



(51) International Patent Classification:

C07K 14/745 (2006.01) C12N 5/0783 (2010.01)

(21) International Application Number:

PCT/US2021/017714

(22) International Filing Date:

11 February 2021 (11.02.2021)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/975,141 11 February 2020 (11.02.2020) US

(71) Applicant: **HCW BIOLOGICS, INC.** [US/US]; 2929 North Commerce Parkway, Miramar, Florida 33025 (US).

(72) Inventor: **WONG, Hing C.**; 2929 North Commerce Parkway, Miramar, Florida 33025 (US).

(74) Agent: **YOON, Sohee Kim et al.**; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH,

GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(88) Date of publication of the international search report:

23 September 2021 (23.09.2021)

(54) Title: METHODS OF TREATING AGE-RELATED AND INFLAMMATORY DISEASES

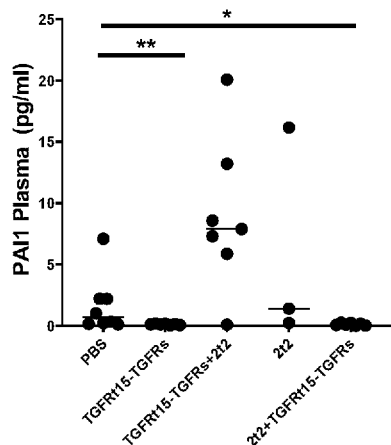


Figure 110A

(57) Abstract: Provided herein are methods of treating an aging-related disease or inflammatory disease in a subject that include (i) a therapeutically effective amount of an NK cell activating agent and/or an NK cell and/or monoclonal antibody; and (ii) a therapeutically effective amount of a Treg cell activating agent and/or a Treg cell and/or a monoclonal antibody and/or AGE inhibitor.

METHODS OF TREATING AGE-RELATED AND INFLAMMATORY DISEASES

CROSS-REFERENCE TO RELATED APPLICATION

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

TECHNICAL FIELD

10 The present disclosure relates to the field of biotechnology, and more specifically, to methods of treating inflammatory and age-related diseases.

BACKGROUND

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)).
15 The process that connects inflammation with aging has been termed inflamm-aging (Franceschi et al., *Ann N Y Acad Sci* 908: 244-254, 2000). Inflamm-aging is characterized by a state of chronic, low-grade, sterile inflammation, and it causes accumulation of senescent cells and persistent activation of inflammasomes. The process of aging is connected with major changes that affect the immune system and results in a
20 variety aging-associated pathologies.

Senescent cells have been implicated for a role in the pathogenesis of a number of different diseases including, e.g., glaucoma (Liton et al., 2005), idiopathic pulmonary fibrosis (IPF) (Yanai et al., 2015; Schafer et al., 2017), atherosclerosis (Uryga and Bennett, 2016; Childs et al., 2016), liver cirrhosis/NAFLD (Krizhanovsky et al., 2008;
25 Kim et al., 2013; Ogrodnik et al., 2017; Wiemann et al., 2002), glomerulosclerosis (Melk et al., 2003; Melk et al., 2004; Maker et al., 2016), type 2 diabetes (Chen et al., 2009; Helman et al., 2016), cachexia (Berry et al., 2017; Xu et al., 2015; Baker et al., 2016), sarcopenia (Sousa-Victor et al., 2014; Cosgrove et al., 2014; Chang et al., 2016), osteoarthritis (Price et al., 2002; Kuyinu et al., 2016; Jeon et al., 2017), cancer (Latz et

al., *Sem. Immunol.* 40:61-73, 2018), arthritis (Latz et al., *Sem. Immunol.* 40:61-73, 2018), and neurodegenerative diseases (Latz et al., *Sem. Immunol.* 40:61-73, 2018).

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 (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 senescence 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 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.

