAGCTAAAGGGATCTGAAACAACATTCATGTGTGA
 ATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGATGGATTACCTT
 5 TTGTCAAAGCATCATCTCAACACTAACT (SEQ ID NO: 12).

Exemplary CD3/CD38 Binding Agents that are Single-Chain Chimeric Polypeptides

In some embodiments where the single-chain chimeric polypeptide is an
 CD3/CD28 binding agent, the first target-binding domain binds specifically to CD3 (e.g.,
 10 one or more of CD3 δ (e.g., human CD3 δ), CD3 ϵ (e.g., human CD3 ϵ), CD3 γ (e.g.,
 human CD3 γ), and CD3 ζ (e.g., human CD3 ζ)) and the second target-binding domain
 binds specifically to CD28 (e.g., human CD28).

In some embodiments, the first target-binding domain that binds specifically to
 CD3 (e.g., one or more of CD3 δ (e.g., human CD3 δ), CD3 ϵ (e.g., human CD3 ϵ), CD3 γ
 15 (e.g., human CD3 γ), and CD3 ζ (e.g., human CD3 ζ)) is an antigen-binding domain (e.g.,
 any of the exemplary antigen-binding domains described herein, e.g., an anti-CD3 scFv).

In some embodiments, the first target binding domain 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
 20 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: 21) and/or a light chain variable domain including a

25 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:

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLA
 SGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINR

30 (SEQ ID NO: 22). 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

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:

CAAGTTCAGCTCCAGCAAAGCGGCGCCGAACCTCGCTCGGCCCCGGCGCTTCCG
TGAAGATGTCTTGTAAGGCCTCCGGCTATACCTTCACCCGGTACACAATGCAC
TGGGTCAAGCAACGGCCCCGGTCAAGGTTTAGAGTGGATTGGCTATATCAACC
10 CCTCCCGGGGCTATACCAACTACAACCAGAAGTTCAAGGACAAAGCCACCCT
CACCACCGACAAGTCCAGCAGCACCGCTTACATGCAGCTGAGCTCTTTAACA
TCCGAGGATTCCGCCGTGTACTACTGCGCTCGGTACTACGACGATCATTACTG
CCTCGATTACTGGGGCCAAGGTACCACCTTAACAGTCTCCTCC (SEQ ID NO:
23), and/or a light chain variable domain encoded by a nucleic acid including a sequence
15 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:

CAGATCGTGCTGACCCAGTCCCCCGCTATTATGAGCGCTAGCCCCGGTGAAA
AGGTGACTATGACATGCAGCGCCAGCTCTTCCGTGAGCTACATGAACTGGTA
20 TCAGCAGAAGTCCGGCACCAGCCCTAAAAGGTGGATCTACGACACCAGCAA
GCTGGCCAGCGGCGTCCCCGCTCACTTTCGGGGCTCCGGCTCCGGAACAAGC
TACTCTCTGACCATCAGCGGCATGGAAGCCGAGGATGCCGCTACCTATTACT
GTCAGCAGTGGAGCTCCAACCCCTTCACCTTTGGATCCGGCACCAAGCTCGA
GATTAATCGT (SEQ ID NO: 24).

25 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:

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLA
SGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRGG
30 GGSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQ
RPGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAV

YYCARYYDDHYCLDYWGQGTTLTVSS (SEQ ID NO: 5). In some embodiments, an anti-CD3 scFv can include the six CDRs present in SEQ ID NO: 5.

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:

CAGATCGTGCTGACCCAGTCCCCGCTATTATGAGCGCTAGCCCCGGTGAAA
 AGGTGACTATGACATGCAGCGCCAGCTCTTCCGTGAGCTACATGAACTGGTA
 TCAGCAGAAGTCCGGCACCAGCCCTAAAAGGTGGATCTACGACACCAGCAA
 10 GCTGGCCAGCGGCGTCCCCGCTCACTTTCGGGGCTCCGGCTCCGGAACAAGC
 TACTCTCTGACCATCAGCGGCATGGAAGCCGAGGATGCCGCTACCTATTACT
 GTCAGCAGTGGAGCTCCAACCCCTTCACCTTTGGATCCGGCACCAAGCTCGA
 GATTAATCGTGGAGGCGGAGGTAGCGGAGGAGGCGGATCCGGCGGTGGAGG
 TAGCCAAGTTCAGCTCCAGCAAAGCGGCGCCGAACCTCGCTCGGCCCGGCGCT
 15 TCCGTGAAGATGTCTTGTAAGGCCTCCGGCTATACCTTCACCCGGTACACAAT
 GCACTGGGTCAAGCAACGGCCCCGGTCAAGGTTTAGAGTGGATTGGCTATATC
 AACCCCTCCCGGGGCTATACCAACTACAACCAGAAGTTCAAGGACAAAGCCA
 CCCTCACCACCGACAAGTCCAGCAGCACCGCTTACATGCAGCTGAGCTCTTT
 AACATCCGAGGATTCCGCCGTGTACTACTGCGCTCGGTACTACGACGATCATT
 20 ACTGCCTCGATTACTGGGGCCAAGGTACCACCTTAACAGTCTCCTCC (SEQ ID
 NO: 25).

In some embodiments, the second target-binding domain that binds specifically to CD28 (e.g., human CD28) is an antigen-binding domain (e.g., any of the exemplary antigen-binding domains described herein, e.g., an anti-CD28 scFv).

25 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:

DIEMTQSPAIMASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYSTSNLA
 30 SGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR (SEQ
 ID NO: 26) 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
 5 TTLTVSS (SEQ ID NO: 27).

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:

10 GACATCGAGATGACACAGTCCCCCGCTATCATGAGCGCCTCTTTAGGAGAAC
 GTGTGACCATGACTTGTACAGCTTCCTCCAGCGTGAGCAGCTCCTATTTCCAC
 TGGTACCAGCAGAAACCCGGCTCCTCCCCTAACTGTGTATCTACTCCACAA
 GCAATTTAGCTAGCGGCGTGCCTCCTCGTTTTAGCGGCTCCGGCAGCACCTCT
 TACTCTTTAACCATTAGCTCTATGGAGGCCGAAGATGCCGCCACATACTTTTG
 15 CCATCAGTACCACCGGTCCCCTACCTTTGGCGGAGGCACAAAGCTGGAGACC
 AAGCGG (SEQ ID NO: 28), 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:

20 GTGCAGCTGCAGCAGTCCGGACCCGAAGTGGTCAAGCCCGGTGCCTCCGTGA
 AAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCAATGG
 GTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAATCCCT
 ACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTCTGAC
 AAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACTTCTG
 25 AGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTGGGGC
 CGGGGAAGTACTTTAACAGTGAGCTCC (SEQ ID NO: 29).

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:

30 VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYN
 DYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRG

TTLTVSSGGGSGGGGSGGGGSDIEMTQSPAIMASASLGERVTMTCTASSSVSSSY
 FHWYQQKPGSSPKLCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCH
 QYHRSPTFGGGTKLETKR (SEQ ID NO: 6). In some embodiments, an anti-CD28
 scFv can include the six CDRs present in SEQ ID NO: 6.

- 5 In some embodiments, an anti-CD28 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:

GTGCAGCTGCAGCAGTCCGGACCCGAACCTGGTCAAGCCCGGTGCCTCCGTGA
 10 AAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCAATGG
 GTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAATCCCT
 ACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTCTGAC
 AAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACTTCTG
 AGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTGGGGC
 15 CGGGGAACACTTTAACAGTGAGCTCCGGCGGCGGCGGAAGCGGAGGTGGA
 GGATCTGGCGGTGGAGGCAGCGACATCGAGATGACACAGTCCCCCGCTATCA
 TGAGCGCCTCTTTAGGAGAACGTGTGACCATGACTTGTACAGCTTCCTCCAGC
 GTGAGCAGCTCCTATTTCCACTGGTACCAGCAGAAACCCGGCTCCTCCCCTAA
 ACTGTGTATCTACTCCACAAGCAATTTAGCTAGCGGCGTGCCTCCTCGTTTTA
 20 GCGGCTCCGGCAGCACCTCTTACTCTTTAACCATTAGCTCTATGGAGGCCGAA
 GATGCCGCCACATACTTTTGCCATCAGTACCACCGGTCCCCTACCTTTGGCGG
 AGGCACAAAGCTGGAGACCAAGCGG (SEQ ID NO: 30).

- In some embodiments, a single-chain chimeric polypeptide that is a CD3/CD28-
 binding agent can include a sequence that is at least 70% identical (e.g., at least 75%
 25 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
 GSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQ
 30 RPGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAV
 YYCARYYDDHYCLDYWGQGTTLTVSSSGTTNTVAAYNLTWKSTNFKTILEWEP

KPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAG
NVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR
NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQ
AVIPSRTVNRKSTDSPVECMGQEKGEFREVQLQQSGPELVKPGASVKMSCKASG
5 YTFTSYVIQWVKQKPGQGLEWIGSINPYNDYTKYNEKFKGKATLTSDKSSITAY
MEFSSLTSEDSALYYCARWGDGNYWGRGTTLTVSSGGGGSGGGGSGGGGSDIE
MTQSPAIMSASLGERVTMTCTASSSVSSSYFHWYQKPGSSPKLCIYSTSNLASG
VPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR (SEQ ID
NO: 7).

- 10 In some embodiments, a single-chain chimeric polypeptide that is a CD3/CD28-
binding agent 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
CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
15 AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACTGGTAT
CAGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAG
CTGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCACCAGCTA
CTCTTTAACCATTTCCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCC
AGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAAT
20 CAATCGTGGAGGAGGTGGCAGCGCGGCGGTGGATCCGGCGGAGGAGGAAG
CCAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCGGCGCCTCC
GTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCA
TTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAAC
CCTTCCCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTT
25 TAACCACTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACC
AGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTG
TTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGCTCCGGCACC
ACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGA
CAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGAT
30 CTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACC
GAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGG

CTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGC
 GAGCCTCTCTACGAGAATTCCCCGAATTCACCCCTTATTTAGAGACCAATTT
 AGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTC
 ACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCC
 5 TCCGGGATGTGTTCGGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCC
 AGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTG
 ACGTGGACAAAGGCGAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTC
 TCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAA
 GAAAAGGGCGAGTTCCGGGAGGTCCAGCTGCAGCAGAGCGGACCCGAATC
 10 GTGAAACCCGGTGCTTCCGTGAAAATGTCTTGTAAGGCCAGCGGATACACCT
 TCACCTCCTATGTGATCCAGTGGGTCAAACAGAAGCCCGGACAAGGTCTCGA
 GTGGATCGGCAGCATCAACCCTTACAACGACTATACCAAATACAACGAGAAG
 TTTAAGGGAAAGGCTACTTTAACCTCCGACAAAAGCTCCATCACAGCCTACA
 TGGAGTTCAGCTCTTTAACATCCGAGGACAGCGCTCTGTACTATTGCGCCCGG
 15 TGGGGCGACGGCAATTACTGGGGACGGGGCACAACACTGACCGTGAGCAGC
 GGAGGCGGAGGCTCCGGCGGAGGCGGATCTGGCGGTGGCGGCTCCGACATC
 GAGATGACCCAGTCCCCCGCTATCATGTCCGCCTCTTTAGGCGAGCGGGTCA
 CAATGACTTGTACAGCCTCCTCCAGCGTCTCCTCCTCCTACTTCCATTGGTAC
 CAACAGAAACCCGGAAGCTCCCCTAAACTGTGCATCTACAGCACCAGCAATC
 20 TCGCCAGCGGCGTGCCCCCTAGGTTTTCCGGAAGCGGAAGCACCAGCTACTC
 TTTAACCATCTCCTCCATGGAGGCTGAGGATGCCGCCACCTACTTTTGTACC
 AGTACCACCGGTCCCCCACCTTCGGAGGCGGCACCAAACCTGGAGACAAAGA
 GG (SEQ ID NO: 9).

In some embodiments, a single-chain chimeric polypeptide that is a CD3/CD28-
 25 binding agent 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:

MKWVTFISLLFLFSSAYSQIVLTQSPAIMASAPGEKVTMTCSASSSVSYMNWYQQ
 KSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWS
 30 SNPFTFGSGTKLEINRGGGSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCK
 ASGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSS

STAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTTLTVSSSGTTNTVAAY
 NLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVK
 DVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVG
 TKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNE
 5 FLIDVDKGENYCFSVQAVIPSRVTNRKSTDSPVECMGQEKGEFREVQLQQSGPEL
 VKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYNDYTKYNEKF
 KGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRGTTTLTVSSGGG
 GSGGGGSGGGGSDIEMTQSPAIMSASLGERVTMTCTASSSVSSSYFHWYQQKPG
 SSPKLCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGG
 10 GTKLETKR (SEQ ID NO: 8).

In some embodiments, a single-chain chimeric polypeptide that is a CD3/CD28-binding agent 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:

15 ATGAAGTGGGTGACCTTCATCAGCTTATTATTTTATTCAGCTCCGCCTATTCC
 CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
 AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACTGGTAT
 CAGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAG
 CTGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCACCAGCTA
 20 CTCTTTAACCATTTCCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCC
 AGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAAT
 CAATCGTGGAGGAGGTGGCAGCGCGGCGGTGGATCCGGCGGAGGAGGAAG
 CCAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCGGCGCCTCC
 GTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCA
 25 TTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAAC
 CCTTCCCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTT
 TAACCACTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACC
 AGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTG
 TTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGCTCCGGCACC
 30 ACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGA
 CAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGAT

CTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACC
 GAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGG
 CTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGC
 GAGCCTCTCTACGAGAATTCCCCGAATTCACCCCTTATTTAGAGACCAATTT
 5 AGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTC
 ACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCC
 TCCGGGATGTGTTCGGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCC
 AGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTG
 ACGTGGACAAAGGCGGAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTC
 10 TCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAA
 GAAAAGGGCGAGTTCCGGGAGGTCCAGCTGCAGCAGAGCGGACCCGAATC
 GTGAAACCCGGTGCTTCCGTGAAAATGTCTTGTAAGGCCAGCGGATACACCT
 TCACCTCCTATGTGATCCAGTGGGTCAAACAGAAGCCCGGACAAGGTCTCGA
 GTGGATCGGCAGCATCAACCCTTACAACGACTATACCAAATACAACGAGAAG
 15 TTTAAGGGAAAGGCTACTTTAACCTCCGACAAAAGCTCCATCACAGCCTACA
 TGGAGTTCAGCTCTTTAACATCCGAGGACAGCGCTCTGTACTATTGCGCCCGG
 TGGGGCGACGGCAATTACTGGGGACGGGGCACAACACTGACCGTGAGCAGC
 GGAGGCGGAGGCTCCGGCGGAGGCGGATCTGGCGGTGGCGGCTCCGACATC
 GAGATGACCCAGTCCCCCGCTATCATGTCCGCCTCTTTAGGCGAGCGGGTCA
 20 CAATGACTTGTACAGCCTCCTCCAGCGTCTCCTCCTCCTACTTCCATTGGTAC
 CAACAGAAACCCGGAAGCTCCCCTAAACTGTGCATCTACAGCACCAGCAATC
 TCGCCAGCGGCGTGCCCCCTAGGTTTTCCGGAAGCGGAAGCACCAGCTACTC
 TTTAACCATCTCCTCCATGGAGGCTGAGGATGCCGCCACCTACTTTTGTACC
 AGTACCACCGGTCCCCACCTTCGGAGGCGGCACCAAACCTGGAGACAAAGA
 25 GG (SEQ ID NO: 10).

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,
 30 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- 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 exemplary target-binding domains described herein or known in the art), and the one or
20 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 additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) bind specifically to CD28, CD3, or a receptor for IL-2.

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 IL-2.

In some embodiments of any of the multi-chain chimeric polypeptides, the first chimeric polypeptide further includes one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) additional target-binding domain(s) (e.g., any of the exemplary target-binding domains described herein or known in the art), where at least one of the one or more additional antigen-binding domain(s) is positioned between the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein or known in the art) and the first domain of the pair of affinity domains (e.g., any of the exemplary first domains of any of the exemplary pairs of affinity domains described herein). In some embodiments, the first chimeric polypeptide can further include 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 at least one of the one or more additional target-binding domain(s) (e.g., any of the exemplary target-binding domains described herein or known in the art), and/or a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between the at least one of the one or more

additional target-binding domain(s) (e.g., any of the exemplary target-binding domains described herein or known in the art) and the first domain of the pair of affinity domains (e.g., any of the exemplary first domains described herein of any of the exemplary pairs of affinity domains described herein).

5 In some embodiments of any of the multi-chain chimeric polypeptides described herein, the first chimeric polypeptide further includes one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) additional target-binding domains at the N-terminal and/or C-terminal end of the first chimeric polypeptide. In some embodiments, at least one of the one or more additional target-binding domains (e.g., any of the exemplary
10 target-binding domains described herein or known in the art) directly abuts the first domain of the pair of affinity domains (e.g., any of the exemplary first domains described herein of any of the exemplary pairs of affinity domains described herein) in the first chimeric polypeptide. In some embodiments, the first chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linker sequences described herein
15 or known in the art) between the at least one of the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) and the first domain of the pair of affinity domains (e.g., any of the exemplary first domains described herein of any of the exemplary pairs of affinity domains described herein). In some embodiments, the at least one of the one or more additional
20 target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) directly abuts the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) in the first chimeric polypeptide. In some embodiments, the first chimeric polypeptide further comprises a linker sequence (e.g., any of the exemplary linker sequences described herein
25 or known in the art) between the at least one of the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) and the first 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 multi-chain chimeric polypeptides described
30 herein, at least one of 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 disposed at the

N- and/or C-terminus of the first chimeric polypeptide, and at least one of 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 positioned between the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein or known in the art) and the first domain of the pair of affinity domains (e.g., any of the exemplary first domains of any of the exemplary pairs of affinity domains described herein) in the first chimeric polypeptide. In some embodiments, the at least one additional target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) of the one or more additional target-binding domains disposed at the N-terminus directly abuts the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) or the first domain of the pair of affinity domains (e.g., any of the exemplary first domains described herein of any of the exemplary pairs of affinity domains described herein) in the first chimeric polypeptide. In some embodiments, the first chimeric polypeptide further comprises a linker sequence (e.g., any of the linker sequences described herein or known in the art) disposed between the at least one additional target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) and the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) or the first domain of the pair of affinity domains (e.g., any of the exemplary first domains described herein of any of the exemplary pairs of affinity domains described herein) in the first chimeric polypeptide. In some embodiments, the at least one additional target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) of the one or more additional target-binding domains disposed 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) or the first domain of the pair of affinity domains (e.g., any of the exemplary first domains of any of the exemplary pairs of affinity domains described herein) in the first chimeric polypeptide. In some embodiments, the first chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) disposed between the at least one additional target-binding domain (e.g., any of the exemplary target-binding domains

described herein or known in the art) and the first target-binding domain (e.g., any of the exemplary target-binding domains described herein or known in the art) or the first domain of the pair of affinity domains (e.g., any of the exemplary first domains described herein of any of the exemplary pairs of affinity domains described herein) in the first
5 chimeric polypeptide. In some embodiments, the at least one of the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) positioned between the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein) and the first domain of the pair of affinity domains (e.g., any of the first domains described herein or
10 any of the exemplary pairs of affinity domains described herein), directly abuts the soluble tissue factor domain and/or the first domain of the pair of affinity domains. In some embodiments, the first chimeric polypeptide further comprises a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) disposed (i) between the soluble tissue factor domain (e.g., any of the exemplary soluble
15 tissue factor domains described herein) and the at least one of the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) positioned between the soluble tissue factor domain (e.g., any of the exemplary soluble tissue factor domains described herein) and the first domain of the pair of affinity domains (e.g., any of the exemplary first domains of any of the
20 exemplary pairs of affinity domains described herein), and/or (ii) between the first domain of the pair of affinity domains and the at least one of the one or more additional target-binding domains positioned between the soluble tissue factor domain and the first domain of the pair of affinity domains.

In some embodiments of any of the multi-chain chimeric polypeptides described
25 herein, the second chimeric polypeptide further 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 the N-terminal end and/or the C-terminal end of the second chimeric polypeptide. In some embodiments, at least one of the one or more additional target-binding domains (e.g., any
30 of the exemplary target-binding domains described herein or known in the art) directly abuts the second domain of the pair of affinity domains (e.g., any of the exemplary

second domains of any of the exemplary pairs of affinity domains described herein) in the second chimeric polypeptide. In some embodiments, the second chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between at least one of the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) and the second domain of the pair of affinity domains (e.g., any of the second domains described herein of any of the exemplary pairs of affinity domains described herein) in the second chimeric polypeptide. In some embodiments, at least one of the one or more additional target-binding domains (e.g., any of the exemplary target-binding domains described herein or known in the art) directly abuts the second target-binding domain (e.g., any of the target-binding domains described herein or known in the art) in the second chimeric polypeptide. In some embodiments, the second chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linker sequences described herein or known in the art) between at least one of the one or more additional target-binding domains (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 the second chimeric polypeptide.

In some embodiments of any of the multi-chain chimeric polypeptides described herein, two or more (e.g., three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten 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 (e.g., three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten 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 (e.g., three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more) of the first target-binding domain, the second target-binding domain, and the one or more additional target-binding domains include 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 include the same amino acid sequence.

In some embodiments of any of the multi-chain chimeric polypeptides described herein, 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 of any of the multi-chain chimeric polypeptides described herein, one or more (e.g., two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more) of the first target-binding domain, the second target-binding domain, and the one or more additional 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 (e.g., a scFv or a single-domain antibody).

Signal Sequence

In some embodiments, a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide described herein can further include 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, a first chimeric polypeptide, or a second chimeric polypeptide can include a signal sequence having an

amino acid sequence MKWVTFISLLFLFSSAYS (SEQ ID NO: 33). In some embodiments, a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide can include a signal sequence encoded by the nucleic acid sequence:

- 5 ATGAAATGGGTGACCTTTATTTCTTTACTGTTCTCTTTAGCAGCGCCTACTCC
(SEQ ID NO: 34),
ATGAAGTGGGTACATTTATCTCTTTACTGTTCTCTCTCCAGCGCCTACAGC
(SEQ ID NO: 35),
ATGAAATGGGTGACCTTTATTTCTTTACTGTTCTCTTTAGCAGCGCCTACTCC
10 (SEQ ID NO: 36), or
ATGAAATGGGTACCTTCATCTCTTTACTGTTTTTATTTAGCAGCGCCTACAGC
(SEQ ID NO: 101).

- In some embodiments, a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide can include a signal sequence having an amino acid sequence MKCLLYLAFLFLGVNC (SEQ ID NO: 37). In some
15 embodiments, a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide can include a signal sequence having an amino acid sequence:

- MGQIVTMFEALPHIIDEVINIVILIIITSIKAVYNFATCGILALVSFLFLAGRSCG
20 (SEQ ID NO: 38). In some embodiments, a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide can include a signal sequence having an amino acid sequence:

- MPNHQSGSPTGSSDLLLSGKKQRPHLALRRKRRREMRKINRKVRRMNLAPIKEK
TAWQHLQALISEAEVLKTSQTPQNSLTLFLALLSVLGPPVTG (SEQ ID NO: 39).

- 25 In some embodiments, a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide can include a signal sequence having an amino acid sequence MDSKGSSQKGSRLLLLLVVSNNLLCQGVVS (SEQ ID NO: 40). Those of ordinary skill in the art will be aware of other appropriate signal sequences for use in a single-chain chimeric polypeptide.

- 30 In some embodiments, a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide can include 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 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 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.

25 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: 33) 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, the first chimeric polypeptide, or the second chimeric polypeptide to the secretory pathway.

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

10 Peptide Tags

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

Exemplary peptide tags that can be included in a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide include, without limitation: AviTag (GLNDIFEAQKIEWHE; SEQ ID NO: 41), a calmodulin-tag (KRRWKKNFIAVSAANRFKKISSSGAL; SEQ ID NO: 42), a polyglutamate tag (EEEEEE; SEQ ID NO: 43), an E-tag (GAPVPYPDPLEPR; SEQ ID NO: 44), a FLAG-tag (DYKDDDDK; SEQ ID NO: 45), an HA-tag, a peptide from hemagglutinin (YPYDVPDYA; SEQ ID NO: 46), a his-tag (HHHHH (SEQ ID NO: 47); HHHHHH (SEQ ID NO: 48); HHHHHHH (SEQ ID NO: 49); HHHHHHHH (SEQ ID NO: 50); HHHHHHHHH (SEQ ID NO: 51); or HHHHHHHHHH (SEQ ID NO: 52)), a myc-tag (EQKLISEEDL; SEQ ID NO: 53), NE-tag (TKENPRSNQEESYDDNES; SEQ ID NO: 54), S-tag, (KETAAAKFERQHMDS; SEQ ID NO: 55), SBP-tag (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP; SEQ ID NO: 56), Softag 1 (SLAELLNAGLGGS; SEQ ID NO: 57), Softag 3 (TQDPSRVG; SEQ ID NO: 58), Spot-tag (PDRVRAVSHWSS; SEQ ID NO: 59), Strep-tag (WSHPQFEK; SEQ ID NO: 60), TC tag (CCPGCC; SEQ ID NO: 61), Ty tag (EVHTNQDPLD; SEQ ID NO:

62), V5 tag (GKIPNPLLGLDST; SEQ ID NO: 63), VSV-tag (YTDIEMNRLGK; SEQ ID NO: 64), and Xpress tag (DLYDDDDK; SEQ ID NO: 65). In some embodiments, tissue factor protein is a peptide tag.

Peptide tags that can be included in a single-chain chimeric polypeptide, a first
5 chimeric polypeptide, or a second chimeric polypeptide can be used in any of a variety of applications related to the single-chain chimeric polypeptide, the first chimeric polypeptide, or the second chimeric polypeptide. For example, a peptide tag can be used in the purification of a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide. As one non-limiting example, a single-chain chimeric
10 polypeptide, a first chimeric polypeptide, or a second 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, a first chimeric polypeptide, or a second
15 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, a first chimeric polypeptide, or a second chimeric polypeptide. In some embodiments, a peptide tag is removed from the single-chain chimeric polypeptide, the first chimeric polypeptide,
20 or the second chimeric polypeptide after purification. In some embodiments, a peptide tag is not removed from the single-chain chimeric polypeptide, the first chimeric polypeptide, or the second chimeric polypeptide after purification.

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

In some embodiments, a single-chain chimeric polypeptide, a first chimeric polypeptide, or a second chimeric polypeptide can include 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 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 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 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 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, a first chimeric polypeptide, or a second 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, a first chimeric polypeptide, or a second chimeric polypeptide includes two

or more peptide tags, the two or more peptide tags can 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: 53) can include one or more additional amino 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).

10 **CD3/CD28-Binding Agents**

Provided herein are methods that include the use of one or more CD3/CD28-binding agent(s). A CD3/CD28-binding agent can be, for example, a single-chain chimeric polypeptide, a multi-chain chimeric polypeptide, a bi-specific antibody, or an anti-CD3/anti-CD28 bead.

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Single-Chain Chimeric Polypeptides

Non-limiting examples of CD3/CD28-binding agents are single-chain chimeric polypeptides that include a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, where: the first target-binding domain of the single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the single-chain chimeric polypeptide binds to CD28. Non-limiting examples of single-chain chimeric polypeptides that are CD3/CD28-binding agents are described herein.

20

Multi-Chain Chimeric Polypeptides

Non-limiting examples of CD3/CD28-binding agents are multi-chain chimeric polypeptides that include a first chimeric polypeptide including (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and a second chimeric polypeptide comprising: (i) a second domain of a pair of affinity domains; and (ii) a second target-binding domain, wherein: the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains; and wherein the first

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target-binding domain binds to CD3 and the second target-binding domain binds to CD28, or the first target-binding domain binds to CD28 and the second target-binding domain to CD3.

In some embodiments, the liquid culture medium further includes an IgG1
5 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the multi-chain chimeric polypeptide. In some embodiments, the IgG1 antibody construct comprises a heavy chain variable domain comprising CDRs of SEQ ID NOs: 66, 67 or 74, and 68, and a light chain variable domain comprising CDRs of SEQ ID NOs: 69, 70, and 71. In some
10 embodiments, the heavy chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 72, and the light chain variable domain comprises a sequence that is at least 90% identical to SEQ ID NO: 73. In some embodiments, the heavy chain variable domain comprises SEQ ID NO: 72, and the light chain variable domain comprises SEQ ID NO: 73.

15 In some embodiments, the first target-binding domain of the first chimeric polypeptide and the soluble tissue factor domain directly abut each other. In some embodiments, the first chimeric polypeptide further comprises a linker sequence 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 of
20 the second chimeric polypeptide directly abut each other. In some embodiments, the second chimeric polypeptide further comprises a linker sequence between the soluble tissue factor domain and the second target-binding domain.

In some embodiments, the CD3 is human CD3. In some embodiments, the first target-binding domain of the first chimeric polypeptide comprises a sequence that is at
25 least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% identical to SEQ ID NO: 5.

In some embodiments, the soluble tissue factor domain of the first chimeric polypeptide is a soluble human tissue factor domain. In some embodiments, the soluble human tissue factor domain of the first chimeric polypeptide comprises a sequence that is
30 at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% identical to SEQ ID NO: 2. In some embodiments, the soluble tissue factor domain of the first

chimeric polypeptide comprises or consists of a sequence from a wildtype soluble human tissue factor.

In some embodiments, the soluble tissue factor domain of the first chimeric polypeptide does not include any of: a lysine at an amino acid position that corresponds
5 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
10 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.

15 In some embodiments, the soluble tissue factor domain of the first chimeric polypeptide includes 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
20 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
25 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 of the first chimeric polypeptide does not initiate blood coagulation. In some embodiments, the first chimeric polypeptide comprises a sequence that is at least 80%, at least 85%, at least 90%, at least
30 95%, at least 99%, or 100% identical to SEQ ID NO: 102. In some embodiments, the

first chimeric polypeptide comprises a sequence that is at least 80%, least 85%, at least 90%, at least 95%, at least 99%, or 100% identical to SEQ ID NO: 104.

In some embodiments, the CD28 is human CD28. In some embodiments, the second target-binding domain of the second chimeric polypeptide comprises a sequence
 5 that is at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% identical to SEQ ID NO: 6. In some embodiments, the second chimeric polypeptide comprises a sequence that is at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% identical to SEQ ID NO: 106. In some embodiments, the second chimeric polypeptide comprises a sequence that is at least 80%, at least 85%, at least 90%, at least
 10 95%, at least 99%, or 100% identical to SEQ ID NO: 108. In some embodiments, the multi-chain chimeric polypeptide does not initiate blood coagulation.

In some embodiments, the liquid culture medium comprises about 10 nM to about 1000 nM (or any of the subranges of this range described herein) of the multi-chain chimeric polypeptide. In some embodiments, the liquid culture medium comprises about
 15 50 nM to about 300 nM (or any of the subranges of this range described herein) of the multi-chain chimeric polypeptide.

Exemplary Mature First Chimeric Polypeptide (SEQ ID NO: 102)

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLA
 20 SGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRGG
 GGSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQ
 RPGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAV
 YYCARYYDDHYCLDYWGQGTTTLTVSSSGTTNTVAAYNLTWKSTNFKTILEWEP
 KPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAG
 25 NVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR
 NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQ
 AVIPSRTVNRKSTDSPVECMGQEKGEFRENWVNVISNLKKIEDLIQSMHIDATLY
 TESDVHPSCKVTAMKCFLELQVISLESGDASIHDTVENLILANNSLSSNGNVTE
 SGCKECELEEKNIKEFLQSFVHIVQMFINTS

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Exemplary DNA Encoding Mature First Chimeric Polypeptide (SEQ ID NO: 103)

CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACTGGTAT
CAGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAG
5 CTGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCACCAGCTA
CTCTTTAACCATTTCCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCC
AGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAAT
CAATCGTGGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAG
CCAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCGGCGCCTCC
10 GTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCA
TTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAAC
CCTTCCCCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTT
TAACCACTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACC
AGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTG
15 TTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGCTCCGGCACC
ACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGA
CAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGAT
CTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACC
GAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGG
20 CTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGC
GAGCCTCTCTACGAGAATTCCCCCGAATTCACCCCTTATTTAGAGACCAATTT
AGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTC
ACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCC
TCCGGGATGTGTTTCGGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCC
25 AGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTG
ACGTGGACAAAGGCGAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTC
TCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAA
GAAAAGGGCGAGTTCCGGGAGAACTGGGTGAACGTCATCAGCAATTAAAG
AAGATCGAAGATTTAATTCAGTCCATGCATATCGACGCCACTTTATACACAG
30 AATCCGACGTGCACCCCTCTTGTAAGGTGACCGCCATGAAATGTTTTTACTG
GAGCTGCAAGTTATCTCTTTAGAGAGCGGAGACGCTAGCATCCACGACACCG

TGGAGAATTTAATCATTTTAGCCAATAACTCTTTATCCAGCAACGGCAACGTG
ACAGAGTCCGGCTGCAAGGAGTGCGAAGAGCTGGAGGAGAAGAACATCAAG
GAGTTTCTGCAATCCTTTGTGCACATTGTCCAGATGTTTCATCAATACCTCC

5 Exemplary Precursor First Chimeric Polypeptide (SEQ ID NO: 104)

MKWVTFISLLFLFSSAYSQIVLTQSPAIMASAPGEKVTMTCSASSSVSYMNWYQQ
KSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWS
SNPFTFGSGTKLEINRGGGSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCK
ASGYTFTRYTMHWVKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTTDKSS
10 STAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSSGTTNTVAAY
NLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTDTDTECDLTDEIVK
DVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVG
TKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNE
FLIDVDKGENYCFSVQAVIPSRVTNRKSTDSPVECMGQEKGEFRENWVNVISNL
15 KKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLESGDASIHTVEN
LILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

Exemplary DNA Encoding Precursor First Chimeric Polypeptide (SEQ ID NO: 105)

ATGAAGTGGGTGACCTTCATCAGCTTATTATTTTATTTCAGCTCCGCCTATTCC
20 CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACTGGTAT
CAGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAG
CTGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCACCAGCTA
CTCTTTAACCATTTCCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCC
25 AGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAAT
CAATCGTGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAG
CCAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCGGCGCCTCC
GTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCA
TTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAAC
30 CCTTCCCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTT
TAACCACTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACC

AGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTG
TTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGCTCCGGCACC
ACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGA
CAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGAT
5 CTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACC
GAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGG
CTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGC
GAGCCTCTCTACGAGAATTCCCCGAATTCACCCCTTATTTAGAGACCAATTT
AGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTC
10 ACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCC
TCCGGGATGTGTTTCGGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCC
AGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTG
ACGTGGACAAAGGCGAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTC
TCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAA
15 GAAAAGGGCGAGTTCCGGGAGAACTGGGTGAACGTCATCAGCAATTTAAAG
AAGATCGAAGATTTAATTCAGTCCATGCATATCGACGCCACTTTATACACAG
AATCCGACGTGCACCCCTCTTGTAAGGTGACCGCCATGAAATGTTTTTTACTG
GAGCTGCAAGTTATCTCTTTAGAGAGCGGAGACGCTAGCATCCACGACACCG
TGGAGAATTTAATCATTTTAGCCAATAACTCTTTATCCAGCAACGGCAACGTG
20 ACAGAGTCCGGCTGCAAGGAGTGCGAAGAGCTGGAGGAGAAGAACATCAAG
GAGTTTCTGCAATCCTTTGTGCACATTGTCCAGATGTTTCATCAATACCTCC

Exemplary Mature Second Chimeric Polypeptide (SEQ ID NO: 106)

VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYN
25 DYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRG
TTLTVSSGGGSGGGGSGGGGSDIEMTQSPAIMSASLGERVTMTCTASSSVSSSY
FHWYQQKPGSSPKLCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCH
QYHRSPTFGGGTKLETKRITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKA
GTSSLTECVLNKATNVAHWTTPSLKCIR

30

Exemplary DNA Encoding Mature Second Chimeric Polypeptide (SEQ ID NO: 107)

GTGCAGCTGCAGCAGTCCGGACCCGAACTGGTCAAGCCCGGTGCCTCCGTGA
AAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCAATGG
GTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAATCCCT
5 ACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTCTGAC
AAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACTTCTG
AGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTGGGGG
CGGGGAACACTTTAACAGTGAGCTCCGGCGGCGGCGGAAGCGGAGGTGGA
GGATCTGGCGGTGGAGGCAGCGACATCGAGATGACACAGTCCCCCGCTATCA
10 TGAGCGCCTCTTTAGGAGAACGTGTGACCATGACTTGTACAGCTTCCTCCAGC
GTGAGCAGCTCCTATTTCCACTGGTACCAGCAGAAACCCGGCTCCTCCCCTAA
ACTGTGTATCTACTCCACAAGCAATTTAGCTAGCGGCGTGCCTCCTCGTTTTA
GCGGCTCCGGCAGCACCTCTTACTCTTTAACCATTAGCTCTATGGAGGCCGAA
GATGCCGCCACATACTTTTGCCATCAGTACCACCGGTCCCCTACCTTTGGCGG
15 AGGCACAAAGCTGGAGACCAAGCGGATTACATGCCCCCCTCCCATGAGCGTG
GAGCACGCCGACATCTGGGTGAAGAGCTATAGCCTCTACAGCCGGGAGAGGT
ATATCTGTAACAGCGGCTTCAAGAGGAAGGCCGGCACCAGCAGCCTCACCGA
GTGCGTGCTGAATAAGGCTACCAACGTGGCTCACTGGACAACACCCTCTTTA
AAGTGCATCCGG

20

Exemplary Precursor Second Chimeric Polypeptide (SEQ ID NO: 108)

MKWVTFISLLFLFSSAYSVQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWV
KQKPGQGLEWIGSINPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSA
LYYCARWGDGNYWGRGTTTLTVSSGGGSGGGGSGGGGSDIEMTQSPAIMSASL
25 GERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYSTSNLASGVPPRFSGSGSTSY
SLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKRITCPPPMSVEHADIWVKSY
SLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCIR

30

Exemplary DNA Encoding Precursor Second Chimeric Polypeptide (SEQ ID NO: 109)

ATGAAATGGGTCACCTTCATCTCTTTACTGTTTTTATTTAGCAGCGCCTACAG
 CGTGCAGCTGCAGCAGTCCGGACCCGAAGTGGTCAAGCCCGGTGCCTCCGTG
 5 AAAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCAATG
 GGTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAATCCC
 TACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTCTGA
 CAAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACTTCT
 GAGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTGGGG
 10 CCGGGGAAGTACTTTAACAGTGAGCTCCGGCGGCGGCGGAAGCGGAGGTGG
 AGGATCTGGCGGTGGAGGCAGCGACATCGAGATGACACAGTCCCCCGCTATC
 ATGAGCGCCTCTTTAGGAGAACGTGTGACCATGACTTGTACAGCTTCCTCCAG
 CGTGAGCAGCTCCTATTTCCACTGGTACCAGCAGAAACCCGGCTCCTCCCCTA
 AACTGTGTATCTACTCCACAAGCAATTTAGCTAGCGGCGTGCCTCCTCGTTTT
 15 AGCGGCTCCGGCAGCACCTCTTACTCTTTAACCATTAGCTCTATGGAGGCCGA
 AGATGCCGCCACATACTTTTGCCATCAGTACCACCGGTCCCCTACCTTTGGCG
 GAGGCACAAAGCTGGAGACCAAGCGGATTACATGCCCCCCTCCCATGAGCGT
 GGAGCACGCCGACATCTGGGTGAAGAGCTATAGCCTCTACAGCCGGGAGAG
 GTATATCTGTAACAGCGGCTTCAAGAGGAAGGCCGGCACCAGCAGCCTCACC
 20 GAGTGCGTGCTGAATAAGGCTACCAACGTGGCTCACTGGACAACACCTCTT
 TAAAGTGCATCCGG

Anti-CD3/anti-CD28 Beads

In some embodiments, a CD3/CD28-binding agent can be an anti-CD3/anti-CD28
 25 bead. An anti-CD3/anti-CD28 bead can include an anti-CD3 antibody or an antigen-
 binding fragment thereof and an anti-CD28 antibody or an antigen-binding fragment
 thereof, both of which are covalently or non-covalently linked to the beads. In some
 embodiments, the anti-CD3 antibody or antigen-binding fragment thereof is an anti-CD3
 scFv (e.g., any of the exemplary anti-CD3 scFvs described herein). In some embodiments,
 30 the anti-CD28 antibody or antigen-binding fragment thereof is an anti-CD28 scFv (e.g.,
 any anti-CD28 scFvs described herein). The beads can be any suitable spherical polymer

particles known in the art. An average diameter of the beads can be about 0.5 to about 20 micrometers (e.g., about 0.5 to about 10, about 0.8 to about 8, or about 1 to about 5 micrometers). In some embodiments, the beads can include one or more paramagnetic material, such as but not limited to dynabeads. Non-limiting examples of anti-CD3/anti-
5 CD28 beads include anti-CD3/anti-CD28 dynabeads, such as those developed by ThermoFisher Scientific, Gibco, and Invitrogen. Additional commercial sources of anti-CD3/anti-CD28 beads are known in the art.