Atherosclerosis is a chronic inflammatory disease arising from an imbalance in lipid metabolism and a maladaptive immune response driven by the accumulation of cholesterol-laden macrophages in the artery wall. Hyperlipidemia increases the number of circulating monocytes recruited to mouse atherosclerotic plaques in a multistep process involving chemokine-chemokine receptor pairs and endothelial adhesion molecules, including selectins and adhesion molecules. The recruited monocytes differentiate into macrophages or dendritic cells in the intima, where they take up atherogenic lipoproteins via macropinocytosis or scavenger receptor-mediated pathways. The resulting foam cells secrete proinflammatory cytokines and chemokines, as well as retention factors that amplify the inflammatory response and promote macrophage chemostasis. These accumulating macrophages experience endoplasmic reticulum stress, which, if prolonged, results in apoptosis. This cell death, coupled with defective efferocytosis, results in the formation of the necrotic core that is characteristic of advanced plaques (Moore et al., *Nat Rev Immunol* 13: 709-721, 2013).

In humans, low levels of circulating Tregs are associated with an increased risk of acute coronary syndrome, and higher numbers of Tregs are found in stable versus unstable plaques (Dietel et al., *Atherosclerosis* 230: 92–99, 2013). These findings are supported by studies in hypercholesterolemic mouse models, which have shown a decline in Treg numbers in the circulation and plaque and reduced suppressive function during atherosclerosis progression (Maganto-García et al., *Circulation* 124: 185–195, 2011). Notably, depletion of Tregs in mouse models of atherosclerosis progression using anti-CD25 antibodies or diphtheria toxin-targeted depletion of FoxP3⁺ cells exacerbates disease (Klingenberg et al., *J Clin Invest.* 123: 1323–1334, 2013), whereas adoptive transfer of Tregs halts disease progression (Ait-Oufella et al., *Nat Med.* 12: 178–180, 2006). Recent studies suggest that Tregs enable atherosclerosis regression through suppression of ongoing macrophage and T-cell proinflammatory responses, and re-education of macrophages to a proresolving state that facilitates tissue repair and plaque contraction. Tregs are essential for the enrichment of M2-like macrophages in regressing plaques and to license key proresolving macrophage functions, including clearance of apoptotic cells, production of specialized proresolving lipid mediators, and upregulation of the receptors that sense these mediators of resolution. Treg-derived cytokines, such as IL-10 and TGF- β , can dampen macrophage inflammatory responses, promote alternative activation, and increase efferocytosis. M2 macrophages can also secrete IL-10 and TGF- β , which may, in turn, sustain iTregs, and this mutual interaction may be synergistic in promoting tissue repair in the plaque. Treg-dependent increase in efferocytosis and reduction of necrotic core area in regressing plaques that corresponded with an increase in smooth muscle cells in the fibrous cap, suggested that Tregs are key participants in enabling tissue reparative functions that promote plaque stability (Sharma et al., *Circ Res.* 127: 335–353, 2020).

Regulatory T (Treg) cells are essential mediators of peripheral tolerance 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 that inhibit cells of innate immunity, antigen-presenting cell (APC) functions, as well as adaptive B, CD4⁺ or CD8⁺ effector T (Teff) cell responses (Sakaguchi et al., *Int Immunol* 21(10): 1105–1111, 2009). Treg cells

play central roles in the global immunoregulatory potential in hosts. 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). Excellent safety profiles have been shown in patients receiving Tregs 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 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).

SUMMARY

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., 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 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
5 receptors A1, A2A, A2B, and A3 displayed on immune cells. A2A and A2B receptors (A2AR and 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
10 as immune-promoting adenosine receptors. In humans, A1R, A2AR and A3R display high 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
15 (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
20 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*
25 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
30 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 a method that utilizes an approach to reduce inflammation by suppressing the activity of inflammasomes (e.g., using 2t2, anti-tissue factor antibody, RAGE (advanced glycation end product trap), anti-CD36 antibody, or adoptive cell therapy (e.g., immune cells treated with 2t2 and 3t28, anti-tissue factor CAR-Treg cells, or anti-CD36 CAR Treg cells) and reducing the accumulation of senescent cells (e.g., using anti-tissue factor antibody, anti-CD26 antibody, or anti-CD36 antibody, optionally further using TGFRt15-TGFRs; TGFRt15-TGFRs; adoptive cell therapy (e.g., immune cells treated with 18t15-12s, immune cells treated with 18t15-12s and 7t15-21s, anti-tissue factor CAR NK cells, or anti-CD26 CAR NK cells)). The method includes various combinations of immunotherapies, mAbs directed at inflammation and senescence, and activated and/or engineered NK and T-cells, to attack inflamm-aging through multiple channels to eliminate the direct causes of inflammation and the underlying development and sustainment of these causes. In one example, the anti-inflammatory approach for inflammasomes is driven by regulatory T cells. Additionally, the senescent cell approach may be NK-cell-mediated. The method addresses both key factors in inflamm-aging, the chronic inflammatory activities and the underlying contribution of accumulating senescent cells that produce senescence-associated secretory phenotype (SASP) factors that sustain the inflammasome activity.