IL-2 Receptor-Activating Agents

10 Provided herein are methods that include the use of one or more IL-2 receptor-activating agent(s). Non-limiting examples of the IL-2 receptor-activating agents include single-chain chimeric polypeptides, multi-chain chimeric polypeptides, soluble IL-2 (e.g., recombinant human IL-2), and an agonistic antibody that binds specifically to an IL-2 receptor (e.g., a human IL-2 receptor).

15

Single-chain chimeric polypeptides

In some embodiments, the IL-2 receptor-activating agents are single-chain chimeric polypeptides that include a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, wherein the first target-binding domain and
20 the second target-binding domain bind to a receptor for IL-2. Non-limiting examples of single-chain chimeric polypeptides that are IL-2 receptor activating agents are described herein.

Soluble IL-2

25 In some examples, an IL-2 receptor-activating agent can be a soluble IL-2 (e.g., any of the soluble IL-2 proteins described herein). In some embodiments, a soluble IL-2 protein can include a sequence that is 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%
30 identical, at least 99% identical, or 100% identical to SEQ ID NO: 1.

Agonistic Antibodies

In some examples, an IL-2 receptor activating agent can be an agonistic antibody that binds specifically to an IL-2 receptor (e.g., a human IL-2 receptor) (see, e.g., those described in Gaulton et al., *Clinical Immunology and Immunopathology* 36(1):18-29, 5 1985), or an antigen-binding fragment thereof.

IgG1 Antibody Constructs

An IgG1 antibody construct can be an IgG1 antibody (e.g., a monoclonal or a polyclonal IgG1 antibody that binds specifically to a soluble tissue factor domain, e.g., 10 any of the soluble tissue factor domains described herein). In some embodiments, an IgG1 antibody construct can be an antibody or an antibody fragment that includes an IgG1 Fc region (e.g., a human IgG1 Fc region). As is known in the art, the IgG1 Fc region binds to CD16a (FcRgammaIII) (e.g., human CD16a) and induces its intracellular signaling. In some embodiments, an IgG1 antibody construct can be a single-chain or a 15 multi-chain polypeptide that includes an Fc region that is capable of binding specifically to CD16a (FcRgammaIII) (e.g., human CD16a) and is capable of inducing its intracellular signaling in a natural killer cell (e.g., a human natural killer cell), and specifically binds to the soluble tissue factor domain. In some embodiments, an IgG1 antibody construct can be a single chain or a multi-chain polypeptide that includes an Fc 20 region that includes 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, or at least 99% identical) to a wildtype IgG1 Fc domain (e.g., a wildtype human IgG1 Fc domain, e.g., SEQ ID NO: 98) and is capable of binding specifically to 25 CD16a (FcRgammaIII) (e.g., human CD16a), and is capable of inducing its intracellular signaling in a natural killer cell (e.g., a human natural killer cell), and specifically binds to the soluble tissue factor domain. In some embodiments, the IgG1 antibody construct binds specifically to a soluble tissue factor domain (e.g., any of the soluble tissue factor domains described herein) and includes a non-human Fc region (e.g., a Fc region from a 30 non-human antibody) that has been altered (e.g., by substituting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids in the wildtype non-human Fc

region) such that the non-human Fc region is capable of binding to human CD16a (human FcRgammaIII) and inducing its intracellular signaling in a natural killer cell (e.g., a human natural killer cell).

5 Wildtype Human IgG1 Fc Region (SEQ ID NO: 98)

PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEETISKAKGQPREPQVYTLPPSRDELTKNQV
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVSMHEA
LHNHYTQKSLSLSPGK

10

In some examples, the IgG1 antibody construct is a humanized or fully human anti-tissue factor antibody (see, e.g., the anti-tissue factor antibodies described in U.S. Patent No. 7,968,094 and U.S. Patent No. 8,007,795).

15 In some embodiments, the IgG1 antibody construct can include a heavy chain variable domain that includes the following set of CDR sequences: a CDR1 including DYNVY (SEQ ID NO: 66); a CDR2 including YIDPYNGITIYDQNFKG (SEQ ID NO: 67); and a CDR3 including DVTTALDF (SEQ ID NO: 68).

In some embodiments, the IgG1 antibody construct can include a heavy chain variable domain that includes the following set of CDR sequences: a CDR1 including
20 DYNVY (SEQ ID NO: 66); a CDR2 including YIDPYNGITIYDQNLKG (SEQ ID NO: 74); and a CDR3 including DVTTALDF (SEQ ID NO: 68).

Exemplary heavy chain variable domain (SEQ ID NO: 72)

QIQLVQSGGEVKKPGASVRVSCASGYSFTDYNVYWVRQSPGKGLEWIGYIDPY
25 NGITIYDQNFKGKATLTVDKSTSTAYMELSSLRSEDVAVYFCARDVTTALDFWG
QGTTVTVSS

In some embodiments, the IgG1 antibody construct can include a heavy chain variable domain that includes a sequence that is at least 70% identical (e.g., at least 72%,
30 at least 74%, at least 75%, at least 76%, at least 78%, at least 80%, at least 82%, at least 84%, at least 85%, at least 86%, at least 88%, at least 90%, at least 92%, at least 94%, at

least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical) to SEQ ID NO: 72.

In some embodiments, the IgG1 antibody construct can include a light chain variable domain that includes the following set of CDR sequences: a CDR1 including
 5 LASQTIDTWLA (SEQ ID NO: 69); a CDR2 including AATNLAD (SEQ ID NO: 70);
 and a CDR3 including QQVYSSPFT (SEQ ID NO: 71).

Exemplary light chain variable domain (SEQ ID NO: 73)

DIQMTQSPASLSASVGDRVTITCLASQTIDTWLAWYLQKPGKSPQLLIYAATNLA
 10 DGVPSRFSGSGSGTDFSFSTISLQPEDFATYYCQQVYSSPFTFGQGGTKLEIK

In some embodiments, the IgG1 antibody construct can include a light chain variable domain that includes a sequence that is at least 70% identical (e.g., at least 72%, at least 74%, at least 75%, at least 76%, at least 78%, at least 80%, at least 82%, at least
 15 84%, at least 85%, at least 86%, at least 88%, at least 90%, at least 92%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical) to SEQ ID NO: 73.

Non-limiting examples of the IgG1 antibody construct can include: a heavy chain variable domain including a sequence that is at least 70% identical (e.g., at least 72%, at
 20 least 74%, at least 75%, at least 76%, at least 78%, at least 80%, at least 82%, at least 84%, at least 85%, at least 86%, at least 88%, at least 90%, at least 92%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical) to SEQ ID NO: 72, and a light chain variable domain including a sequence that is at least
 25 70% identical (e.g., at least 72%, at least 74%, at least 75%, at least 76%, at least 78%, at least 80%, at least 82%, at least 84%, at least 85%, at least 86%, at least 88%, at least 90%, at least 92%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical) to SEQ ID NO: 73.

mTOR Inhibitors

30 Non-limiting examples of mTOR inhibitors include rapamycin (also called sirolimus), Zortress (also called everolimus and RAD001), Torisel (also called

temsirolimus and CCI-779), Afinitor (everolimus), dactolisib (also called NVP-BEZ235), GSK2126458, XL765, AZD8055, INK128 (also called MLN0128), OSI027, and RapaLinks.

5 Exemplary Methods of Stimulating or Increasing Proliferation of a T_{reg} Cell

Provided herein are methods of stimulating or increasing the proliferation of a T_{reg} cell that include culturing a T_{reg} cell in a liquid culture medium over a period of time, where at the beginning of the period of time, the liquid culture medium includes: a CD3/CD28-binding agent (e.g., any of the exemplary CD3/CD28-binding agents described herein); and a single-chain chimeric polypeptide including a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, where the first target-binding domain and the second target-binding domain bind to a receptor for IL-2. In some examples, the liquid culture medium further includes an IgG1 antibody construct that includes at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the single-chain chimeric polypeptide (e.g., any of the exemplary IgG1 antibody constructs described herein). In some examples, the single-chain chimeric polypeptide includes 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 SEQ ID NO: 3. In some examples, the single-chain chimeric polypeptide includes 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 SEQ ID NO: 4.

In some embodiments, the liquid culture medium includes about 10 nM to about 500 nM (e.g., about 10 nM to about 450 nM, about 10 nM to about 400 nM, about 10 nM to about 350 nM, about 10 nM to about 300 nM, about 10 nM to about 250 nM, about 10 nM to about 200 nM, about 10 nM to about 150 nM, about 10 nM to about 100 nM, about 10 nM to about 50 nM, about 50 nM to about 500 nM, about 50 nM to about 450 nM, about 50 nM to about 400 nM, about 50 nM to about 350 nM, about 50 nM to about 300

nM, about 50 nM to about 250 nM, about 50 nM to about 200 nM, about 50 nM to about 150 nM, about 50 nM to about 100 nM, about 100 nM to about 500 nM, about 100 nM to about 450 nM, about 100 nM to about 400 nM, about 100 nM to about 350 nM, about 100 nM to about 300 nM, about 100 nM to about 250 nM, about 100 nM to about 200 nM, about 100 nM to about 150 nM, about 150 nM to about 500 nM, about 150 nM to about 450 nM, about 150 nM to about 400 nM, about 150 nM to about 350 nM, about 150 nM to about 300 nM, about 150 nM to about 250 nM, about 150 nM to about 200 nM, about 200 nM to about 500 nM, about 200 nM to about 450 nM, about 200 nM to about 400 nM, about 200 nM to about 350 nM, about 200 nM to about 300 nM, about 200 nM to about 250 nM, about 250 nM to about 500 nM, about 250 nM to about 450 nM, about 250 nM to about 400 nM, about 250 nM to about 350 nM, about 250 nM to about 300 nM, about 300 nM to about 500 nM, about 300 nM to about 450 nM, about 300 nM to about 400 nM, about 300 nM to about 350 nM, about 350 nM to about 500 nM, about 350 nM to about 450 nM, about 350 nM to about 400 nM, about 400 nM to about 500 nM, about 400 nM to about 450 nM, or about 450 nM to about 500 nM), of the single-chain chimeric polypeptide.

In some embodiments, the CD3/CD28-binding agent is an anti-CD3/anti-CD28 bead (e.g., any of the exemplary anti-CD3/anti-CD28 beads described herein or known in the art). In some embodiments the liquid culture medium includes the anti-CD3/anti-CD28 bead at a ratio of about 1:1 to about 6:1 beads/cell (e.g., about 1:1 to about 5:1, about 1:1 to about 4:1, about 1:1 to about 3:1, about 1:1 to about 2:1, about 2:1 to about 6:1, about 2:1 to about 5:1, about 2:1 to about 4:1, about 2:1 to about 3:1, about 3:1 to about 6:1, about 3:1 to about 5:1, about 3:1 to about 4:1, about 4:1 to about 6:1, about 4:1 to about 5:1, about 5:1 to about 6:1 beads/cell).

In some embodiments, the CD3/CD28-binding agent is an additional single-chain chimeric polypeptide including a first antigen-binding domain, a soluble tissue factor domain, and a second target-binding domain, where the first target-binding domain of the additional single-chain chimeric polypeptide specifically binds to CD3 and the second target-binding domain of the additional single-chain chimeric polypeptide specifically binds to CD28. In some embodiments, the liquid culture medium further includes an IgG1 antibody construct (e.g., any of the exemplary IgG1 antibody constructs described

herein) that includes at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the additional single-chain chimeric polypeptide.

In some embodiments, the additional single-chain chimeric polypeptide comprises 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 SEQ ID NO: 7. In some embodiments, the additional single-chain chimeric polypeptide comprises 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 SEQ ID NO: 8. In some embodiments, the liquid culture medium includes about 10 nM to about 1000 nM (e.g., about 10 nM to about 950 nM, about 10 nM to about 900 nM, about 10 nM to about 850 nM, about 10 nM to about 800 nM, about 10 nM to about 750 nM, about 10 nM to about 700 nM, about 10 nM to about 650 nM, about 10 nM to about 600 nM, about 10 nM to about 550 nM, about 10 nM to about 500 nM, about 10 nM to about 450 nM, about 10 nM to about 400 nM, about 10 nM to about 350 nM, about 10 nM to about 300 nM, about 10 nM to about 250 nM, about 10 nM to about 200 nM, about 10 nM to about 150 nM, about 10 nM to about 100 nM, about 10 nM to about 50 nM, about 25 nM to about 1000 nM, about 25 nM to about 950 nM, about 25 nM to about 900 nM, about 25 nM to about 850 nM, about 25 nM to about 800 nM, about 25 nM to about 750 nM, about 25 nM to about 700 nM, about 25 nM to about 650 nM, about 25 nM to about 600 nM, about 25 nM to about 550 nM, about 25 nM to about 500 nM, about 25 nM to about 450 nM, about 25 nM to about 400 nM, about 25 nM to about 350 nM, about 25 nM to about 300 nM, about 25 nM to about 250 nM, about 25 nM to about 200 nM, about 25 nM to about 150 nM, about 25 nM to about 100 nM, about 25 nM to about 50 nM, about 50 nM to about 1000 nM, about 50 nM to about 950 nM, about 50 nM to about 900 nM, about 50 nM to about 850 nM, about 50 nM to about 800 nM, about 50 nM to about 750 nM, about 50 nM to about 700 nM, about 50 nM to about 650 nM, about 50 nM to about 600 nM, about 50 nM to about 550 nM, about 50 nM to about 500 nM, about 50 nM to about 450 nM, about 50 nM to about 400 nM, about 50 nM to about 350 nM,

about 50 nM to about 300 nM, about 50 nM to about 250 nM, about 50 nM to about 200 nM, about 50 nM to about 150 nM, about 50 nM to about 100 nM, about 100 nM to about 1000 nM, about 100 nM to about 950 nM, about 100 nM to about 900 nM, about 100 nM to about 850 nM, about 100 nM to about 800 nM, about 100 nM to about 750 nM, about
5 100 nM to about 700 nM, about 100 nM to about 650 nM, about 100 nM to about 600 nM, about 100 nM to about 550 nM, about 100 nM to about 500 nM, about 100 nM to about 450 nM, about 100 nM to about 400 nM, about 100 nM to about 350 nM, about 100 nM to about 300 nM, about 100 nM to about 250 nM, about 100 nM to about 200 nM, about 100 nM to about 150 nM, about 150 nM to about 1000 nM, about 150 nM to
10 about 950 nM, about 150 nM to about 900 nM, about 150 nM to about 850 nM, about 150 nM to about 800 nM, about 150 nM to about 750 nM, about 150 nM to about 700 nM, about 150 nM to about 650 nM, about 150 nM to about 600 nM, about 150 nM to about 550 nM, about 150 nM to about 500 nM, about 150 nM to about 450 nM, about 150 nM to about 400 nM, about 150 nM to about 350 nM, about 150 nM to about 300
15 nM, about 150 nM to about 250 nM, about 150 nM to about 200 nM, about 200 nM to about 1000 nM, about 200 nM to about 950 nM, about 200 nM to about 900 nM, about 200 nM to about 850 nM, about 200 nM to about 800 nM, about 200 nM to about 750 nM, about 200 nM to about 700 nM, about 200 nM to about 650 nM, about 200 nM to about 600 nM, about 200 nM to about 550 nM, about 200 nM to about 500 nM, about
20 200 nM to about 450 nM, about 200 nM to about 400 nM, about 200 nM to about 350 nM, about 200 nM to about 300 nM, about 200 nM to about 250 nM, about 250 nM to about 1000 nM, about 250 nM to about 950 nM, about 250 nM to about 900 nM, about 250 nM to about 850 nM, about 250 nM to about 800 nM, about 250 nM to about 750 nM, about 250 nM to about 700 nM, about 250 nM to about 650 nM, about 250 nM to
25 about 600 nM, about 250 nM to about 550 nM, about 250 nM to about 500 nM, about 250 nM to about 450 nM, about 250 nM to about 400 nM, about 250 nM to about 350 nM, about 250 nM to about 300 nM, about 300 nM to about 1000 nM, about 300 nM to about 950 nM, about 300 nM to about 900 nM, about 300 nM to about 850 nM, about 300 nM to about 800 nM, about 300 nM to about 750 nM, about 300 nM to about 700
30 nM, about 300 nM to about 650 nM, about 300 nM to about 600 nM, about 300 nM to about 550 nM, about 300 nM to about 500 nM, about 300 nM to about 450 nM, about

300 nM to about 400 nM, about 300 nM to about 350 nM, about 350 nM to about 1000 nM, about 350 nM to about 950 nM, about 350 nM to about 900 nM, about 350 nM to about 850 nM, about 350 nM to about 800 nM, about 350 nM to about 750 nM, about 350 nM to about 700 nM, about 350 nM to about 650 nM, about 350 nM to about 600 nM, about 350 nM to about 550 nM, about 350 nM to about 500 nM, about 350 nM to about 450 nM, about 350 nM to about 400 nM, about 400 nM to about 1000 nM, about 400 nM to about 950 nM, about 400 nM to about 900 nM, about 400 nM to about 850 nM, about 400 nM to about 800 nM, about 400 nM to about 750 nM, about 400 nM to about 700 nM, about 400 nM to about 650 nM, about 400 nM to about 600 nM, about 400 nM to about 550 nM, about 400 nM to about 500 nM, about 400 nM to about 450 nM, about 450 nM to about 1000 nM, about 450 nM to about 950 nM, about 450 nM to about 900 nM, about 450 nM to about 850 nM, about 450 nM to about 800 nM, about 450 nM to about 750 nM, about 450 nM to about 700 nM, about 450 nM to about 650 nM, about 450 nM to about 600 nM, about 450 nM to about 550 nM, about 450 nM to about 500 nM, about 500 nM to about 1000 nM, about 500 nM to about 950 nM, about 500 nM to about 900 nM, about 500 nM to about 850 nM, about 500 nM to about 800 nM, about 500 nM to about 750 nM, about 500 nM to about 700 nM, about 500 nM to about 650 nM, about 500 nM to about 600 nM, about 500 nM to about 550 nM, about 550 nM to about 1000 nM, about 550 nM to about 950 nM, about 550 nM to about 900 nM, about 550 nM to about 850 nM, about 550 nM to about 800 nM, about 550 nM to about 750 nM, about 550 nM to about 700 nM, about 550 nM to about 650 nM, about 550 nM to about 600 nM, about 600 nM to about 1000 nM, about 600 nM to about 950 nM, about 600 nM to about 900 nM, about 600 nM to about 850 nM, about 600 nM to about 800 nM, about 600 nM to about 750 nM, about 600 nM to about 700 nM, about 600 nM to about 650 nM, about 650 nM to about 1000 nM, about 650 nM to about 950 nM, about 650 nM to about 900 nM, about 650 nM to about 850 nM, about 650 nM to about 800 nM, about 650 nM to about 750 nM, about 650 nM to about 700 nM, about 700 nM to about 1000 nM, about 700 nM to about 950 nM, about 700 nM to about 900 nM, about 700 nM to about 850 nM, about 700 nM to about 800 nM, about 700 nM to about 750 nM, about 750 nM to about 1000 nM, about 750 nM to about 950 nM, about 750 nM to about 900 nM, about 750 nM to about 850 nM, about 750 nM to about 800

nM, about 800 nM to about 1000 nM, about 800 nM to about 950 nM, about 800 nM to about 900 nM, about 800 nM to about 850 nM, about 850 nM to about 1000 nM, about 850 nM to about 950 nM, about 850 nM to about 900 nM, about 900 nM to about 1000 nM, about 900 nM to about 950 nM, or about 950 nM to about 1000 nM) of the
5 additional single-chain chimeric polypeptide.

In some embodiments, the CD3/CD28-binding agent is multi-chain chimeric polypeptide that includes a first chimeric polypeptide including (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and a second chimeric polypeptide comprising: (i) a second domain of a pair of
10 affinity domains; and (ii) a second target-binding domain, wherein: the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains; and wherein the first target-binding domain binds to CD3 and the second target-binding domain binds to CD28, or the first target-binding domain binds to CD28 and the second target-binding
15 domain to CD3. In some embodiments, the liquid culture medium further includes an IgG1 antibody construct (e.g., any of the exemplary IgG1 antibody constructs described herein) that includes at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the multi-chain chimeric polypeptide.

In some embodiments, the first chimeric polypeptide comprises a sequence that is
20 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 SEQ ID NO: 102. In some embodiments, the first chimeric polypeptide comprises a sequence that is at least 80% identical (e.g., at least 82%
25 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 SEQ ID NO: 104. In some embodiments, the second chimeric polypeptide comprises a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical,
30 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 SEQ ID NO: 106. In some embodiments, the second chimeric polypeptide comprises 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 SEQ ID NO: 108. In some embodiments, the liquid culture medium includes about 10 nM to about 1000 nM (e.g., or any of the subranges of this range described herein) of the multi-chain chimeric polypeptide. In some embodiments, the liquid culture medium further comprises an mTOR inhibitor (e.g., any of the mTOR inhibitors described herein, e.g., rapamycin). In some
5 embodiments, the liquid culture medium includes about 10 nM to about 500 nM (e.g., or any of the subranges of this range described herein) of the mTOR inhibitor. In some
10 embodiments, the liquid culture medium does not include an mTOR inhibitor.

Some embodiments further include periodically adding to the liquid culture medium, after each 36 hours to about 60 hours (e.g., about 36 to about 56 hours, about 36
15 to about 52 hours, about 36 to about 48 hours, about 36 to about 44 hours, about 36 to about 40 hours, about 40 to about 60 hours, about 40 to about 56 hours, about 40 to about 52 hours, about 40 to about 48 hours, about 40 to about 44 hours, about 44 to about 60 hours, about 44 to about 56 hours, about 44 to about 52 hours, about 44 to about 48 hours, about 48 to about 60 hours, about 48 to about 56 hours, about 48 to about 52
20 hours, about 52 to about 60 hours, about 52 to about 56 hours, or about 56 to about 60 hours) after the start of the period of time, the single-chain chimeric polypeptide (e.g., any of the single-chain chimeric polypeptides described herein). In some embodiments, the periodic addition of the single-chain chimeric polypeptide is performed to result in a concentration of about 10 nM to about 500 nM (e.g., or any of the subranges of this range
25 described herein) of the single-chain chimeric polypeptide in the liquid culture medium.

Some embodiments further include periodically adding to the liquid culture medium, after each about 4 days to about 10 days (e.g., after each about 4 days to about 9 days, after each about 4 days to about 8 days, after each about 4 days to about 7 days, after each about 4 days to about 6 days, after each about 4 days to about 5 days, after
30 each about 5 days to about 10 days, after each about 5 days to about 9 days, after each about 5 days to about 8 days, after each about 5 days to about 7 days, after each about 5

days to about 6 days, after each about 6 days to about 10 days, after each about 6 days to about 9 days, after each about 6 days to about 8 days, after each about 6 days to about 7 days, after each about 7 days to about 10 days, after each about 7 days to about 9 days, after each about 7 days to about 8 days, after each about 8 days to about 10 days, after each about 8 days to about 9 days, or after each about 9 days to about 10 days) after the start of the period of time, the CD3/CD28-binding agent.

In some embodiments of any of the methods of stimulating or increasing the proliferation of a T_{reg} cell described herein, the liquid culture medium can further include an mTOR inhibitor (e.g., any of the mTOR inhibitors described herein). In some
10 embodiments, the liquid culture medium includes about 10 nM to about 500 nM (e.g., or any of the subranges of this range described herein) of the mTOR inhibitor (e.g., any of the mTOR inhibitors described herein).

In some examples of these methods, the liquid culture medium is a serum-free liquid culture medium. In some embodiments of any of the methods described herein, the
15 liquid culture medium is a chemically-defined liquid culture medium.

In some embodiments, the period of time is about 7 days to about 56 days (e.g., about 7 days to about 56 days, about 7 days to about 54 days, about 7 days to about 46 days, about 7 days to about 38 days, about 7 days to about 32 days, about 7 days to about 28 days, about 7 days to about 25 days, about 7 days to about 23 days, about 7 days to about 21 days, about 7 days to about 19 days, about 7 days to about 17 days, about 7 days to about 15 days, about 7 days to about 13 days, about 7 days to about 11 days, about 7 days to about 9 days, about 9 days to about 56 days, about 9 days to about 54 days, about 9 days to about 46 days, about 9 days to about 38 days, about 9 days to about 32 days, about 9 days to about 28 days, about 9 days to about 25 days, about 9 days to about 23 days, about 9 days to about 21 days, about 9 days to about 19 days, about 9 days to about 17 days, about 9 days to about 15 days, about 9 days to about 13 days, about 9 days to about 11 days, about 11 days to about 56 days, about 11 days to about 54 days, about 11 days to about 46 days, about 11 days to about 38 days, about 11 days to about 32 days, about 11 days to about 28 days, about 11 days to about 25 days, about 11 days to about 23 days, about 11 days to about 21 days, about 11 days to about 19 days, about 11 days to about 17 days, about 11 days to about 15 days, about 11 days to about 13 days, about 13
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days to about 56 days, about 13 days to about 54 days, about 13 days to about 46 days,
about 13 days to about 38 days, about 13 days to about 32 days, about 13 days to about
28 days, about 13 days to about 25 days, about 13 days to about 23 days, about 13 days to
about 21 days, about 13 days to about 19 days, about 13 days to about 17 days, about 13
5 days to about 15 days, about 15 days to about 56 days, about 15 days to about 54 days,
about 15 days to about 46 days, about 15 days to about 38 days, about 15 days to about
32 days, about 15 days to about 28 days, about 15 days to about 25 days, about 15 days to
about 23 days, about 15 days to about 21 days, about 15 days to about 19 days, about 15
days to about 17 days, about 17 days to about 56 days, about 17 days to about 54 days,
10 about 17 days to about 46 days, about 17 days to about 38 days, about 17 days to about
32 days, about 17 days to about 28 days, about 17 days to about 25 days, about 17 days to
about 23 days, about 17 days to about 21 days, about 17 days to about 19 days, about 19
days to about 56 days, about 19 days to about 54 days, about 19 days to about 46 days,
about 19 days to about 38 days, about 19 days to about 32 days, about 19 days to about
15 28 days, about 19 days to about 25 days, about 19 days to about 23 days, about 19 days to
about 21 days, about 21 days to about 56 days, about 21 days to about 54 days, about 21
days to about 46 days, about 21 days to about 38 days, about 21 days to about 32 days,
about 21 days to about 28 days, about 21 days to about 25 days, about 21 days to about
23 days, about 23 days to about 56 days, about 23 days to about 54 days, about 23 days to
20 about 46 days, about 23 days to about 38 days, about 23 days to about 32 days, about 23
days to about 28 days, about 23 days to about 25 days, about 25 days to about 56 days,
about 25 days to about 54 days, about 25 days to about 46 days, about 25 days to about
38 days, about 25 days to about 32 days, about 25 days to about 28 days, about 28 days to
about 56 days, about 28 days to about 54 days, about 28 days to about 50 days, about 28
25 days to about 46 days, about 28 days to about 38 days, about 28 days to about 32 days,
about 32 days to about 56 days, about 32 days to about 54 days, about 32 days to about
46 days, about 32 days to about 38 days, about 38 days to about 56 days, about 38 days to
about 54 days, about 38 days to about 46 days, about 46 days to about 56 days, about 46
days to about 54 days, about 54 days to about 56 days).

Some embodiments further include, before the culturing step, a step of isolating the T_{reg} cell from a sample obtained from a subject (e.g., using any of the methods of isolating described herein).

5 Exemplary Methods of Stimulating or Increasing Proliferation of a T_{reg} Cell

Also provided are methods of stimulating or increasing the proliferation of a T_{reg} cell that include: culturing a T_{reg} cell in a liquid culture medium over a period of time, where at the beginning of the period of time, the liquid culture medium includes: an IL-2 receptor-activating agent (e.g., any of the IL-2 receptor activating agents described
10 herein); and a single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, where: the first target-binding domain of the single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the single-chain chimeric polypeptide binds to CD28. In some examples, the liquid culture medium further includes an IgG1 antibody construct
15 that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the single-chain chimeric polypeptide (e.g., any of the exemplary IgG1 antibody constructs described herein).

In some embodiments, the single-chain chimeric polypeptide includes a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least
20 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 SEQ ID NO: 7. In some embodiments, the single-chain chimeric polypeptide includes 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%
25 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 SEQ ID NO: 8.

In some embodiments, the liquid culture medium includes about 10 nM to about 1000 nM (e.g., or any of the subranges of this range described herein) of the single-chain chimeric polypeptide (e.g., any of the single-chain chimeric polypeptides described
30 herein)

In some embodiments, the IL-2 receptor-activating agent is a soluble IL-2. In some embodiments, the human soluble IL-2 includes 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 SEQ ID NO: 1. In some embodiments, the liquid culture medium includes about 100 IU/mL to about 800 IU/mL (e.g., about 100 IU/mL to about 800 IU/mL, about 100 IU/mL to about 700 IU/mL, about 100 IU/mL to about 600 IU/mL, about 100 IU/mL to about 500 IU/mL, about 100 IU/mL to about 400 IU/mL, about 100 IU/mL to about 300 IU/mL, about 100 IU/mL to about 200 IU/mL, about 200 IU/mL to about 800 IU/mL, about 200 IU/mL to about 700 IU/mL, about 200 IU/mL to about 600 IU/mL, about 200 IU/mL to about 500 IU/mL, about 200 IU/mL to about 400 IU/mL, about 200 IU/mL to about 300 IU/mL, about 300 IU/mL to about 800 IU/mL, about 300 IU/mL to about 700 IU/mL, about 300 IU/mL to about 600 IU/mL, about 300 IU/mL to about 500 IU/mL, about 300 IU/mL to about 400 IU/mL, about 400 IU/mL to about 800 IU/mL, about 400 IU/mL to about 700 IU/mL, about 400 IU/mL to about 600 IU/mL, about 400 IU/mL to about 500 IU/mL, about 500 IU/mL to about 800 IU/mL, about 500 IU/mL to about 700 IU/mL, about 500 IU/mL to about 600 IU/mL, about 600 IU/mL to about 800 IU/mL, about 600 IU/mL to about 700 IU/mL, or about 700 IU/mL to about 800 IU/mL) of the human soluble IL-2.

In some embodiments, the liquid culture medium further includes an mTOR inhibitor (e.g., any of the exemplary mTOR inhibitors described herein, e.g., rapamycin). In some embodiments, the liquid culture medium includes about 10 nM to about 500 nM (e.g., or any of the subranges of this range described herein) of the mTOR inhibitor.

Some embodiments of these methods further include periodically adding to the liquid culture medium, after each 36 hours to about 60 hours (e.g., about 36 to about 56 hours, about 36 to about 52 hours, about 36 to about 48 hours, about 36 to about 44 hours, about 36 to about 40 hours, about 40 to about 60 hours, about 40 to about 56 hours, about 40 to about 52 hours, about 40 to about 48 hours, about 40 to about 44 hours, about 44 to about 60 hours, about 44 to about 56 hours, about 44 to about 52 hours, about 44 to about 48 hours, about 48 to about 60 hours, about 48 to about 56

hours, about 48 to about 52 hours, about 52 to about 60 hours, about 52 to about 56 hours, or about 56 to about 60 hours) after the start of the period of time, the IL-2 receptor-activating agent (e.g., any of the IL-2 receptor-activating agents described herein). In some embodiments, the periodic addition of the IL-2 receptor activating agent
5 is performed to result in a concentration of about 10 nM to about 1000 nM (e.g., or any of the subranges of this range described herein) of the IL-2 receptor-activating agent in the liquid culture medium.

Some embodiments of these methods further include periodically adding to the liquid culture medium, after each about 4 days to about 10 days (e.g., after each about 4
10 days to about 9 days, after each about 4 days to about 8 days, after each about 4 days to about 7 days, after each about 4 days to about 6 days, after each about 4 days to about 5 days, after each about 5 days to about 10 days, after each about 5 days to about 9 days, after each about 5 days to about 8 days, after each about 5 days to about 7 days, after each about 5 days to about 6 days, after each about 6 days to about 10 days, after each
15 about 6 days to about 9 days, after each about 6 days to about 8 days, after each about 6 days to about 7 days, after each about 7 days to about 10 days, after each about 7 days to about 9 days, after each about 7 days to about 8 days, after each about 8 days to about 10 days, after each about 8 days to about 9 days, or after each about 9 days to about 10 days) after the start of the period of time, the single-chain chimeric polypeptide (e.g., any of the
20 exemplary single-chain chimeric polypeptides described herein).

In some embodiments of any of these methods, the period of time is about 7 to about 56 days (e.g., or any of the subranges of this range described herein).

Some embodiments further include, before the culturing step, a step of isolating the T_{reg} cell from a sample obtained from a subject (e.g., using any of the methods of
25 isolating described herein).

Exemplary Methods of Stimulating or Increasing Proliferation of a T_{reg} Cell

Also provided are methods of stimulating or increasing the proliferation of a T_{reg} cell that include: culturing a T_{reg} cell in a liquid culture medium over a period of time,
30 where at the beginning of the period of time, the liquid culture medium includes: a CD3/CD28-binding agent (e.g., any of the CD3/CD28-binding agents described herein);

and a multi-chain chimeric polypeptide that includes: (a) a first chimeric polypeptide including: (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and (b) a second chimeric polypeptide including: (i) a second domain of a pair of affinity domains; and (ii) a second target-binding domain, where the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains (e.g., any of the multi-chain chimeric polypeptides described herein). In some embodiments, the liquid culture medium further includes an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the multi-chain chimeric polypeptide (e.g., any of the IgG1 antibody constructs described herein).

In some embodiments, the CD3/CD28-binding agent is an anti-CD3/anti-CD28 bead (e.g., any of the anti-CD3/anti-CD28 beads described herein). In some embodiments of any of the methods described herein, the liquid culture medium includes the anti-CD3/anti-CD28 bead at a ratio of about 1:1 to about 6:1 beads/cell (e.g., about 1:1 to about 5:1, about 1:1 to about 4:1, about 1:1 to about 3:1, about 1:1 to about 2:1, about 2:1 to about 6:1, about 2:1 to about 5:1, about 2:1 to about 4:1, about 2:1 to about 3:1, about 3:1 to about 6:1, about 3:1 to about 5:1, about 3:1 to about 4:1, about 4:1 to about 6:1, about 4:1 to about 5:1, about 5:1 to about 6:1 beads/cell).

In some embodiments, the CD3/CD28-binding agent is a single-chain chimeric polypeptide including a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, where: the first target-binding domain of the single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the single-chain chimeric polypeptide binds to CD28 (e.g., any of the exemplary single-chain chimeric polypeptides described herein). In some embodiments, the single-chain chimeric polypeptide includes 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 SEQ ID NO: 7. In some embodiments, the single-chain chimeric polypeptide includes 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 SEQ ID NO: 8. In some embodiments, the liquid culture medium includes about 10 nM to about 1000 nM (e.g., or any of the subranges of this range described
5 herein) of the single-chain chimeric polypeptide.

In some embodiments, the CD3/CD28-binding agent is multi-chain chimeric polypeptide that includes a first chimeric polypeptide including (i) a first target-binding domain; (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and a second chimeric polypeptide comprising: (i) a second domain of a pair of
10 affinity domains; and (ii) a second target-binding domain, wherein: the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains; and wherein the first target-binding domain binds to CD3 and the second target-binding domain binds to CD28, or the first target-binding domain binds to CD28 and the second target-binding
15 domain to CD3. In some embodiments, the liquid culture medium further includes an IgG1 antibody construct (e.g., any of the exemplary IgG1 antibody constructs described herein) that includes at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the multi-chain chimeric polypeptide.

In some embodiments, the first chimeric polypeptide comprises a sequence that is
20 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 SEQ ID NO: 102. In some embodiments, the first chimeric polypeptide comprises a sequence that is at least 80% identical (e.g., at least 82%
25 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 SEQ ID NO: 104. In some embodiments, the second chimeric polypeptide comprises a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical,
30 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 SEQ ID NO: 106. In some embodiments, the second chimeric polypeptide comprises 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, 5 at least 99% identical, or 100% identical) to SEQ ID NO: 108. In some embodiments, the liquid culture medium includes about 10 nM to about 1000 nM (e.g., or any of the subranges of this range described herein) of the multi-chain chimeric polypeptide.

In some embodiments, the liquid culture medium further includes an mTOR inhibitor (e.g., any of the mTOR inhibitors described herein, e.g., rapamycin). In some 10 embodiments, the liquid culture medium includes about 10 nM to about 500 nM of the mTOR inhibitor (e.g., or any of the subranges of this range described herein). In some embodiments, the liquid culture medium does not include an mTOR inhibitor.

Some embodiments of these methods further include periodically adding to the liquid culture medium, after each 36 hours to about 60 hours (e.g., about 36 to about 56 15 hours, about 36 to about 52 hours, about 36 to about 48 hours, about 36 to about 44 hours, about 36 to about 40 hours, about 40 to about 60 hours, about 40 to about 56 hours, about 40 to about 52 hours, about 40 to about 48 hours, about 40 to about 44 hours, about 44 to about 60 hours, about 44 to about 56 hours, about 44 to about 52 hours, about 44 to about 48 hours, about 48 to about 60 hours, about 48 to about 56 20 hours, about 48 to about 52 hours, about 52 to about 60 hours, about 52 to about 56 hours, or about 56 to about 60 hours) after the start of the period of time, the multi-chain chimeric polypeptide (e.g., any of the multi-chain chimeric polypeptides described herein). In some embodiments, the periodic addition of the multi-chain chimeric polypeptide is performed to result in a concentration of about 10 nM to about 500 nM 25 (e.g., or any of the subranges of this range described herein) of the multi-chain chimeric polypeptide in the liquid culture medium.

Some embodiments of these methods further include periodically adding to the liquid culture medium, after each about 4 days to about 10 days (e.g., after each about 4 days to about 9 days, after each about 4 days to about 8 days, after each about 4 days to 30 about 7 days, after each about 4 days to about 6 days, after each about 4 days to about 5 days, after each about 5 days to about 10 days, after each about 5 days to about 9 days,

after each about 5 days to about 8 days, after each about 5 days to about 7 days, after each about 5 days to about 6 days, after each about 6 days to about 10 days, after each about 6 days to about 9 days, after each about 6 days to about 8 days, after each about 6 days to about 7 days, after each about 7 days to about 10 days, after each about 7 days to about 9 days, after each about 7 days to about 8 days, after each about 8 days to about 10 days, after each about 8 days to about 9 days, or after each about 9 days to about 10 days) after the start of the period of time, the CD3/CD28-binding agent (e.g., any of the exemplary CD3/CD28-binding agents described herein).

In some embodiments of any of these methods, the period of time is about 7 to about 56 days (e.g., or any of the subranges of this range described herein).

Some embodiments further include, before the culturing step, a step of isolating the Treg cell from a sample obtained from a subject (e.g., using any of the methods of isolating described herein).

15 **Methods of Isolating T_{reg} cells from a Sample**

Any of the methods of stimulating or increasing the proliferation of a T_{reg} cell as described herein can further include, before the culturing step, a step of isolating the T_{reg} cell from a sample obtained from a subject.

The sample can be a blood sample (e.g., a peripheral blood sample or a cord blood sample), lymphoid tissue, or a thymus sample (e.g., pediatric thymus sample). Some embodiments of any of the methods described herein further include obtaining a sample from the subject.

Methods of isolating T_{reg} cells from samples obtained from a subject are well known in the art. For example, magnetic-activated cell sorting (MACS) and/or fluorescence-activated cell sorting (FACS) can be used to isolate T_{reg} cells based on marker expression. Non-limiting examples of FACS instruments include FACS Aria (Becton Dickinson), FX500 fluidics cell sorter (Sony), and MACSQuant Tyto cell sorter (Miltenyi Biotec). Exemplary cell surface proteins for isolating T_{reg} cells from samples include CD4, CD25, and CD127. An intracellular marker for T_{reg} cells is Foxp3. For example, expression levels of CD4 and CD25 can be used to isolate T_{reg} cells from a sample (e.g., a cord blood sample). Expression levels of CD127 can be used as an

additional marker for isolating T_{reg} cells from a sample (e.g., a peripheral blood sample). In some instances, isolating T_{reg} cells from samples include sorting for T_{reg} cells that are CD4⁺CD25⁺Foxp3⁺ and/or are CD4⁺CD25⁺CD127^{dim}. Kits for isolating T_{reg} cells include CD4⁺CD25⁺CD127^{dim} regulatory T cell isolation kit II reagent (Miltenyi Biotec).

- 5 Additional kits for isolating a T_{reg} cell are commercially available from StemCell Technologies (EasySep[™] Human CD4⁺CD127^{low}, CD25⁺ Regulatory T cell Isolation Kit), Miltenyi Biotec (Human CD4⁺CD25⁺ Regulatory T Cell Isolation Kit), and R&D Systems (MagCollect Human CD4⁺CD25⁺ Regulatory T Cell Isolation Kit).