Provided is a method of treating inflammasome-related diseases and senescent cell-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.

Provided herein are methods of treating an aging-related disease or inflammatory disease in a subject that include administering to the subject: (i) a therapeutically effective amount of an NK cell activating agent and/or an NK cell and/or a monoclonal antibody; and (ii) a therapeutically effective amount of a Treg cell activating agent and/or a Treg cell and/or a monoclonal antibody and/or an advanced glycation end product (AGE) inhibitor.

In some embodiments of any of the methods described herein, the aging-related disease is inflamm-aging related. In some embodiments of any of the methods described herein, (i) is administered to the subject at substantially the same time as (ii). In some embodiments of any of the methods described herein, (i) is administered to the subject
5 prior to administration of (ii) to the subject. In some embodiments of any of the methods described herein, (ii) is administered to the subject prior to administration of (i) to the subject.

In some embodiments of any of the methods described herein, the method includes administering a therapeutically effective amount of an NK cell to the subject. In
10 some embodiments of any of the methods described herein, the NK cell is an autologous, haploidentical or allogeneic NK cell isolated from peripheral blood, umbilical cord blood, or isolated and differentiated from iPSC. Some embodiments of any of the methods described herein further include: isolating the NK cell from the subject; and culturing the isolated NK cell in a liquid culture medium under conditions sufficient to induce or
15 increase proliferation of the NK cell, where following the isolating and culturing steps, the NK cell is administered to the subject. In some embodiments of any of the methods described herein, the liquid culture medium includes a multi-chain chimeric polypeptide. In some embodiments of any of the methods described herein, the NK cell comprises a chimeric antigen receptor. In some embodiments of any of the methods described herein,
20 the chimeric antigen receptor comprises an extracellular domain that binds specifically to tissue factor or CD26.

In some embodiments of any of the methods described herein, the method comprises administering a therapeutically effective amount of an NK cell activating agent and/or monoclonal antibody to the subject. In some embodiments of any of the methods
25 described herein, the NK cell activating agent is one or more multi-chain chimeric polypeptide(s). In some embodiments of any of the methods described herein, the monoclonal antibody is one or more of an anti-tissue factor antibody and/or an anti-CD26 antibody. In some embodiments of any of the methods described herein, the NK cell activating agent comprises one or more multi-chain chimeric polypeptide(s) and the
30 monoclonal antibody comprises one or more of an anti-tissue factor antibody and/or an anti-CD26 antibody.

In some embodiments of any of the methods described herein, the method includes administering a therapeutically effective amount of a Treg cell to the subject. In some embodiments of any of the methods described herein, the Treg cell is an autologous Treg cell, a haploidentical Treg cell, or an allogeneic Treg cell isolated from peripheral blood or umbilical cord blood. In some embodiments of any of the methods described herein, the method further includes: isolating the Treg cell from the subject; culturing the isolated Treg cell in a liquid culture medium under conditions sufficient to induce or increase proliferation of the Treg cell, where following the isolating and culturing steps, the Treg cell is administered to the subject.

In some embodiments of any of the methods described herein, the step of isolating the Treg cell from the subject comprises obtaining a sample comprising Treg cells from the subject, and isolating the Treg cell from the sample using an antibody or ligand capable of binding CD39. In some embodiments of any of the methods described herein, the step of isolating the Treg cell from the sample comprises: mixing the sample with the 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 components in the sample, thereby isolating the Treg cell. In some embodiments of any of the methods 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 described herein, the 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 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 described herein, the Treg cell is a CD4⁺CD25⁺Foxp3⁺ cell. In some embodiments of any of the methods described herein,

the Treg cell is a CD4⁺CD25⁺CD127dim⁻ cell. In some embodiments of any of the methods described herein, the Treg cell is immunosuppressive in vitro and in vivo.