- Also provided herein are methods of isolating a Treg cell from a sample obtained
- 10 from a subject that include separating the Treg cell from the sample based on its expression of CD39, thereby isolating the Treg cell. Some embodiments of these methods include mixing the sample with an antibody or ligand capable of binding CD39 under conditions that allow binding of the antibody or ligand to Treg cells expressing CD39; and separating the Treg cell bound to the antibody or ligand from other
- 15 components in the sample, thereby isolating the Treg cell. In some embodiments of these methods, the antibody is a mouse, a humanized, or a human antibody or antigen-binding fragment thereof; and/or the antibody or the ligand is labeled with at least one of biotin, avidin, streptavidin, or a fluorochrome, or is bound to a particle, bead, resin, or solid support. In some embodiments of these methods, the separating comprises the use of
- 20 flow cytometry, fluorescence-activated cell sorting (FACS), centrifugation, or column, plate, particle, or bead-based methods. Some embodiments of these methods further include: mixing the sample with a biotinylated antibody or ligand capable of binding CD39 under conditions that allow binding of the antibody or the ligand to the Treg cell; capturing the Treg cell bound to the biotinylated antibody or the ligand using
- 25 streptavidin-coated magnetic particles; separating the magnetic particle-bound Treg cell using a magnet; washing the magnet particle-bound Treg cell to remove other components in the sample; and releasing the magnetic particle-bound Treg cell from the magnet into a solution, thereby isolating the Treg cell.

- In some examples, the Treg cell is an autologous Treg cell, a haploidentical Treg
- 30 cell, or an allogeneic Treg cell isolated from a sample comprising fresh or frozen peripheral blood, umbilical cord blood, peripheral blood mononuclear cells, lymphocytes,

CD4⁺ T cells or Treg cells. In some examples, the Treg cell is a CD4⁺CD25⁺Foxp3⁺ cell. In some examples, the Treg cell is a CD4⁺CD25⁺CD127^{dim} cell. In some examples, the Treg cells comprises a chimeric antigen receptor. In some examples, the Treg cells are immunosuppressive in vitro and in vivo.

5 Also provided herein are populations of isolated Treg cells generated and/or isolated using any of the methods described here. In some examples, the isolated population of Treg cells are greater than 70% (e.g., greater than 75%, greater than 80%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, or greater than 99% CD39⁺ cells). In some examples, the isolated Treg cell or populations of isolated
10 Treg cells provided herein are further expanded in vitro. Also provided are methods of expanding any of the populations of isolated Treg cells described herein that include culturing the population of isolated Treg cells under conditions that allow for proliferation of the isolated Treg cells.

 Also provided herein are compositions comprising any of the populations of
15 isolated Treg cells described herein and a pharmaceutically acceptable carrier. Also provided herein are methods of treating a subject in need thereof that include administering to the subject a therapeutically effective amount of any of the compositions described herein. In some examples, the subject has been identified or diagnosed as having an aging-related disease or an inflammatory disease. In some examples, the
20 aging-related disease is selected from the group 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,
25 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, neurodegeneration, stroke, dementia, vascular
30 disease, infection susceptibility, chronic inflammation, and renal dysfunction. In some examples, the inflammatory disease is selected from the group of: rheumatoid arthritis,

inflammatory bowel disease, lupus erythematosus, lupus nephritis, diabetic nephropathy, CNS injury, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Crohn's disease, multiple sclerosis, Guillain-Barre syndrome, psoriasis, Grave's disease, ulcerative colitis, nonalcoholic steatohepatitis, and mood disorders.

5

T_{reg} Cells

In some embodiments of any of the methods, compositions, and kits described herein, the T_{reg} cell can be an autologous T_{reg} cell, a haploidentical T_{reg} cell, or an allogeneic T_{reg} cell isolated from peripheral blood or umbilical cord blood. In some
10 embodiments of any of the methods, compositions, and kits described herein, the T_{reg} cell can be a CD4⁺CD25⁺Foxp3⁺ cell. In some embodiments of any of the methods, compositions, and kits described herein, the T_{reg} cell can be a CD4⁺CD25⁺CD127^{dim} cell.

In some embodiments of any of the methods, compositions, and kits described herein, the T_{reg} cell can include a chimeric antigen receptor or a recombinant T-cell
15 receptor. In some examples, the T_{reg} cell has previously been genetically-modified to express a chimeric antigen receptor or a recombinant T-cell receptor. In some examples, the T_{reg} cell has previously been genetically-modified to express a recombinant T-cell receptor recognizing a peptide of interest in the target tissue or a co-stimulatory molecule (e.g., ICOS).

20 Some embodiments of these methods can further include, after the culturing step, introducing into the T_{reg} cell a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor. Some embodiments of these methods can further include, after the culturing step, introducing into the T_{reg} cell a nucleic acid encoding a co-stimulatory molecule (e.g., ICOS).

25 Some embodiments of these methods can further include, before the culturing step, introducing into the T_{reg} cell a nucleic acid encoding a chimeric antigen-receptor or a recombinant T-cell receptor. Some embodiments of these methods can further include, before the culturing step, introducing into the T_{reg} cell a nucleic acid encoding a co-stimulatory molecule (e.g., ICOS).

30 Detection of the proliferation of a T_{reg} cell can be performed using methods known in the art, e.g., cytometry (e.g., fluorescence-assisted flow cytometry),

microscopy, and immunofluorescence microscopy. For example, proliferation of T_{reg} cells can be determined by staining with dyes (e.g., trypan blue) and counting the number of viable cells. In some examples, proliferation and/or activation of T_{reg} cells can be determined by detecting the levels of surface markers on the T_{reg} cells such as but not limited to: Helios, CTLA-4, CD39, CD62L, CD25, CD103, CD69, PD1 and CD49b. The detection of these surface markers can be performed using FACS. Activation of T_{reg} cells can also be determined by detecting the levels of glucose metabolism in the T_{reg} cells. In some examples, glucose metabolism can be determined by measuring the extracellular acidification rate (ECAR), which involves the stages of non-glycolytic acidification (without drugs), glycolysis (addition of glucose), glycolytic capacity (addition of oligomycin), and glycolytic reserve (addition of 2-deoxyglucose). An extracellular flux analyzer (Agilent) can be used for measuring ECAR.

In some examples, the proliferation and/or activation of T_{reg} cells can be determined by detecting an increase in the level of IL-10 secretion. For example, the methods provided herein 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

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about 20% to about 90%, about 20% to about 80%, about 20% to about 70%, about 20%

to about 60%, about 20% to about 50%, about 20% to about 45%, about 20% to about 40%, about 20% to about 35%, about 20% to about 30%, about 20% to about 25%, about 25% to about 800%, about 25% to about 750%, about 25% to about 700%, about 25% to about 650%, about 25% to about 600%, about 25% to about 550%, about 25% to about 500%, about 25% to about 450%, about 25% to about 400%, about 25% to about 350%, about 25% to about 300%, about 25% to about 280%, about 25% to about 260%, about 25% to about 240%, about 25% to about 220%, about 25% to about 200%, about 25% to about 180%, about 25% to about 160%, about 25% to about 140%, about 25% to about 120%, about 25% to about 100%, about 25% to about 90%, about 25% to about 80%, about 25% to about 70%, about 25% to about 60%, about 25% to about 50%, about 25% to about 45%, about 25% to about 40%, about 25% to about 35%, about 35% to about 800%, about 35% to about 750%, about 35% to about 700%, about 35% to about 650%, about 35% to about 600%, about 35% to about 550%, about 35% to about 500%, about 35% to about 450%, about 35% to about 400%, about 35% to about 350%, about 35% to about 300%, about 35% to about 280%, about 35% to about 260%, about 35% to about 240%, about 35% to about 220%, about 35% to about 200%, about 35% to about 180%, about 35% to about 160%, about 35% to about 140%, about 35% to about 120%, about 35% to about 100%, about 35% to about 90%, about 35% to about 80%, about 35% to about 70%, about 35% to about 60%, about 35% to about 50%, about 35% to about 45%, about 35% to about 40%, about 40% to about 800%, about 40% to about 750%, about 40% to about 700%, about 40% to about 650%, about 40% to about 600%, about 40% to about 550%, about 40% to about 500%, about 40% to about 450%, about 40% to about 400%, about 40% to about 350%, about 40% to about 300%, about 40% to about 280%, about 40% to about 260%, about 40% to about 240%, about 40% to about 220%, about 40% to about 200%, about 40% to about 180%, about 40% to about 160%, about 40% to about 140%, about 40% to about 120%, about 40% to about 100%, about 40% to about 90%, about 40% to about 80%, about 40% to about 70%, about 40% to about 60%, about 40% to about 50%, about 40% to about 45%, about 45% to about 800%, about 45% to about 750%, about 45% to about 700%, about 45% to about 650%, about 45% to about 600%, about 45% to about 550%, about 45% to about 500%, about 45% to about 450%, about 45% to about 400%, about 45% to about 350%, about 45% to about 300%, about

45% to about 280%, about 45% to about 260%, about 45% to about 240%, about 45% to about 220%, about 45% to about 200%, about 45% to about 180%, about 45% to about 160%, about 45% to about 140%, about 45% to about 120%, about 45% to about 100%, about 45% to about 90%, about 45% to about 80%, about 45% to about 70%, about 45% to about 60%, about 45% to about 50%, about 50% to about 800%, about 50% to about 750%, about 50% to about 700%, about 50% to about 650%, about 50% to about 600%, about 50% to about 550%, about 50% to about 500%, about 50% to about 450%, about 50% to about 400%, about 50% to about 350%, about 50% to about 300%, about 50% to about 280%, about 50% to about 260%, about 50% to about 240%, about 50% to about 220%, about 50% to about 200%, about 50% to about 180%, about 50% to about 160%, about 50% to about 140%, about 50% to about 120%, about 50% to about 100%, about 50% to about 90%, about 50% to about 80%, about 50% to about 70%, about 50% to about 60%, about 60% to about 800%, about 60% to about 750%, about 60% to about 700%, about 60% to about 650%, about 60% to about 600%, about 60% to about 550%, about 60% to about 500%, about 60% to about 450%, about 60% to about 400%, about 60% to about 350%, about 60% to about 300%, about 60% to about 280%, about 60% to about 260%, about 60% to about 240%, about 60% to about 220%, about 60% to about 200%, about 60% to about 180%, about 60% to about 160%, about 60% to about 140%, about 60% to about 120%, about 60% to about 100%, about 60% to about 90%, about 60% to about 80%, about 60% to about 70%, about 70% to about 800%, about 70% to about 750%, about 70% to about 700%, about 70% to about 650%, about 70% to about 600%, about 70% to about 550%, about 70% to about 500%, about 70% to about 450%, about 70% to about 400%, about 70% to about 350%, about 70% to about 300%, about 70% to about 280%, about 70% to about 260%, about 70% to about 240%, about 70% to about 220%, about 70% to about 200%, about 70% to about 180%, about 70% to about 160%, about 70% to about 140%, about 70% to about 120%, about 70% to about 100%, about 70% to about 90%, about 70% to about 80%, about 80% to about 800%, about 80% to about 750%, about 80% to about 700%, about 80% to about 650%, about 80% to about 600%, about 80% to about 550%, about 80% to about 500%, about 80% to about 450%, about 80% to about 400%, about 80% to about 350%, about 80% to about 300%, about 80% to about 280%, about 80% to about 260%, about 80% to about 240%, about 80% to

about 220%, about 80% to about 200%, about 80% to about 180%, about 80% to about 160%, about 80% to about 140%, about 80% to about 120%, about 80% to about 100%, about 80% to about 90%, about 90% to about 800%, about 90% to about 750%, about 90% to about 700%, about 90% to about 650%, about 90% to about 600%, about 90% to about 550%, about 90% to about 500%, about 90% to about 450%, about 90% to about 400%, about 90% to about 350%, about 90% to about 300%, about 90% to about 280%, about 90% to about 260%, about 90% to about 240%, about 90% to about 220%, about 90% to about 200%, about 90% to about 180%, about 90% to about 160%, about 90% to about 140%, about 90% to about 120%, about 90% to about 100%, about 100% to about 800%, about 100% to about 750%, about 100% to about 700%, about 100% to about 650%, about 100% to about 600%, about 100% to about 550%, about 100% to about 500%, about 100% to about 450%, about 100% to about 400%, about 100% to about 350%, about 100% to about 300%, about 100% to about 280%, about 100% to about 260%, about 100% to about 240%, about 100% to about 220%, about 100% to about 200%, about 100% to about 180%, about 100% to about 160%, about 100% to about 140%, about 100% to about 120%, about 120% to about 800%, about 120% to about 750%, about 120% to about 700%, about 120% to about 650%, about 120% to about 600%, about 120% to about 550%, about 120% to about 500%, about 120% to about 450%, about 120% to about 400%, about 120% to about 350%, about 120% to about 300%, about 120% to about 280%, about 120% to about 260%, about 120% to about 240%, about 120% to about 220%, about 120% to about 200%, about 120% to about 180%, about 120% to about 160%, about 120% to about 140%, about 140% to about 800%, about 140% to about 750%, about 140% to about 700%, about 140% to about 650%, about 140% to about 600%, about 140% to about 550%, about 140% to about 500%, about 140% to about 450%, about 140% to about 400%, about 140% to about 350%, about 140% to about 300%, about 140% to about 280%, about 140% to about 260%, about 140% to about 240%, about 140% to about 220%, about 140% to about 200%, about 140% to about 180%, about 140% to about 160%, about 160% to about 800%, about 160% to about 750%, about 160% to about 700%, about 160% to about 650%, about 160% to about 600%, about 160% to about 550%, about 160% to about 500%, about 160% to about 450%, about 160% to about 400%, about 160% to about

350%, about 160% to about 300%, about 160% to about 280%, about 160% to about 260%, about 160% to about 240%, about 160% to about 220%, about 160% to about 200%, about 160% to about 180%, about 180% to about 800%, about 180% to about 750%, about 180% to about 700%, about 180% to about 650%, about 180% to about 600%, about 180% to about 550%, about 180% to about 500%, about 180% to about 450%, about 180% to about 400%, about 180% to about 350%, about 180% to about 300%, about 180% to about 280%, about 180% to about 260%, about 180% to about 240%, about 180% to about 220%, about 180% to about 200%, about 200% to about 800%, about 200% to about 750%, about 200% to about 700%, about 200% to about 650%, about 200% to about 600%, about 200% to about 550%, about 200% to about 500%, about 200% to about 450%, about 200% to about 400%, about 200% to about 350%, about 200% to about 300%, about 200% to about 280%, about 200% to about 260%, about 200% to about 240%, about 200% to about 220%, about 220% to about 800%, about 220% to about 750%, about 220% to about 700%, about 220% to about 650%, about 220% to about 600%, about 220% to about 550%, about 220% to about 500%, about 220% to about 450%, about 220% to about 400%, about 220% to about 350%, about 220% to about 300%, about 220% to about 280%, about 220% to about 260%, about 220% to about 240%, about 240% to about 800%, about 240% to about 750%, about 240% to about 700%, about 240% to about 650%, about 240% to about 600%, about 240% to about 550%, about 240% to about 500%, about 240% to about 450%, about 240% to about 400%, about 240% to about 350%, about 240% to about 300%, about 240% to about 280%, about 240% to about 260%, about 260% to about 800%, about 260% to about 750%, about 260% to about 700%, about 260% to about 650%, about 260% to about 600%, about 260% to about 550%, about 260% to about 500%, about 260% to about 450%, about 260% to about 400%, about 260% to about 350%, about 260% to about 300%, about 260% to about 280%, about 280% to about 800%, about 280% to about 750%, about 280% to about 700%, about 280% to about 650%, about 280% to about 600%, about 280% to about 550%, about 280% to about 500%, about 280% to about 450%, about 280% to about 400%, about 280% to about 350%, about 280% to about 300%, about 300% to about 800%, about 300% to about 750%, about 300% to about 700%, about 300% to about 650%, about 300% to about

600%, about 300% to about 550%, about 300% to about 500%, about 300% to about 450%, about 300% to about 400%, about 300% to about 350%, about 350% to about 800%, about 350% to about 750%, about 350% to about 700%, about 350% to about 650%, about 350% to about 600%, about 350% to about 550%, about 350% to about 500%, about 350% to about 450%, about 350% to about 400%, about 400% to about 800%, about 400% to about 750%, about 400% to about 700%, about 400% to about 650%, about 400% to about 600%, about 400% to about 550%, about 400% to about 500%, about 400% to about 450%, about 450% to about 800%, about 450% to about 750%, about 450% to about 700%, about 450% to about 650%, about 450% to about 600%, about 450% to about 550%, about 450% to about 500%, about 500% to about 800%, about 500% to about 750%, about 500% to about 700%, about 500% to about 650%, about 500% to about 600%, about 500% to about 550%, about 550% to about 800%, about 550% to about 750%, about 550% to about 700%, about 550% to about 650%, about 550% to about 600%, about 600% to about 800%, about 600% to about 750%, about 600% to about 700%, about 600% to about 650%, about 650% to about 800%, about 650% to about 750%, about 650% to about 700%, about 700% to about 800%, about 700% to about 750%, or about 750% to about 800%) in the level of secreted IL-10 by the T_{reg} cells (e.g., at the end of the period of time as compared to the start of the period of time).

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 concentration or number of the T_{reg} cells (e.g., at the end of the period of time as compared to the start of the period of time).

In some embodiments, the methods provided herein can result in an increase in the concentration or number of T_{reg} cells (e.g., about a 0.1-fold increase to about a 100-fold increase, about a 0.1-fold increase to about a 90-fold increase, about a 0.1-fold increase to about a 80-fold increase, about a 0.1-fold increase to about a 70-fold increase, about a 0.1-fold increase to about a 60-fold increase, about a 0.1-fold increase to about a 50-fold increase, about a 0.1-fold increase to about a 45-fold increase, about a 0.1-fold increase to about a 40-fold increase, about a 0.1-fold increase to about a 35-fold increase, about a 0.1-fold increase to about a 30-fold increase, about a 0.1-fold increase to about a

[illegible]

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fold increase, about a 6-fold increase to about a 60-fold increase, about a 6-fold increase
to about a 50-fold increase, about a 6-fold increase to about a 45-fold increase, about a 6-
fold increase to about a 40-fold increase, about a 6-fold increase to about a 35-fold
increase, about a 6-fold increase to about a 30-fold increase, about a 6-fold increase to
5 about a 25-fold increase, about a 6-fold increase to about a 20-fold increase, about a 6-
fold increase to about a 15-fold increase, about a 6-fold increase to about a 10-fold
increase, about a 6-fold increase to about a 8-fold increase, about a 8-fold increase to
about a 100-fold increase, about a 8-fold increase to about a 90-fold increase, about a 8-
fold increase to about a 80-fold increase, about a 8-fold increase to about a 70-fold
10 increase, about a 8-fold increase to about a 60-fold increase, about a 8-fold increase to
about a 50-fold increase, about a 8-fold increase to about a 45-fold increase, about a 8-
fold increase to about a 40-fold increase, about a 8-fold increase to about a 35-fold
increase, about a 8-fold increase to about a 30-fold increase, about a 8-fold increase to
about a 25-fold increase, about a 8-fold increase to about a 20-fold increase, about a 8-
15 fold increase to about a 15-fold increase, about a 8-fold increase to about a 10-fold
increase, about a 10-fold increase to about a 100-fold increase, about a 10-fold increase to
about a 90-fold increase, about a 10-fold increase to about a 80-fold increase, about a 10-
fold increase to about a 70-fold increase, about a 10-fold increase to about a 60-fold
increase, about a 10-fold increase to about a 50-fold increase, about a 10-fold increase to
20 about a 45-fold increase, about a 10-fold increase to about a 40-fold increase, about a 10-
fold increase to about a 35-fold increase, about a 10-fold increase to about a 30-fold
increase, about a 10-fold increase to about a 25-fold increase, about a 10-fold increase to
about a 20-fold increase, about a 10-fold increase to about a 15-fold increase, about a 15-
fold increase to about a 100-fold increase, about a 15-fold increase to about a 90-fold
25 increase, about a 15-fold increase to about a 80-fold increase, about a 15-fold increase to
about a 70-fold increase, about a 15-fold increase to about a 60-fold increase, about a 15-
fold increase to about a 50-fold increase, about a 15-fold increase to about a 45-fold
increase, about a 15-fold increase to about a 40-fold increase, about a 15-fold increase to
about a 35-fold increase, about a 15-fold increase to about a 30-fold increase, about a 15-
30 fold increase to about a 25-fold increase, about a 15-fold increase to about a 20-fold
increase, about a 20-fold increase to about a 100-fold increase, about a 20-fold increase to

[illegible]

50-fold increase to about a 80-fold increase, about a 50-fold increase to about a 70-fold increase, about a 50-fold increase to about a 60-fold increase, about a 60-fold increase to about a 100-fold increase, about a 60-fold increase to about a 90-fold increase, about a 60-fold increase to about a 80-fold increase, about a 60-fold increase to about a 70-fold increase, about a 70-fold increase to about a 100-fold increase, about a 70-fold increase to about a 90-fold increase, about a 70-fold increase to about a 80-fold increase, about a 80-fold increase to about a 100-fold increase, about a 80-fold increase to about a 90-fold increase, or about a 90-fold increase to about a 100-fold increase) (e.g., at the end of the period of time as compared to the start of the period of time).