In some embodiments of any of the methods described herein, the liquid culture medium comprises one or more single-chain chimeric polypeptide(s). In some
5 embodiments of any of the methods described herein, the Treg cell comprises a chimeric antigen receptor. In some embodiments of any of the methods described herein, the chimeric antigen receptor comprises an extracellular domain that binds specifically to tissue factor or CD36.

In some embodiments of any of the methods described herein, the method
10 comprises administering a therapeutically effective amount of a Treg cell activating agent and/or monoclonal antibody and/or AGE inhibitor to the subject. In some embodiments of any of the methods described herein, the Treg cell activating agent is one or more single-chain chimeric polypeptide(s). In some embodiments of any of the methods described herein, the monoclonal antibody is one or both of an anti-tissue factor antibody
15 and/or an anti-CD36 antibody. In some embodiments of any of the methods described herein, the AGE inhibitor is a soluble RAGE trap. In some embodiments of any of the methods described herein, the Treg cell activating agent comprises one or more single-chain chimeric polypeptide(s), the monoclonal antibody comprises one or more of an anti-tissue factor antibody and/or an anti-CD36 antibody, and the AGE inhibitor
20 comprises one or more soluble RAGE trap.

In some embodiments of any of the methods described herein, the multi-chain chimeric polypeptide comprises: (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
25 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.

In some embodiments of any of the methods described herein, the single-chain chimeric polypeptide comprises: (i) a first target-binding domain; (ii) a soluble tissue
30 factor domain; and (iii) a second target-binding domain.

In some embodiments of any of the methods described herein, the aging-related disorder 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, 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, cancer, dementia, vascular disease, infection susceptibility, chronic inflammation, and renal dysfunction.

In some embodiments of any of the methods described herein, 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, and non-alcoholic steatohepatitis.

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., an 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.

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

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

The term “soluble interleukin receptor” is used herein in the broadest sense to refer to a polypeptide that lacks a transmembrane domain (and optionally an intracellular domain) that is capable of binding one or more of its natural ligands (e.g., under

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

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

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

“Affinity” refers to the strength of the sum total of non-covalent interactions between an antigen-binding site and its binding partner (e.g., an antigen or epitope). Unless indicated otherwise, as used herein, “affinity” refers to intrinsic binding affinity, which reflects a 1:1 interaction between members of an antigen-binding domain and an antigen or epitope. The affinity of a molecule X for its partner Y can be represented by

the dissociation equilibrium constant (K_D). The kinetic components that contribute to the dissociation equilibrium constant are described in more detail below. Affinity can be measured by common methods known in the art, including those described herein.

Affinity can be determined, for example, using surface plasmon resonance (SPR)

5 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.

A “multi-chain polypeptide” as used herein refers to a polypeptide comprising
10 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 “pair of affinity domains” is two different protein domain(s) that bind specifically to each other with a K_D of less than of less than 1×10^{-7} M (e.g., less than 1×10^{-8} M, less than 1×10^{-9} M, less than 1×10^{-10} M, or less than 1×10^{-11} M). In some
15 examples, a pair of affinity domains can be a pair of naturally-occurring proteins. In some embodiments, a pair of affinity domains can be a pair of synthetic proteins. Non-limiting examples of pairs of affinity domains are described herein.

The term “epitope” means a portion of an antigen that specifically binds to an
20 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
25 amino acid residues that are directly involved in the binding, and other amino acid residues, which are not directly involved in the binding. Methods for identifying an epitope to which an antigen-binding domain binds are known in the art.

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
30 symptom of cancer includes reducing aberrant proliferation, gene expression, signaling, translation, and/or secretion of factors. Generally, the methods of treatment include

administering a therapeutically effective amount of composition that reduces at least one symptom of a disorder to a subject who is in need of, or who has been determined to be in need of such treatment.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

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

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows exemplary diagrams for a multi-chain chimeric polypeptide: (i) a first chimeric polypeptide including a first target-binding domain (A), a soluble tissue factor domain, a first domain of an affinity pair of domains (soluble interleukin IL-15), and an additional target-binding domain (B); and (ii) second chimeric polypeptide including a second domain of an affinity pair of domains (IL-15 receptor alpha sushi domain), a second target-binding domain (C), and an additional antigen-binding domain (D). The top cartoon diagram depicts the association of the first and the second chimeric polypeptides through the pair of affinity domains. The bottom schematic diagrams show the order of the domains in the first and second chimeric polypeptides.

Figure 2 shows exemplary diagrams for a multi-chain chimeric polypeptide: (i) a first chimeric polypeptide including a first target-binding domain (A), a soluble tissue factor domain including five amino acid substitutions in order to remove binding of the soluble tissue factor domain to FVIIa, a first domain of an affinity pair of domains (soluble interleukin IL-15 including a D8N or D8A amino acid substitution), and an additional target-binding domain (B); and (ii) second chimeric polypeptide including a second domain of an affinity pair of domains (IL-15 receptor alpha sushi domain), a

second target-binding domain (C), and an additional antigen-binding domain (D). The top cartoon diagram depicts the association of the first and the second chimeric polypeptides through the pair of affinity domains. The bottom schematic diagrams show the order of the domains in the first and second chimeric polypeptides. In other

5 embodiments of any of the multi-chain chimeric polypeptides described herein the soluble tissue factor domain can comprise or consists of a soluble wildtype human tissue factor domain (comprising or consisting of a contiguous sequence within wildtype human tissue factor).

10 Figure 3 shows a schematic diagram of an exemplary IL-12/IL-15R α Su DNA construct.

Figure 4 shows a schematic diagram of an exemplary IL-18/TF/IL-15 DNA construct.

Figure 5 shows a schematic diagram of the interaction between the exemplary IL-12/IL-15R α Su and IL-18/TF/IL-15 DNA constructs.

15 Figure 6 shows a schematic diagram of the interaction between the exemplary IL-12/IL-15R α Su and IL-18/TF/IL-15 fusion proteins resulting in IL-18/TF/IL-15:IL-12/IL-15R α Su complex (18t15-12s).

Figure 7 shows a chromatograph of 18t15-12s purification elution from an anti-TF antibody affinity column.

20 Figure 8 shows an exemplary chromatographic profile of anti-TF Ab / SEC-purified 18t15-12s protein following elution on an analytical size exclusion column, demonstrating separation of monomeric multiprotein 18t15-12s complexes from protein aggregates.

25 Figure 9 shows an example of a 4-12% SDS-PAGE of the 18t15-12s complex following disulfide bond reduction. Lane 1: SeeBlue Plus2 marker; Lane 2: an anti-tissue factor antibody affinity column-purified 18t15-12s (0.5 μ g); Lane 3: an anti-tissue factor antibody affinity column-purified 18t15-12s (1 μ g).

30 Figure 10 shows SDS PAGE analysis of deglycosylated and non-deglycosylated 18t15-12s. Lane 1: an anti-tissue factor antibody affinity column-purified 18t15-12s (0.5 μ g), non-deglycosylated; Lane 2: anti-TF Ab-purified 18t15-12s (1 μ g), non-

deglycosylated; Lane 3: 18t15-12s (1 µg), deglycosylated, Lane 4: Mark12 unstained maker.

Figure 11 shows a sandwich ELISA for the 18t15-12s complex, comprising an anti-human tissue factor capture antibody and a biotinylated anti-human IL-12 detection antibody (BAF 219).

Figure 12 shows a sandwich ELISA for the 18t15-12s complex, comprising an anti-human tissue factor capture antibody and a biotinylated anti-human IL-15 detection antibody (BAM 247).

Figure 13 shows a sandwich ELISA for the 18t15-12s complex, comprising an anti-human tissue factor capture antibody and a biotinylated anti-human IL-18 detection antibody (D045-6).