Compositions and Kits

Also provided herein populations of T_{reg} cells produced using any of the methods described herein. Also provided herein are compositions (e.g., pharmaceutical compositions) that include a T_{reg} cell produced using any of the methods described herein or a population of T_{reg} cells produced using any of the methods described herein. In some embodiments, the pharmaceutical compositions can include a pharmaceutically acceptable carrier (e.g., phosphate buffered saline). The compositions can be used to treat a subject in need thereof (e.g., any of the exemplary subjects described herein).

Single or multiple administrations of pharmaceutical compositions can be given to 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 T_{reg} cells to effectively treat, prevent, or ameliorate conditions, diseases, or symptoms in the subject.

Also provided herein are kits that include (i) a CD3/CD28-binding agent (e.g., any of the CD3/CD28-binding agents described herein); (ii) a single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, wherein the first target-binding domain and the second target-binding domain bind to a receptor for IL-2 (e.g., any of such single-chain chimeric polypeptides described herein); and (iii) an mTOR inhibitor (e.g., any mTOR inhibitors described herein). Some embodiments of these kits can further include an IgG1 antibody construct that includes at least one antigen-binding domain that binds specifically to the

soluble tissue factor domain of the single-chain chimeric polypeptide (e.g., any of the exemplary IgG1 antibody constructs described herein). In some embodiments of these kits, the CD3/CD28-binding agent is an anti-CD3/anti-CD28 bead (e.g., any of the exemplary anti-CD3/anti-CD28 beads described herein). In some embodiments, the

5 CD3/CD28-binding agent is an additional single-chain chimeric polypeptide comprising a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, where: the first target-binding domain of the additional single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the additional single-chain chimeric polypeptide binds to CD28 (e.g., any of the examples of such single-chain

10 chimeric polypeptides described herein). In some embodiments, the kit can further include an IgG1 antibody construct that includes at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the additional single-chain chimeric polypeptide (e.g., any of the exemplary IgG1 antibody constructs described herein).

15 Also provided herein are kits that include (i) an interleukin-2 receptor-activating agent (e.g., any of the interleukin-2 receptor-activating agents described herein); (ii) a single-chain chimeric polypeptide including a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, where: the first target-binding domain binds to CD3 and the second target-binding domain binds to CD28; or the first

20 target-binding domain binds to CD28 and the second target-binding domain binds to CD3 (e.g., any of such single-chain chimeric polypeptides described herein); and (iii) an mTOR inhibitor (e.g., any mTOR inhibitors described herein). In some embodiments, the kits can further include an IgG1 antibody construct that includes at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the single-

25 chain chimeric polypeptide (e.g., any of the exemplary IgG1 antibody constructs described herein).

Also provided herein are kits that include: a CD3/CD28-binding agent (e.g., any of the CD3/CD28-binding agents described herein); a multi-chain chimeric polypeptide comprising: (a) a first chimeric polypeptide comprising: (i) a first target-binding domain;

30 (ii) a soluble tissue factor domain; and (iii) a first domain of a pair of affinity domains; and (b) a second chimeric polypeptide comprising: (i) a second domain of a pair of

affinity domains; and (ii) a second target-binding domain, where the first chimeric polypeptide and the second chimeric polypeptide associate through the binding of the first domain and the second domain of the pair of affinity domains (e.g., any of the exemplary multi-chain chimeric polypeptides described herein); and an mTOR inhibitor (e.g., any of the exemplary mTOR inhibitors described herein). Some embodiments of these kits further include an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the multi-chain chimeric polypeptide (e.g., any of the exemplary IgG1 antibody constructs described herein). In some embodiments, the CD3/CD28-binding agent is an anti-CD3/anti-CD28 bead (e.g., any of the anti-CD3/anti-CD28 beads described herein). In some embodiments, the CD3/CD28-binding agent is a single-chain chimeric polypeptide including a first target-binding domain, a soluble tissue factor domain, and a second target-binding domain, where the first target-binding domain of the additional single-chain chimeric polypeptide binds to CD3 and the second target-binding domain of the additional single-chain chimeric polypeptide binds to CD28 (e.g., any of the examples of such single-chain chimeric polypeptides described herein). In some embodiments, the kit further includes an IgG1 antibody construct that comprises at least one antigen-binding domain that binds specifically to the soluble tissue factor domain of the single-chain chimeric polypeptide (e.g., any of the exemplary IgG1 antibody constructs described herein).

Nucleic Acids/Vectors

Also provided herein are nucleic acids that encode any of the single-chain chimeric polypeptides or multi-chain chimeric polypeptides described herein. Also provided herein are vectors that include any of the nucleic acids encoding any of the single-chain chimeric polypeptides or multi-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 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® vectors. A vector can, e.g., include sufficient cis-acting elements for expression; other
5 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 or multi-chain chimeric polypeptides described herein.

10 Cells

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 or any of the multi-chain chimeric polypeptides described herein.

15 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 single-chain chimeric polypeptides or any of the multi-chain chimeric polypeptides described herein.

In some embodiments of any of the methods described herein, the cell can be a
20 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 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,
25 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 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,
30 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 transduction), and nanoparticle transfection.

Methods of Producing Single-Chain or Multi-Chain Chimeric Polypeptides

5 Also provided herein are methods of producing any of the single-chain chimeric polypeptides or multi-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 chimeric polypeptide or a multi-chain chimeric polypeptide; and recovering the single-chain chimeric polypeptide or the multi-chain
10 chimeric polypeptide from the cell and/or the culture medium.

 The recovery of the single-chain chimeric polypeptide or the multi-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 chromatography (anion or cation),
15 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 can be cultured by contacting a cell (e.g., any cell) with a cell culture medium that
20 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) or multi-chain chimeric polypeptides (e.g., any of the multi-chain chimeric polypeptides) produced by any of the
25 methods described herein.

Methods of Treatment

 Also provided herein are methods of treating a subject in need thereof (e.g., any of the exemplary subjects described herein) that include administering to the subject a
30 therapeutically effective amount of a population of T_{reg} cells generated using any of the methods described herein or any of the compositions (e.g., pharmaceutical compositions)

comprising a T_{reg} cell or a population of T_{reg} cells generated using any of the methods described herein.

In some embodiments of these methods, the subject has been identified or diagnosed as having an aging-related disease or an inflammatory disease. In some
5 embodiments, the aging-related disease is selected from the group 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,
10 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,
15 neurodegeneration, stroke, dementia, vascular disease, infection susceptibility, chronic inflammation, and renal dysfunction.

In some embodiments, the inflammatory disease is selected from the group of: rheumatoid arthritis, inflammatory bowel disease, lupus erythematosus, lupus nephritis, diabetic nephropathy, CNS injury, Alzheimer's disease, Parkinson's disease, amyotrophic
20 lateral sclerosis, Crohn's disease, multiple sclerosis, Guillain-Barre syndrome, psoriasis, Grave's disease, ulcerative colitis, nonalcoholic steatohepatitis, and mood disorders.

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
25 aging-related disease or the inflammatory disease 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, about 1% decrease to about 80% decrease, about 1% decrease to about 75% decrease, about 1% to about 70%
30 decrease, about 1% decrease to about 65% decrease, about 1% decrease to about 60% decrease, about 1% decrease to about 55% decrease, about 1% decrease to about 50%

decrease, about 1% decrease to about 45% decrease, about 1% decrease to about 40% decrease, about 1% decrease to about 35% decrease, about 1% decrease to about 30% decrease, about 1% decrease to about 25% decrease, about 1% decrease to about 20% decrease, about 1% decrease to about 15% decrease, about 1% decrease to about 10% decrease, about 1% decrease to about 5% decrease, about 5% decrease to about 99% decrease, an about 5% decrease to about 95% decrease, about 5% decrease to about 90% decrease, about 5% decrease to about 85% decrease, about 5% decrease to about 80% decrease, about 5% decrease to about 75% decrease, about 5% to about 70% decrease, about 5% decrease to about 65% decrease, about 5% decrease to about 60% decrease, about 5% decrease to about 55% decrease, about 5% decrease to about 50% decrease, about 5% decrease to about 45% decrease, about 5% decrease to about 40% decrease, about 5% decrease to about 35% decrease, about 5% decrease to about 30% decrease, about 5% decrease to about 25% decrease, about 5% decrease to about 20% decrease, about 5% decrease to about 15% decrease, about 5% decrease to about 10% decrease, about 10% decrease to about 99% decrease, an about 10% decrease to about 95% decrease, about 10% decrease to about 90% decrease, about 10% decrease to about 85% decrease, about 10% decrease to about 80% decrease, about 10% decrease to about 75% decrease, about 10% to about 70% decrease, about 10% decrease to about 65% decrease, about 10% decrease to about 60% decrease, about 10% decrease to about 55% decrease, about 10% decrease to about 50% decrease, about 10% decrease to about 45% decrease, about 10% decrease to about 40% decrease, about 10% decrease to about 35% decrease, about 10% decrease to about 30% decrease, about 10% decrease to about 25% decrease, about 10% decrease to about 20% decrease, about 10% decrease to about 15% decrease, about 15% decrease to about 99% decrease, an about 15% decrease to about 95% decrease, about 15% decrease to about 90% decrease, about 15% decrease to about 85% decrease, about 15% decrease to about 80% decrease, about 15% decrease to about 75% decrease, about 15% to about 70% decrease, about 15% decrease to about 65% decrease, about 15% decrease to about 60% decrease, about 15% decrease to about 55% decrease, about 15% decrease to about 50% decrease, about 15% decrease to about 45% decrease, about 15% decrease to about 40% decrease, about 15% decrease to about 35% decrease, about 15% decrease to about 30% decrease, about 15% decrease to about 25% decrease,

about 15% decrease to about 20% decrease, about 20% decrease to about 99% decrease,
an about 20% decrease to about 95% decrease, about 20% decrease to about 90%
decrease, about 20% decrease to about 85% decrease, about 20% decrease to about 80%
decrease, about 20% decrease to about 75% decrease, about 20% to about 70% decrease,
5 about 20% decrease to about 65% decrease, about 20% decrease to about 60% decrease,
about 20% decrease to about 55% decrease, about 20% decrease to about 50% decrease,
about 20% decrease to about 45% decrease, about 20% decrease to about 40% decrease,
about 20% decrease to about 35% decrease, about 20% decrease to about 30% decrease,
about 20% decrease to about 25% decrease, about 25% decrease to about 99% decrease,
10 an about 25% decrease to about 95% decrease, about 25% decrease to about 90%
decrease, about 25% decrease to about 85% decrease, about 25% decrease to about 80%
decrease, about 25% decrease to about 75% decrease, about 25% to about 70% decrease,
about 25% decrease to about 65% decrease, about 25% decrease to about 60% decrease,
about 25% decrease to about 55% decrease, about 25% decrease to about 50% decrease,
15 about 25% decrease to about 45% decrease, about 25% decrease to about 40% decrease,
about 25% decrease to about 35% decrease, about 25% decrease to about 30% decrease,
about 30% decrease to about 99% decrease, an about 30% decrease to about 95%
decrease, about 30% decrease to about 90% decrease, about 30% decrease to about 85%
decrease, about 30% decrease to about 80% decrease, about 30% decrease to about 75%
20 decrease, about 30% to about 70% decrease, about 30% decrease to about 65% decrease,
about 30% decrease to about 60% decrease, about 30% decrease to about 55% decrease,
about 30% decrease to about 50% decrease, about 30% decrease to about 45% decrease,
about 30% decrease to about 40% decrease, about 30% decrease to about 35% decrease,
about 35% decrease to about 99% decrease, an about 35% decrease to about 95%
25 decrease, about 35% decrease to about 90% decrease, about 35% decrease to about 85%
decrease, about 35% decrease to about 80% decrease, about 35% decrease to about 75%
decrease, about 35% to about 70% decrease, about 35% decrease to about 65% decrease,
about 35% decrease to about 60% decrease, about 35% decrease to about 55% decrease,
about 35% decrease to about 50% decrease, about 35% decrease to about 45% decrease,
30 about 35% decrease to about 40% decrease, about 40% decrease to about 99% decrease,
an about 40% decrease to about 95% decrease, about 40% decrease to about 90%

decrease, about 40% decrease to about 85% decrease, about 40% decrease to about 80% decrease, about 40% decrease to about 75% decrease, about 40% to about 70% decrease, about 40% decrease to about 65% decrease, about 40% decrease to about 60% decrease, about 40% decrease to about 55% decrease, about 40% decrease to about 50% decrease, 5 about 40% decrease to about 45% decrease, about 45% decrease to about 99% decrease, an about 45% decrease to about 95% decrease, about 45% decrease to about 90% decrease, about 45% decrease to about 85% decrease, about 45% decrease to about 80% decrease, about 45% decrease to about 75% decrease, about 45% to about 70% decrease, about 45% decrease to about 65% decrease, about 45% decrease to about 60% decrease, 10 about 45% decrease to about 55% decrease, about 45% decrease to about 50% decrease, about 50% decrease to about 99% decrease, an about 50% decrease to about 95% decrease, about 50% decrease to about 90% decrease, about 50% decrease to about 85% decrease, about 50% decrease to about 80% decrease, about 50% decrease to about 75% decrease, about 50% to about 70% decrease, about 50% decrease to about 65% decrease, 15 about 50% decrease to about 60% decrease, about 50% decrease to about 55% decrease, about 55% decrease to about 99% decrease, an about 55% decrease to about 95% decrease, about 55% decrease to about 90% decrease, about 55% decrease to about 85% decrease, about 55% decrease to about 80% decrease, about 55% decrease to about 75% decrease, about 55% to about 70% decrease, about 55% decrease to about 65% decrease, 20 about 55% decrease to about 60% decrease, about 60% decrease to about 99% decrease, an about 60% decrease to about 95% decrease, about 60% decrease to about 90% decrease, about 60% decrease to about 85% decrease, about 60% decrease to about 80% decrease, about 60% to about 70% decrease, about 60% decrease to about 65% decrease, about 65% decrease to about 99% decrease, 25 an about 65% decrease to about 95% decrease, about 65% decrease to about 90% decrease, about 65% decrease to about 85% decrease, about 65% decrease to about 80% decrease, about 65% decrease to about 75% decrease, about 65% to about 70% decrease, about 70% decrease to about 99% decrease, an about 70% decrease to about 95% decrease, about 70% decrease to about 90% decrease, about 70% decrease to about 85% decrease, 30 decrease, about 70% decrease to about 80% decrease, about 70% decrease to about 75% decrease, about 75% decrease to about 99% decrease, an about 75% decrease to about

95% decrease, about 75% decrease to about 90% decrease, about 75% decrease to about 85% decrease, about 75% decrease to about 80% decrease, about 80% decrease to about 99% decrease, an about 80% decrease to about 95% decrease, about 80% decrease to about 90% decrease, about 80% decrease to about 85% decrease, about 85% decrease to about 99% decrease, an about 85% decrease to about 95% decrease, about 85% decrease to about 90% decrease, about 90% decrease to about 99% decrease, an about 90% decrease to about 95% decrease, or about 95% decrease to about 99% decrease) in the number of senescent cells in the subject (e.g., a decrease in the number of senescent cells in one or more specific tissues involved and/or implicated in the aging-related disease or the inflammatory disease in the subject), e.g., as compared to the number of senescent cells 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.

Senescent Cells

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 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 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 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 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) 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). 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 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.

The secretion profile of senescent cells is context dependent. For instance, the mitochondrial dysfunction-associated senescence (MiDAS), induced by different
5 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 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
10 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 senescent phenotype in human fibroblasts without an inflammatory SASP indicating that
15 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 (histone coding gene) and 53BP1 (involved in DNA damage response) in chromatin: this
20 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 and CDK6). This eventually leads to hypophosphorylation of Retinoblastoma protein
25 (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 nature, the senescent cells are normally removed by the innate immune cells. Induction
30 of senescence not only prevents the potential proliferation and transformation of damaged/altered 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 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 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
5 additional therapeutic agents can be administered to the subject at substantially the same time as a T_{reg} cell generated by any of the methods described herein or any of the compositions (e.g., pharmaceutical compositions) comprising a population of T_{reg} cells produced using any of the methods described herein. In some embodiments, one or more additional therapeutic agents can be administered to the subject prior to administration of
10 a T_{reg} cell or a composition (e.g., a pharmaceutical composition) comprising a population of T_{reg} cells produced using any of the methods described herein. In some embodiments, one or more additional therapeutic agents can be administered to the subject after administration of a T_{reg} cell or a composition (e.g., a pharmaceutical composition) comprising a population of T_{reg} cells produced using any of the methods described herein
15 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, Glucocorticoid Synthase Kinase (GSK) 3 inhibitors, and antibodies.

20 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, vindesine, camptothecin, 9-methoxycamptothecin, coronaridine, taxol, nucleosides,
25 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, topotecan, or acridone alkaloids), proteasome inhibitors (e.g., lactacystin, disulfiram,
30 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, 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, 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, temozolomide, carmustine, lumustine, streptozocin, carboplatin, cisplatin, and oxaliplatin.

EXAMPLES

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

20

Example 1. Production of α CD3scFv/TF/ α CD28scFv

The nucleic acid and amino acid sequences of α CD3scFv/TF/ α CD28scFv are shown below.

25 Nucleic Acid Encoding α CD3scFv/TF/ α CD28scFv (SEQ ID NO: 10)

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCTTATTATTTTATTCAGCTCCGCCT
ATTCC

(α CD3 light chain variable region)

30 CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGT
GAGAAGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACT

GGTATCAGCAGAAAAGCGGAACCCAGCCCCAAAAGGTGGATCTACGACACCA
GCAAGCTGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCAC
CAGCTACTCTTTAACCATTTCCGGCATGGAAGCTGAAGACGCTGCCACCTACT
ATTGCCAGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCT
5 CGAAATCAATCGT
(*Linker*)
GGAGGAGGTGGCAGCGGCGGGTGGATCCGGCGGAGGAGGAAGC
(*αCD3 heavy chain variable region*)
CAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCCGGCGC
10 CTCCGTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAA
TGCATTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATAT
CAACCCTTCCCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCC
ACTTTAACCAGTACAAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTT
AACCAGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCAC
15 TACTGTTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGC
(*Human tissue factor 219 form*)
TCCGGCACCAACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGC
ACCAACTTCAAGACAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTT
ACACCGTGCAGATCTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTA
20 CACAACAGACACCGAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAG
CAAACCTATCTGGCTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCAC
CGGCTCCGCTGGCGAGCCTCTCTACGAGAATTCCCCGAATTCACCCCTTATT
TAGAGACCAATTTAGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCAC
CAAGGTGAACGTCACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAAC
25 ACATTTTTATCCCTCCGGGATGTGTTTCGGCAAAGACCTCATCTACACACTGTA
CTATTGGAAGTCCAGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAAC
GAGTTTTTAATTGACGTGGACAAAGGCGAGAACTACTGCTTCAGCGTGCAAG
CCGTGATCCCTTCTCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGA
GTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG
30 (*αCD28 light chain variable region*)

GTCCAGCTGCAGCAGAGCGGACCCGAACTCGTGAAACCCGGTGCTTCC
 GTGAAAATGTCTTGTAAGGCCAGCGGATACACCTTCACCTCCTATGTGATCCA
 GTGGGTCAAACAGAAAGCCCGGACAAGGTCTCGAGTGGATCGGCAGCATCAA
 CCCTTACAACGACTATACCAAATACAACGAGAAGTTTAAGGGAAAGGCTACT
 5 TTAACCTCCGACAAAAGCTCCATCACAGCCTACATGGAGTTCAGCTCTTTAAC
 ATCCGAGGACAGCGCTCTGTACTATTGCGCCCGGTGGGGCGACGGCAATTAC
 TGGGGACGGGGCACAACACTGACCGTGAGCAGC

(Linker)

GGAGGCGGAGGCTCCGGCGGAGGCGGATCTGGCGGTGGCGGCTCC
 10 *(αCD28 light chain variable region)*
 GACATCGAGATGACCCAGTCCCCCGCTATCATGTCCGCCTCTTTAGGCGAGC
 GGGTCACAATGACTTGTACAGCCTCCTCCAGCGTCTCCTCCTCCTACTTCCAT
 TGGTACCAACAGAAACCCGGAAGCTCCCCTAAACTGTGCATCTACAGCACCA
 GCAATCTCGCCAGCGGCGTGCCCCCTAGGTTTTCCGGAAGCGGAAGCACCAG
 15 CTACTCTTTAACCATCTCCTCCATGGAGGCTGAGGATGCCGCCACCTACTTTT
 GTCACCAGTACCACCGGTCCCCCACCTTCGGAGGCGGCACCAAACCTGGAGAC
 AAAGAGG

αCD3scFv/TF/αCD28scFv (SEQ ID NO: 8)

20 *(Signal peptide)*
 MKWVTFISLLFLFSSAYS
(αCD3 light chain variable region)
 QIVLTQSPAISASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDT
 SKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEI
 25 NR
(Linker)
 GGGGSGGGGSGGGGS
(αCD3 heavy chain variable region)
 QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLEWI
 30 GYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDD
 HYCLDYWGQGTTTLTVSS

(Human tissue factor 219)

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
 FYTTDTECDLTDEIVKDKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
 ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYW
 5 KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
 KGEFRE

(α CD28 light chain variable region)

VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGS
 INPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNY
 10 WGRGTTTLTVSS

(Linker)

GGGGSGGGGSGGGGS

(α CD28 heavy chain variable region)

DIEMTQSPAIMASASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYSTSNLA
 15 SGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR

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
 20 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 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
 25 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 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
 30 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

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.