Figure 14 shows a sandwich ELISA for the 18t15-12s complex, comprising an anti-human tissue factor (I43) capture antibody and an anti-human tissue factor detection antibody.

Figure 15 shows proliferation of IL-15-dependent 32Dβ cells mediated by the 18t15-12s complex (open squares) and recombinant IL-15 (black squares).

Figure 16 shows biological activity of IL-18 within the 18t15-12s complex (open squares), where recombinant IL-18 (black squares) and recombinant IL-12 (black circles) serve as positive and negative controls, respectively.

Figure 17 shows biological activity of IL-12 within the 18t15-12s complex (open squares), where recombinant IL-12 (black circles) and recombinant IL-18 (open squares) serve as positive and negative controls, respectively.

Figures 18A and 18B show cell-surface expression of CD25 on NK cells induced by the 18t15-12s complex and cell-surface CD69 expression of NK cells induced by the 18t15-12s complex.

Figure 19 shows a flow cytometry graph of intracellular interferon gamma expression of NK cells induced by the 18t15-12s complex.

Figure 20 shows cytotoxicity of 18t15-12s induced human NK cells against K562 cells.

Figure 21 shows a schematic diagram of an exemplary IL-7/IL-15RαSu DNA construct.

Figure 22 shows a schematic diagram of an exemplary IL-21/TF/IL-15 DNA construct.

Figure 23 shows a schematic diagram of the interaction between the exemplary IL-7/IL-15R α Su and IL-21/TF/IL-15 DNA constructs.

5 Figure 24 shows a schematic diagram of the interaction between the exemplary IL-7/IL-15R α Su and IL-21/TF/IL-15 fusion proteins resulting in an IL-21/TF/IL-15:IL-7/IL-15R α Su complex (21t15-7s).

Figure 25 shows cytotoxic activity of expanded NK cells against K562 human tumor cells, wherein NK cells stimulated with 21t15-7s + anti-TF IgG1 antibody is
10 demonstrated to exhibit greater specific lysis of K562 cells than NK cells not stimulated with 21t15-7s + anti-TF IgG1 antibody.

Figure 26 shows a schematic diagram of an exemplary IL-21/IL-15R α Su DNA construct.

15 Figure 27 shows a schematic diagram of an exemplary IL-7/TF/IL-15 DNA construct.

Figure 28 shows a schematic diagram of the interaction between the exemplary IL-21/IL-15R α Su and IL-7/TF/IL-15 DNA constructs.

Figure 29 shows a schematic diagram of the interaction between the exemplary IL-21/IL-15R α Su and IL-7/TF/IL-15 fusion proteins resulting in an IL-7/TF/IL-15:IL-
20 21/IL-15R α SU complex (7t15-21s).

Figure 30 shows size exclusion chromatography (SEC) profiles of anti-TF IgG1 antibody, 7t15-21s and the complex containing equal amounts of anti-TF IgG1 antibody and 7t15-21s.

Figure 31 shows the oxygen consumption rate (OCR) in pmoles/min for human
25 NK cells isolated from blood (2×10^6 cells/mL) of two different donors.

Figure 32 shows the extracellular acidification rate (ECAR) in mpH/minute for human NK cells isolated from blood (2×10^6 cells/mL) of two different donors.

Figure 33 shows a schematic of the TGFRT15-TGFRs construct.

30 Figure 34 shows an additional schematic of the TGFRT15-TGFRs construct.

Figure 35 shows results of TGF β 1 inhibition by TGFRT15-TGFRs and TGFRT15-TGFR-Fc.

Figures 36A and 36B show results of detecting IL-15 and TGF β RII in TGFRt15-TGFRs with corresponding antibodies using ELISA.

Figure 37 is a line graph showing the chromatographic profile of TGFRt15-TGFRs protein containing cell culture supernatant following binding and elution on anti-TF antibody resin.

Figure 38 shows the analytical SEC profile of TGFRt15-TGFRs.

Figure 39 shows TGFRt15-TGFRs before and after deglycosylation as analyzed by reduced SDS-PAGE.

Figures 40A and 40B show spleen weight and the percentages of immune cell types in TGFRt15-TGFRs-treated and control-treated mice. Figure 40A shows spleen weight in mice treated with TGFRt15-TGFRs as compared to PBS control. Figure 40B shows the percentage of CD4⁺ T cells, CD8⁺ T cells, and NK cells in mice treated with TGFRt15-TGFRs as compared to PBS control.

Figure 41A and 41B show the spleen weight and immunostimulation over 92 hours in mice treated with TGFRt15-TGFRs. Figure 41A shows spleen weight of mice treated with TGFRt15-TGFRs at 16, 24, 48, 72, and 92 hours after treatment. Figure 41B shows the percentages of immune cells in mice treated with TGFRt15-TGFRs at 16, 24, 48, 72, and 92 hours after treatment.

Figure 42A and 42B show Ki67 and Granzyme B expression in mice treated with TGFRt15-TGFRs over time.

Figure 43 shows enhancement of cytotoxicity of splenocytes by TGFRt15-TGFRs in C57BL/6 Mice.

Figure 44 shows changes in tumor size in response to PBS treatment, chemotherapy alone, TGFRt15-TGFRs alone, or chemotherapy and TGFRt15-TGFRs combination, in a pancreatic cancer mouse model.

Figure 45 shows the cytotoxicity of NK cells isolated from mice treated with TGFRt15-TGFRs.

Figure 46 shows changes in the surface phenotype of lymphocyte populations after stimulation with 18t15-12s, 18t15-12s16, and 7t15-21s.

Figure 47 shows an increase in phospho-STAT4 and phospho-STAT5 levels in NK cells after stimulation with 18t15-12s.

Figures 48A-48C show *in vivo* stimulation of Tregs, NK cells, and CD8⁺ T cells in ApoE^{-/-} mice fed with a Western diet and treated with TGFRT15-TGFRs.

Figures 49A-49C show immunostimulation in C57BL/6 mice following treatment with TGFRT15-TGFRs.

5 Figures 50A and 50B show *in vivo* induction of proliferation of NK cells and CD8⁺ T cells in ApoE^{-/-} mice fed with a Western diet and treated with TGFRT15-TGFRs.

Figures 51A and 51B show enhancement of cytotoxicity of NK cells following treatment of NK cells with TGFRT15-TGFRs.

10 Figures 52A and 52B show enhancement of ADCC activity of NK cells following treatment of NK cells with TGFRT15-TGFRs.

Figures 53A-53H show antitumor activity of TGFRT15-TGFRs plus anti-TRP1 antibody (TA99) in combination with chemotherapy in a melanoma mouse model.

Figures 54A-54C show amelioration of the Western diet-induced hyperglycemia in ApoE^{-/-} mice by TGFRT15-TGFRs.

15 Figure 55 shows cell surface staining summarizing the differentiation of NK cells into cytokine-induced memory like NK Cells (CIML-NK Cells) after stimulation with 18t15-12s and cultured in rhIL15.

Figure 56 shows upregulation shows upregulation of CD44^{hi} memory T cells upon treatment with TGFRT15-TGFRs.

20 Figure 57 are schematic diagrams of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide.

Figure 58 is a chromatograph showing the elution of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide from an anti-tissue factor antibody affinity column.

25 Figure 59 is a chromatograph showing the elution of a Superdex 200 Increase 10/300 GL gel filtration column loaded with an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide.

Figure 60 is a sodium dodecyl sulfate polyacrylamide gel (4-12% NuPage Bis-Tris gel) of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide purified using an anti-tissue factor antibody affinity column.

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Figure 61 is a graph showing the ELISA quantitation of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide performed using the methods described in Example 38. Purified tissue factor was used as the control.

Figure 62 is a graph showing the ability of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide to stimulate CD25 expression in CD4⁺ T-cells isolated from blood from two donors. The experiments were performed as described in Example 39.

Figure 63 is a graph showing the ability of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide to stimulate CD25 expression in CD8⁺ T-cells isolated from blood from two donors. The experiments were performed as described in Example 39.

Figure 64 is a graph showing the ability of an exemplary α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide to stimulate CD69 expression in CD4⁺ T-cells isolated from blood from two donors. The experiments were performed as described in Example 39.