- 5 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 using 10 column volumes of PBS, 0.05% NaN₃, and stored at 2-8 °C.

Example 2. Production of IL-2/TF/IL-2

- 10 The nucleic acid and amino acid sequences of IL-2/TF/IL-2 are shown below.

Nucleic Acid Encoding IL-2/TF/IL-2 (SEQ ID NO: 12)

(Signal peptide)

- ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCCTGTTCTCCAGCGCCT
15 ACTCC

(First IL-2 fragment)

- GCCCCACCTCCTCCTCCACCAAGAAGACCCAGCTGCAGCTGGAGCAT
TTACTGCTGGATTTACAGATGATTTTAAACGGCATCAACAACCTACAAGAACC
CCAAGCTGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCAC
20 CGAGCTGAAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAG
GTGCTGAATTTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAAT
CAGCAACATCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTC
ATGTGCGAGTACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTT
GGATCACCTTCTGCCAGTCCATCATCTCCACTTTAACC

- 25 *(Human tissue factor 219 form)*

- AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAG
CACCAACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTT
TACACCGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCT
ATACCACCGACACCGAGTGCATCTCACCAGTATGAGATCGTGAAAGATGTGAA
30 ACAGACCTACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGC
ACTGGTTCCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTA

CCTCGAGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGC
 ACAAAGGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAAC
 AACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCAAAGATTTAATCTACACACT
 GTATTACTGGAAGTCCTCTTCTCCGGCAAGAAGACAGCTAAAACCAACACA
 5 AACGAGTTTTTAATCGACGTGGATAAAGGCGAAAACACTACTGTTTCAGCGTGC
 AAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGT
 TGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(Second IL-2 fragment)

GCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCAT
 10 TTACTGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCC
 CAAACTCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACA
 GAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAG
 TGCTAAATTTAGCTCAAAGCAAAAACCTTCACTTAAGACCCAGGGACTTAAT
 CAGCAATATCAACGTAATAGTTCTGGAATAAAGGGATCTGAAACAACATTC
 15 ATGTGTGAATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGAT
 GGATTACCTTTTGTCAAAGCATCATCTCAACACTAACT

IL-2/TF/IL-2 (SEQ ID NO: 4)

(Signal peptide)

20 MKWVTFISLLFLFSSAYS

(Human IL-2)

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA
 TELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCE
 YADETATIVEFLNRWITFCQSIISTLT

25 *(Human Tissue Factor 219)*

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
 FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
 ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYW
 KSSSSGKKTAKTNTNEFLIDVDKGENYCFVQAVIPSRTVNRKSTDSPVECMGQE
 30 KGEFRE

(Human IL-2)

APTSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA
 TELKHLQCLEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCE
 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 IL-2/TF/IL-2 single-chain chimeric polypeptide (referred to as 2t2), which can be
 10 purified by anti-TF antibody affinity and other chromatography methods.

Example 3: Stimulation of Foxp3⁺ T_{reg} cells by 2t2 with anti-CD3/anti-CD28 beads and rapamycin compared with commercial recombinant human IL-2

Fresh human leukocytes (3 donors) were obtained from the blood bank and
 15 CD4⁺CD25⁺CD127^{dim} T_{reg} cells were isolated with the CD4⁺CD25⁺CD127^{dim} regulatory T cell isolation kit II reagent (Miltenyi Biotec). The purity of Foxp3⁺ T_{reg} cells was >35% and confirmed by staining with CD4-Alexa Fluor 488, CD25-APC, CD3 APC-Cy7, Foxp3-PE (Intracellular staining, Invitrogen), and CD127-Alex Fluor 700 (BioLegend). The cells were counted and resuspended in 1x10⁶/mL in a 48-well flat-
 20 bottom plate in 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 either (1) hIL-2 cytokine (500 IU/mL) (Proleukin), anti-CD3/anti-CD28 beads (4:1, Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM)
 25 (Sigma) or (2) 100 nM of 2t2, anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma) and incubated for 21 days at 37 °C, 5% CO₂. During the expansion period, the cells were re-stimulated with either (1) hIL-2 cytokine (500 IU/mL) (Proleukin) and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2 and rapamycin (100 nM) (Sigma) every alternate day. Every 7 days, the cells were re-
 30 stimulated with anti-CD3/anti-CD28 beads (1:1 Beads:Cells) (Dynabeads, Life Technologies). The cells were maintained for 21 days at 1x10⁶/mL concentration with

fresh complete media containing either (1) hIL-2 cytokine (500 IU/mL) (Proleukin) and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2 and rapamycin (100 nM) (Sigma) every alternate day. At Days 7, 14, and 21, the cells were stained with CD4-Alexa Fluro 488, CD25-PE, CD3 APC-Cy7, Foxp3-PE (intracellular staining using Foxp3 staining
 5 kit, Invitrogen), and CD127-Alex Fluro 700 (BioLegend) to assess the expansion of Foxp3⁺ T_{reg} cells by flow cytometry. Fold-expansion of Foxp3⁺ T_{reg} cells was measured by counting in a trypan blue (0.4%, Invitrogen) viability assay. FIG. 1 shows a 145-fold increase in Foxp3⁺ T_{reg} cells which were stimulated with 100 nM of 2t2, anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM)
 10 (Sigma) compared to a 100-fold increase with hIL-2 (500 IU/mL) (Proleukin), anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma).

**Example 4: Detection of Helios, CTLA-4, CD39, CD62L, CD25, CD103, CD69, PD1
 15 and CD49b markers**

Fresh human leukocytes (3 donors) were obtained from the blood bank and CD4⁺CD25⁺CD127^{dim} T_{reg} cells were isolated with the CD4⁺CD25⁺CD127^{dim} regulatory T cell isolation kit II reagent (Miltenyi Biotec). The purity of Foxp3⁺ T_{reg} cells was >35% and confirmed by staining with CD4-Alexa Fluro 488, CD25-APC, CD3 APC-
 20 Cy7, Foxp3-PE (Intracellular staining, Invitrogen), and CD127-Alex Fluro 700 (BioLegend). The cells were counted and resuspended in 1x10⁶/mL in a 48-well flat-bottom plate in 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
 25 stimulated with either (1) hIL-2 cytokine (500 IU/mL) (Proleukin), anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2, anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma), then incubated for 21 days at 37 °C, 5% CO₂. During the expansion period, the cells were re-stimulated with either (1)
 30 hIL-2 (500 IU/mL) (Proleukin) and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2 and rapamycin (100 nM) (Sigma) every alternate day. Every 7 days, the cells were re-

stimulated with anti-CD3/anti-CD28 beads (1:1, Beads:Cells) (Dynabeads, Life Technologies). The cells were maintained for 21 days at 1×10^6 /mL concentration with fresh complete media containing either (1) hIL-2 (500 IU/mL) (Proleukin) and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2 and rapamycin (100 nM) (Sigma), every alternate day. The cells were stained with CD4-Alexa Fluoro 488, CD25-PE, CD3 APC-Cy7, Fcγ3-PE (Intracellular staining using Fcγ3 staining kit, Invitrogen), and CD127-Alexa Fluoro 700 (BioLegend) to assess the expansion of Fcγ3⁺ T_{reg} cells by flow cytometry. Fold-expansion of Fcγ3⁺ T_{reg} cells was measured by counting in a trypan blue (0.4%, Invitrogen) viability assay. On day 21, the cells were harvested, and surface stained for CD4-Alexa Fluoro 488, CD25-PE, CD3 APC-Cy7, Fcγ3-PE (Intracellular staining using Fcγ3 staining kit, Invitrogen), CD127-Alexa Fluoro 700, Helios PE-Cy7, CTLA-4 Brilliant Violet 605, CD39 APC, CD62L PECy7, CD25 PE, CD103 PerCP.Cy5.5, CD69 APC Fire 750, PD1 Alexa Fluoro 750, and CD49b APC, and intracellularly stained using Fcγ3-Pacific Blue (BioLegend) (Intracellular staining kit, Invitrogen). For 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 analyzed by Flow Cytometry (Celesta-BD Bioscience). For intracellularly cell staining, the cells were fixed in fixative buffer (Invitrogen) for 20 minutes at room temperature. After fixation steps, the cells were washed (1500 RPM for 5 minutes in room temperature) in 1x Permeabilized Buffer (eBioscience) and stained using Fcγ3-Pacific Blue (Biolegend) for 30 minutes at room temperature. The cells were washed once again with 1x Permeabilized Buffer and then washed with FACS buffer. The cell pellets were resuspended in 300 μL of FACS Buffer for analysis by Flow Cytometry (Celesta-BD Bioscience). (The cells were gated on CD4⁺CD25⁺Fcγ3⁺ cells and plotted Data-%+ of surface markers, 3 healthy donors). FIGs. 2A-2I show comparable surface markers on Fcγ3⁺ T_{reg} cells which were stimulated with either (1) 100 nM of 2t2, anti-CD3/anti-CD28 beads (4:1 Beads:Cells)(Dynabeads, Life Technologies) and rapamycin (100 nM) (Sigma) or (2) hIL-2 (500 IU/mL) (Proleukin), anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma) for 21 days.

Example 5: Activation of human regulatory T cells displays a high level of glucose metabolism.

Fresh human leukocytes (3 donors) were obtained from the blood bank and CD4⁺CD25⁺CD127^{dim} T_{reg} cells were isolated using CD4⁺CD25⁺CD127^{dim} T_{reg} cell isolation kit II (Miltenyi Biotec). The purity of Foxp3⁺ T_{reg} cells was >35% and confirmed by staining with CD4-Alexa Fluor 488, CD25-APC, CD3 APC-Cy7, Foxp3-PE (Intracellular staining, Invitrogen), and CD127-Alex Fluor 700 (BioLegend). The cells were counted and resuspended in 1x10⁶/mL in a 24-well flat-bottom plate in 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 either (1) hIL-2 (500 IU/mL) (Proleukin), anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2, anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma), then incubated overnight at 37 °C at 5% CO₂. The next day, the cells were harvested, counted, and resuspended in 4 x 10⁶/mL in a 96-well plate and 50 µL/well were seeded in Cell-Tak-coated Seahorse Bioanalyzer XFe96 culture plates in Seahorse XF RPMI medium, pH 7.4 supplemented with 2 mM L-glutamine. The cells were allowed to attach to the plate for 30 mins at 37 °C. Additional 130 µL of assay medium was added to each well of the plate (also the background wells). The plate was incubated in 37 °C, non-CO₂ incubator for 1 hr. For the calibration plate: the 10x solution of glucose/oligomycin/2-deoxyglucose were prepared in Seahorse assay media. 20 µL volume of glucose/oligomycin/2-deoxyglucose were added to ports A, B, and C of the extracellular flux plate that was calibrated overnight. Extracellular acidification rate (ECAR) was measured using an XFe96 Extracellular Flux Analyzer. Complete ECAR analysis consisted of four stages: non-glycolytic acidifications (without drugs), glycolysis (10 mM glucose), glycolytic capacity (2 µM oligomycin), and glycolytic reserve (100 mM 2-deoxyglucose) which was calculated by using Seahorse Xfe96 software, wave. The results (FIGs. 3A-3D) showed that the activated human T_{reg} cells displayed a high level of glucose metabolism.

Example 6: Measurement of suppression of autologous T responder cells by expanded regulatory T cell

Fresh human leukocytes (3 donors) were obtained from the blood bank and CD4⁺CD25⁺CD127^{dim} T_{reg} cells were isolated with the CD4⁺CD25⁺CD127^{dim} regulatory T cell isolation kit II reagent (Miltenyi Biotec). The purity of Foxp3⁺ T_{reg} cells was >35% and confirmed by staining with CD4-Alexa Fluro 488, CD25-APC, CD3 APC-Cy7, Foxp3-PE (Intracellular staining, Invitrogen), and CD127-Alex Fluro 700 (BioLegend). The cells were counted and resuspended in 1x10⁶/mL in a 24-well flat-bottom plate in 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 either (1) hIL-2 (500 IU/mL) (Proleukin), anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2, anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma), and then incubated for 21 days at 37 °C, 5% CO₂. During the expansion period, the cells were re-stimulated with either (1) hIL-2 cytokine (500 IU/mL) (Proleukin) and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2 and rapamycin (100 nM) (Sigma), every alternate day. Every 7 days, the cells were re-stimulated with anti-CD3/anti-CD28 beads (1:1 Beads:Cells) (Dynabeads, Life Technologies). The cells were maintained for 21 days at 1x10⁶/mL concentration with fresh complete media containing either (1) hIL-2 (500 IU/mL) (Proleukin) and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2 and rapamycin (100 nM) (Sigma), every alternate day. Fold-expansion of Foxp3⁺ T_{reg} cells was measured by counting in trypan blue (0.4%, Invitrogen) viability assay. On day 21, frozen autologous T responder (Tresp) cells were thawed and labelled with Cell Trace Violet (Invitrogen). Expanded T_{reg} cells were harvested and counted. T_{reg} cells were cultured with T responder cells at various ratios (5:1, 2:1, 1:1, 0.5:1, T_{reg} cells:T responder cells). The cells were re-stimulated with either (1) anti-CD3/anti-CD28 beads (Beads:Cell 1:70) and 2t2 (25nM) or (2) anti-CD3/anti-CD28 beads (Beads:Cell 1:70) and IL2 (50 IU/mL), and cultured for 5 days. The cells were harvested and 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 analyzed by flow cytometry (Celesta-BD Bioscience). (Plotted % of CFSE⁺ proliferating cells). FIG. 4 shows suppression capacity of Foxp3⁺ T_{reg} cells which were stimulated with 100 nM of 2t2, anti-CD3/anti-CD28 beads (4:1 Beads:Cells)(Dynabeads, Life Technologies), and rapamycin (100 nM) compared to cells stimulated with hIL-2 (500 IU/mL) (Proleukin), anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma).

Example 7: Measurement of IL-10 from suppression assay

Fresh human leukocytes (3 donors) were obtained from the blood bank and CD4⁺CD25⁺CD127^{dim} T_{reg} cells were isolated with the CD4⁺CD25⁺CD127^{dim} regulatory T cell isolation kit II reagent (Miltenyi Biotec). The purity of Foxp3⁺ T_{reg} cells was >35% and confirmed by staining with CD4-Alexa Fluro 488, CD25-APC, CD3 APC-Cy7, Foxp3-PE (Intracellular staining, Invitrogen), and CD127-Alex Fluro 700 (BioLegend). The cells were counted and resuspended in 1x10⁶/mL in a 24-well flat-bottom plate in 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 either (1) hIL-2 (500 IU/mL) (Proleukin), anti-CD3/anti-CD28 beads (4:1 Beads:Cells) (Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2, anti-CD3/anti-CD28 beads (4:1 Beads:Cells)(Dynabeads, Life Technologies), and rapamycin (100 nM) (Sigma), and then incubated for 21 days at 37 °C and 5% CO₂. During the expansion period, the cells were re-stimulated with either (1) hIL-2 cytokine (500 IU/mL) (Proleukin) and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2 and rapamycin (100 nM) (Sigma), every alternate day. Every 7 days, the cells were re-stimulated with anti-CD3/anti-CD28 beads (1:1 Beads:Cells) (Dynabeads, Life Technologies). The cells were maintained for 21 days at 1x10⁶/mL concentration with fresh complete media containing either (1) hIL-2 (500 IU/mL) (Proleukin) and rapamycin (100 nM) (Sigma) or (2) 100 nM of 2t2 and rapamycin (100 nM) (Sigma), every alternate day. Fold-expansion of Foxp3⁺ T_{reg} cells was measured by counting in a trypan blue (0.4%, Invitrogen) viability assay. On day 21, frozen autologous T responder (Tresp)

cells were thawed and labelled with Cell Trace Violet (Invitrogen). The expanded T_{reg} cells were harvested and counted. The T_{reg} cells were cultured with T responder cells at various ratios (5:1, 1:1, T_{reg} cells:T responder cells). The cells were re-stimulated with (1) anti-CD3/anti-CD28 beads (Beads:Cell 1:70) and 2t2 (25nM) or (2) anti-CD3/anti-CD28 beads (Beads:Cell 1:70) and IL2 (50 IU/mL), and cultured for 5 days. The cell-free liquid culture medium was collected and analyzed by LEGENDplex multi-analyte flow assay kit (Biolegend) according to the manufacturer's instructions. FIG. 5 shows IL-10 in the cell-free liquid culture medium from a suppression assay where IL-10 secretion was significantly higher in 2t2-expanded T_{reg} cells compared to IL-2 expanded T_{reg} cells, both in the presences or absence of rapamycin.

Example 8. Generation and Characterization of 3t15*-28s Fusion Protein Complex

Construction and production of 3t15*-28s

A fusion protein was generated comprising anti-CD3scFv/TF/IL15D8N mutant and anti-CD28scFv/IL15R α Su fusion proteins. The anti-CD3 antibody (α CD3) variable regions, human tissue factor, IL15D8N mutant, anti-CD28 antibody (α CD28) variable regions, and IL15R α Su sequences were obtained from the UniProt website and DNA for these sequences was synthesized by Genewiz. Specifically, the construct was made linking α CD3 light chain variable region sequence by a (G₄S)₃ linker to α CD3 heavy chain variable region sequence to form an α CD3scFv. The α CD3scFv was linked to the N-terminus coding region of tissue factor 219 followed by a linkage to the N-terminus coding region of IL15D8N mutant (FIGs. 6A-6B). The nucleic acid and protein sequences of a construct are shown below.

The nucleic acid sequence of the α CD3scFv/TF/IL15D8N construct (including signal peptide sequence) is as follows (SEQ ID NO: 105):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCTTATTATTTTATTCAGCTCCGCCT
ATTCC

(α CD3 light chain variable region)

CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGT
GAGAAGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACT
GGTATCAGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCA
GCAAGCTGGCCTCCGGAGTGCCCGCTCATTTCGGGGCTCTGGATCCGGCAC
5 CAGCTACTCTTTAACCATTTCGGCATGGAAGCTGAAGACGCTGCCACCTACT
ATTGCCAGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCT
CGAAATCAATCGT

(Linker)

GGAGGAGGTGGCAGCGGCGGGTGGATCCGGCGGAGGAGGAAGC
10 *(α CD3 heavy chain variable region)*
CAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCCGGCGC
CTCCGTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAA
TGCATTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATAT
CAACCCTTCCCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCC
15 ACTTTAACCCTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTT
AACCAGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCAC
TACTGTTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGC

(Human tissue factor 219 form)

TCCGGCACCAACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGC
20 ACCAACTTCAAGACAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTT
ACACCGTGCAGATCTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTA
CACAACAGACACCGAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAG
CAAACCTATCTGGCTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCAC
CGGCTCCGCTGGCGAGCCTCTCTACGAGAATTCCCCGAATTCACCCCTTATT
25 TAGAGACCAATTTAGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCAC
CAAGGTGAACGTCACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAAC
ACATTTTTATCCCTCCGGGATGTGTTTCGGCAAAGACCTCATCTACACACTGTA
CTATTGGAAGTCCAGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAAC
GAGTTTTTTAATTGACGTGGACAAAGGCGAGAACTACTGCTTCAGCGTGCAAG
30 CCGTGATCCCTTCTCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGA
GTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(Human IL15D8N mutant)

AACTGGGTGAACGTCATCAGCAATTTAAAGAAGATCGAAGATTTAATT
 CAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTC
 TTGTAAGGTGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTTATCTCTT
 5 TAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTT
 AGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAG
 GAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTG
 TGCACATTGTCCAGATGTTTCATCAATACCTCC

10 The amino acid sequence of the α CD3scFv/TF/IL15D8N construct (including
 signal peptide sequence) is as follows (SEQ ID NO: 104):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(α CD3 light chain variable region)

15 QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDT
 SKLASGVP AHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEI
 NR

(Linker)

GGGSGGGSGGGGS

20 *(α CD3 heavy chain variable region)*

QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLEWI
 GYINPSRGYTNYNQKFKDKATLT TDKSSSTAYMQLSSLTSEDSAVYYCARYYDD
 HYCLDYWGQGTTTLTVSS

(Human tissue factor 219)

25 SGTNTNTVAAYNLTKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
 FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
 ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYW
 KSSSSGKK TAKTNTNEFLIDVDKGENYCF SVQAVIPSR TVNRKSTDSPVECMGQE
 KGEFRE

30 *(Human IL15D8N mutant)*

NWVNVISNLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVIS
 LESGDASIHDVTENLIILANNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQ
 MFINTS

5 Another construct was made by linking α CD28scFv which was constructed by linking α CD28 light chain variable region sequence by a (G₄S)₃ linker to α CD28 heavy chain variable region sequence, to the N-terminus coding region of IL15R α Su. The final construct α CD28scFv/IL15R α Su fusion protein sequence was synthesized by Genewiz. The nucleic acid and protein sequences of a construct are shown below.

10 The nucleic acid sequence of the α CD28scFv/IL15R α Su construct (including signal peptide sequence) is as follows (SEQ ID NO: 109):

(Signal peptide)

ATGAAATGGGTCACCTTCATCTCTTTACTGTTTTTATTTAGCAGCGCCT
 ACAGC

15 *(α CD28 light chain variable region)*

GTGCAGCTGCAGCAGTCCGGACCCGAAGTGGTCAAGCCCGGTGCCTCC
 GTGAAAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCA
 ATGGGTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAAT
 CCCTACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTC
 20 TGACAAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACT
 TCTGAGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTG
 GGGCCGGGGAAGTACTTTAACAGTGAGCTCC

(Linker)

GGCGGCGGCGGAAGCGGAGGTGGAGGATCTGGCGGTGGAGGCAGC

25 *(α CD28 heavy chain variable region)*

GACATCGAGATGACACAGTCCCCCGCTATCATGAGCGCCTCTTTAGGA
 GAACGTGTGACCATGACTTGTACAGCTTCCTCCAGCGTGAGCAGCTCCTATTT
 CCACTGGTACCAGCAGAAACCCGGCTCCTCCCCTAAACTGTGTATCTACTCCA
 CAAGCAATTTAGCTAGCGGCGTGCCCTCCTCGTTTTAGCGGCTCCGGCAGCACC
 30 TCTTACTCTTTAACCATTAGCTCTATGGAGGCCGAAGATGCCGCCACATACTT

TTGCCATCAGTACCACCGGTCCCCTACCTTTGGCGGAGGCACAAAGCTGGAG
ACCAAGCGG

(Human IL15R α Su)

ATTACATGCCCCCTCCCATGAGCGTGGAGCACGCCGACATCTGGGTG
5 AAGAGCTATAGCCTCTACAGCCGGGAGAGGTATATCTGTAACAGCGGCTTCA
AGAGGAAGGCCGGCACCAGCAGCCTCACCGAGTGCGTGCTGAATAAGGCTA
CCAACGTGGCTCACTGGACAACACCCTCTTTAAAGTGCATCCGG

The amino acid sequence of the α CD28scFv/IL15R α Su construct (including
10 signal peptide sequence) is as follows (SEQ ID NO: 108):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(α CD28 light chain variable region)

VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGS
15 INPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNY
WGRGTTLTVSS

(Linker)

GGGGSGGGSGGGGS

(α CD28 heavy chain variable region)

DIEMTQSPAISASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYS
20 TSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKAT
NVAHWTTPSLKCIR

25

In some cases, the leader peptide is cleaved from the intact polypeptide to
generate the mature form that may be soluble or secreted.

The α CD3scFv/TF/IL15D8N and α CD28scFv/IL15R α Su constructs were cloned
into a modified retrovirus expression vectors with different antibiotics selection markers
30 as described previously (Hughes et al., *Hum Gene Ther* 16: 457–72, 2005), and the
expression vectors were co-transfected into CHO-K1 cells. Expression of the construct

in CHO-K1 cells allowed for secretion of the soluble α CD3scFv/TF/IL15D8N: α CD28scFv/IL15R α Su fusion protein (referred to as 3t15*-28s), which can be purified by anti-TF antibody affinity chromatography and other chromatographic methods.

5

Purification of 3t15*-28s by immunoaffinity chromatography

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 the elution step, which was 2 mL/min.

10 Cell culture harvest of 3t15*-28s 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 washed with 5 column volumes PBS, followed by elution with 6 column volumes 0.1M Acetic Acid, pH 2.9. A280 elution peak was collected and then neutralized
15 to pH 7.5-8.0 by adding 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 FIG. 7, it is clear that the anti-TF antibody affinity column can bind 3t15*-28s which contains TF as a fusion partner of 3t15*-28s. The buffer-exchanged protein sample is stored at 2-8°C for further biochemical analysis and biological activity
20 test.

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

25 Analytical size exclusion chromatography (SEC) analysis of 3t15*-28s

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 equilibrated with 2 column volumes of PBS. The flow rate was 0.8 mL/min. A 200 μ L sample of 3t15*-28s (1 mg/mL) was injected onto the column using a capillary loop. The
30 injection was then chased with 1.25 column volumes of PBS. The SEC chromatograph

was shown in FIG. 8. The result indicated that there are 4 protein peaks, likely representing a monomer and dimer or other different forms of 3t15*-28s.

SDS PAGE analysis of 3t15*-28s

5 To determine the purity and protein molecular weight, the purified 3t15*-28s protein sample from anti-TF antibody affinity chromatography 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 min, followed by destaining overnight with purified water. FIG. 9 shows the
10 SDS gel of 3t15*-28s purified from anti-TF antibody affinity chromatography. The results indicated that purified 3t15*-28s contains protein bands with expected molecular weight (72 kDa) in reduced SDS PAGE.

In Vitro Characterization of the Activities of 3t15*-28s Fusion Protein Complex

15 ELISA-based methods confirmed the formation of the 3t15*-28s fusion protein complex. To assess whether 3t15*-28s interacts with CD28 and CD3 (FIGs. 10A-10B), 10 µg/mL of human CD28-Fc (Cat# CD8-H525a, Acro Biosystems) or 10 µg/mL of human CD3E/CD3D heterodimer-Llama Fc/Llama Fc (Cat# CDD-H5258, Acro Biosystems) in 50 mM carbonate buffer pH 9.4 (100µl/well) was applied to an ELISA
20 plate (Cat# 80040LE 0910, ThermoFisher) and incubated overnight at 4°C. Next day, the plate was washed 3 times with ELISA washing buffer (phosphate-buffered saline with 0.05% Tween 20) and blocked with the blocking buffer (1% BSA-PBS) for 1 hour. Descending concentrations (3 to 0.00243 µg/mL in blocking buffer) of 3t15*-28s or a negative control fusion protein (HCW9218 which contains the same TF domain) were
25 added to the plate and the plate was incubated for 1 hour at 25°C. The plate was washed for 3 times with ELISA washing buffer. A detection antibody, biotinylated anti-TF antibody (Cat# BAF2339, R&D Systems) at 0.1 µg/mL was added to the plate and incubated at 25°C for 1 hour. The plate was washed and Horseradish peroxidase-streptavidin (code#016-030-084, Jackson ImmunoResearch) at 0.25 µg/mL was added to
30 the plate and incubated at 25°C for 30 minutes. The plate was washed and a substrate of HRP, ABTS (Cat# ABTS-1000-01, Surmodics) was added to the plate and incubated for

20 minutes at 25°C. The plate was read with a microplate reader (Multiscan Sky, Thermo Scientific) at OD405nm. As shown in FIGs. 10A-10B, 3t15*-28s interacted with CD28 and CD3 while the control fusion protein HCW9218 did not. The α CD3scFv and α CD28scFv domains of 3t15*-28s fusion protein complex were capable of binding CD3 and CD28. Binding of the α CD28scFv and TF domains to CD28 and anti-TF antibody, respectively (FIG. 10A), confirmed the purified protein was comprised of the 3t15*-28s heterodimeric complex.

Example 9. Expansion of Foxp3⁺ Regulatory T Cells Using 2t2 and 3t15*-28s

Fresh human leukocytes (3 donors) were obtained from the blood bank and CD4⁺CD25⁺CD127^{low} T regulatory cells were isolated with the EasySep Human CD4⁺CD25⁺CD127^{low} regulatory T cells isolation kit (StemCell Technology). The purity of Foxp3⁺ regulatory T cells was >35% and confirmed by staining with CD4-Alexa Fluro 488, CD25-PE, CD3 APC-Cy7 and Foxp3-PacBlue antibodies (Abs) (BioLegend). Cells were counted and resuspended in 1x10⁶/mL in 24 well flat bottom plates in 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). Cells were stimulated with 2t2 (100 nM) and 3t15*-28s (200 nM) and incubated for 11 days at 37°C, 5% CO₂. During the expansion period, cells were maintained for 11 days at 1x10⁶/mL concentration with fresh complete media containing 2t2 (100 nM) and 3t28 (200 nM) every alternate day. Cells were then stained with CD4-Alexa Fluro 488, CD25-PE, CD3 APC-Cy7 and Foxp3-PacBlue Abs (BioLegend) to assess the expansion of Foxp3⁺ regulatory T cells by flow cytometry. Fold expansion of Foxp3⁺ regulatory T cells was measured by counting using trypan blue (0.4%, Invitrogen) exclusion. FIG. 11 shows 2.5-fold increase in Foxp3⁺ regulatory T cells at 11 days.

Example 10. Expansion of Human Immune Cells Using 2t2 and 3t15*-28s Plus Anti-Tissue Factor Antibody

Fresh human leukocytes (2 donors) were obtained from the blood bank and PBMC (peripheral blood mononuclear cells) were isolated with Ficoll separation. Cells

were counted and resuspended at a density of 1×10^7 cells/mL in PBS. 2 μ M of final concentration of Cell-Trace Violet Dye (Life Technologies) was added in the cells and incubated for 20 minutes in 37°C under an atmosphere of 5% CO₂. Cells were resuspended at 10 times volume in fresh pre-warmed RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine (Thermo Life Technologies), antibiotics (penicillin, 10,000 units/mL; streptomycin, 10,000 μ g/mL; Thermo Life Technologies), and 10% FBS (Hyclone) for 5 minutes at room temperature. Cells were washed 2 times at 1200 rpm for 10 minutes at counted and resuspended at a density of 2×10^6 cells/mL in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine (Thermo Life Technologies), antibiotics (penicillin, 10,000 units/mL; streptomycin, 10,000 μ g/mL; Thermo Life Technologies), and 10% FBS (Hyclone). Cells (0.1 mL) were transferred in a 96 well flat bottom plate and incubated with 2t2 (100 nM) and 3t15*-28s (0.01-1000 nM) with or without the anti-tissue factor antibody (α TF Ab, 0.01-100 nM). Complex of 3t15*-28s and anti-tissue factor antibody was made in 2:1 ratio, 15 minutes prior to stimulation. Half of the media was removed and replaced with fresh 2t2 (100 nM) and 3t15*-28s with or without anti-tissue factor antibody every 48 to 72 hrs. Cells were stained with CD4-Alexa Fluro 488, CD3-PE, CD8-PerCP Cy5.5 Abs (BioLegend) to assess dilution of Cell Trace Violet by flow cytometry.

FIG. 12 shows the percent of CD4⁺ T cells undergoing six or more cell division under different culture conditions. Incubation with 3t15*-28s: α TF Ab complex plus 2t2 was capable of supporting cell proliferation at a lower 3t15*-28s concentration than was observed with 3t15*-28s plus 2t2 (without α TF Ab). This result suggests that immune cells derived from PBMC could be optimally stimulated to proliferate using a complex of anti-tissue factor antibody and 3t28.

Example 11. Purification of CD39⁺Foxp3⁺ Regulatory T cells Using an Anti-CD39 IgG1_k Reagent

Fresh human leukocytes were obtained from the blood bank. Half of the leukocytes were used to purify CD4⁺CD25⁺CD127^{low} T regulatory cells with the EasySep Human CD4⁺CD25⁺CD127^{low} regulatory T cells isolation kit (StemCell Technology). The purity of Foxp3⁺ regulatory T cells was >60% and confirmed by staining with CD4-

Alexa Fluoro 488, CD39-APC, CD3 PE, Foxp3-PacBlue (BioLegend). The other half of the leukocytes were used to purify CD39⁺ Foxp3⁺ regulatory T cells by two step process. First CD4⁺ T cells were isolated with the RosetteSep Human CD4⁺ cells isolation kit (StemCell Technology). After enriching CD4⁺ T cells, CD39⁺ CD4⁺ cells were isolated using a novel biotinylated anti CD39 antibody reagent (HCW Biologic) and magnetic particles per manufacturer's instructions (EasySep Human Biotin Positive Selection Kit, StemCell Technology). Cells were stained with CD4-Alexa Fluoro 488, CD39-APC, CD3 PE, Foxp3-PacBlue Abs (BioLegend) to assess the purity of CD39⁺ Foxp3⁺ regulatory T cells by flow cytometry (Cells were gated on CD3⁺CD4⁺Foxp3⁺). Post-purification purity analysis by flow cytometer showed that cell purified using biotinylated anti CD39 Ab were 84.8% CD39⁺ compared to 62% for cells isolated with the EasySep Human CD4⁺CD25⁺CD127^{low} regulatory T cells isolation kit (StemCell Technology). This data demonstrates a novel strategy to isolated highly pure and phenotypical active regulatory T cells (FIG. 13A). These cells can be expanded in media containing hIL-2, anti-CD3/anti-CD28 beads, rapamycin, 2t2, 3t15*, and or 3t15*-28s:αTF Ab complex by the methods described above.

In a second study, Foxp3⁺ regulatory T cells were first expanded with 2t2 from purified CD4⁺CD25⁺CD127^{low} T regulatory cells, and then frozen. The cells were thawed and maintained at 2x10⁶/mL in T25 flask with 2t2 (100 nM) in 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). CD39⁺CD4⁺ cells were isolated using a novel biotinylated anti CD39 Ab reagent (HCW Biologic) and magnetic particles per manufacturer's instructions (EasySep Human Biotin Positive Selection Kit, StemCell Technology). Cells were stained with CD4-Alexa Fluoro 488, CD39-APC, CD3 PE, Foxp3-PacBlue Abs (BioLegend) to assess the purity of CD39⁺ Foxp3⁺ regulatory T cells by flow cytometry (Cells were gated on CD3⁺CD4⁺Foxp3⁺). Post-purification purity analysis by flow cytometer shows that cell purified using biotinylated anti CD39 antibody yielded a Foxp3⁺ regulatory T cells that are 99.9% enriched for the highly suppressive CD39⁺ subset (FIG. 13B). These cells can be expanded in media containing hIL-2, anti-

CD3/anti-CD28 beads, rapamycin, 2t2, 3t15*, and or 3t15*-28s:αTF Ab complex by the methods described above.

Example 12. Suppression of Autologous T Responder Cells by Purified CD39⁺

5 Regulatory T Cells

Fresh human leukocytes were obtained from the blood bank. Half of the leukocytes were used to purify CD4⁺CD25⁺CD127^{low} T regulatory cells with the EasySep Human CD4⁺CD25⁺CD127^{low} regulatory T cells isolation kit (StemCell Technology). The purity of Foxp3⁺ regulatory T cells was >60% and confirmed by staining with CD4-
 10 Alexa Fluoro 488, CD39-APC, CD3 PE, Foxp3-PacBlue (BioLegend). The other half of the leukocytes were used to purify CD39⁺ Foxp3⁺ regulatory T cells by two step process. First CD4⁺ T cells were isolated with the RosetteSep Human CD4⁺ cells isolation kit (StemCell Technology). After enriching CD4⁺ T cells, CD39⁺ CD4⁺ cells were isolated using a novel biotinylated anti CD39 antibody reagent (HCW Biologics) and magnetic
 15 particles per manufacturer's instructions (EasySep Human Biotin Positive Selection Kit, StemCell Technology). Cells were stained with CD4-Alexa Fluoro 488, CD39-APC, CD3 PE, Foxp3-PacBlue Abs (BioLegend) to assess the purity of CD39⁺ Foxp3⁺ regulatory T cells by flow cytometry (Cells gated on CD3⁺CD4⁺Foxp3⁺). Autologous T responder (Tresp) cells were labelled with Cell Trace Violet (Invitrogen). The purified Foxp3⁺ T
 20 cells were cultured with T responder cells (1:1 regulatory T cells: T responder cells). Cells were re-stimulated with 2t2 (10 nM) and cultured for 5 days. Cells were harvested, 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, cells were analyzed by flow cytometry (Celesta-BD Bioscience) and % of
 25 dividing Tresp cells were determined based on Cell Trace Violet dilution. FIG. 14 shows that purified CD39⁺Foxp3⁺ regulatory T cells were more effective at suppressing T responder cell proliferation when compared to cells isolated based on CD4⁺CD25⁺CD127^{low} T regulatory cells.

Example 13. Suppression of THP-1 Monocyte Cells by Expanded and CD39⁺-Purified T Regulatory Cells

Fresh human leukocytes were obtained from the blood bank.

CD4⁺CD25⁺CD127^{low} T regulatory cells with the EasySep Human CD4⁺CD25⁺CD127^{low} regulatory T cells isolation kit (StemCell Technology). The purity of Foxp3⁺ regulatory T cells was >60% and confirmed by staining with CD4-Alexa Fluro 488, CD39-APC, CD3 PE, Foxp3-PacBlue (BioLegend). Cells were counted and resuspended in 1x10⁶/mL in 24 wells flat bottom plate in 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). Cells were stimulated with 100 nM of 2t2 with CD3/CD28 beads (4:1, Beads:Cells) (Dynabeads, Life Technologies) and incubated for 21 days at 37°, 5% CO₂. During the expansion period, cells were re-stimulated with 100 nM of 2t2 with every alternate day and every 7 days cells were re-stimulated with CD3/CD28 beads (1:1, Beads:Cells) (Dynabeads, Life Technologies). Cells were maintained upto 21 days at 1x10⁶/mL concentration with fresh complete media containing 100 nM of 2t2 every alternate day.

On day 21 cells were stained with CD4-Alexa Fluro 488, CD25-PE, CD3 APC-Cy7, Foxp3-PE (Intracellular staining using Foxp3 staining kit, Invitrogen), CD127-Alexa Fluro 700 (BioLegend) to assess the expansion of Foxp3⁺ regulatory T cells by flow cytometry and frozen at 20 x10⁶/mL. Previously expanded cells were thawed and maintained at 2x10⁶/mL in T25 flask in 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) for an hour and washed before isolating CD39⁺CD4⁺ T reg cells. CD39⁺CD4⁺ cells were isolated using a novel biotinylated anti-CD39 Ab reagent (HCW Biologics; EasySep Human Biotin Positive Selection Kit, StemCell Technology). Cells were stained with CD4-Alexa Fluro 488, CD39-APC, CD3 PE, Foxp3-PacBlue Abs (BioLegend) to assess the purity of CD39⁺ Foxp3⁺ regulatory T cells by flow cytometry (cells were gated on CD3⁺CD4⁺Foxp3⁺). Post-purification purity analysis by flow cytometer shows that cell purified using biotinylated anti CD39 antibody yielded a Foxp3⁺ regulatory T cells that

are 99.9% enriched for the highly suppressive CD39⁺ subset. Suppression assay was setup with THP-1 (Human Monocyte Cells) and purified CD39⁺ Foxp3⁺ T Reg cells or CD39⁻ Foxp3⁺ T Reg cells or Total CD4⁺ T Reg cells (1:1 ratio). Cells were cultured for 3 days in (RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Thermo Life
5 Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone) supplemented with 50 ng/mL LPS. Cells supernatant was collected on day 3 to analyze IL-10 by ELISA (R&D System).

FIG. 15 shows that purified CD39⁺Foxp3⁺ regulatory T cells secret significantly more IL-10 compare to CD39⁻ Foxp3⁺ T Reg cells or Total CD4⁺ T Reg cells. This data
10 shows that CD39⁺ Foxp3⁺ T Reg has more suppressive capacity compare to CD39⁻ Foxp3⁺ T Reg cells or Total CD4⁺ T Reg cells.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction
15 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 following claims.