Figure 65 are schematic diagrams of an exemplary IL-2/TF/IL-2 single-chain chimeric polypeptide.

Figure 66 shows IL-2 activity in IL-2/TF/IL-2 as compared to recombinant IL-2 using a 32D β cell proliferation assay.

Figure 67 shows IL-2 activity in IL-2/TF/IL-2 as compared to recombinant IL-2 using a CTLL-2 cell proliferation assay.

Figure 68 shows the fasting blood glucose levels in ApoE^{-/-} mice fed with standard chow or a high fat diet and treated with a PBS control (untreated) or with IL-2/TF/IL-2.

Figure 69 shows the ratio of CD4⁺CD25⁺FoxP3⁺ T regulatory cells in blood lymphocytes from ApoE^{-/-} mice fed with standard chow or a high fat diet and treated with a PBS control (untreated) or with IL-2/TF/IL-2.

Figure 70 is a line graph showing the chromatographic profile of IL-2/TF/IL-2 protein containing cell culture supernatant following binding and elution on anti-TF antibody resin.

Figure 71 shows an analytical SEC profile of IL-2/TF/IL-2.

Figures 72A and 72B show reduced SDS-PAGE analysis of IL-2/TF/IL-2 before and after deglycosylation. Figure 16A shows reduced SDS-PAGE analysis of IL-2/TF/IL-2 before deglycosylation. Figure 16B shows reduced SDS-PAGE analysis of IL-2/TF/IL-2 after deglycosylation.

5 Figures 73A and 73B show results of immunostimulation in C57BL/6 mice using IL-2/TF/IL-2. Figure 73A shows spleen weight following treatment with IL-2/TF/IL-2. Figure 73B shows the percentages of immune cell types following IL-2/TF/IL-2 treatment.

10 Figure 74 shows upregulation of CD25 expression of CD4⁺ T cells in mice treated with IL-2/TF/IL-2.

Figure 75 shows the pharmacokinetics of IL-2/TF/IL-2 in C57BL/6 mice.

Figures 76A and 76B show effects of IL-2/TF/IL-2 in attenuating the formation of high fat-induced atherosclerotic plaques in ApoE^{-/-} mice. Figure 20A shows a representative view of atherosclerotic plaques from ApoE^{-/-} mice fed with standard chow or a high fat diet and treated with either PBS control or IL-2/TF/IL-2. Figure 76B shows the results of quantitative analysis of atherosclerotic plaques of each group.

Figure 77 shows fasting glucose levels in IL-2/TF/IL-2 treated-mice as compared to control-treated mice.

20 Figure 78 shows the percentage of CD4⁺CD25⁺FoxP3⁺ Tregs in blood lymphocytes from mice treated with IL-2/TF/IL-2 and control-treated mice.

Figures 79A-79C is a set of graphs showing immunostimulation in C57BL/6 mice following treatment with 2t2.

Figures 80A-80C is a set of graphs showing *in vivo* stimulation of Tregs, NK cells, and CD8⁺ T cells in ApoE^{-/-} mice fed with a Western diet and treated with 2t2.

25 Figures 81A and 81B is a set of graphs showing induction of splenocyte proliferation by 2t2 in C57BL/6 mice.

Figures 82A and 82B is a set of graphs showing *in vivo* induction of proliferation of NK cells and CD8⁺ T cells in ApoE^{-/-} mice fed with a Western diet and treated with 2t2.

30 Figures 83A-83C is a set of graphs showing amelioration of the Western diet-induced hyperglycemia in ApoE^{-/-} mice by 2t2.

Figure 84 shows upregulation of CD44 memory T cells upon treatment with 2t2.

Figure 85A-85C show human blood lymphocyte pStat5a responses in CD4⁺CD25^{hi}T_{reg} cells, CD4⁺CD25⁻T_{con} cells, or in CD8⁺ T_{con} cells in response to 2t2 or IL2 treatment. Figure 85A shows pSTAT5 responses in CD4⁺CD25^{hi}T_{reg} cells. Figure 34B shows pSTAT5 responses in CD4⁺CD25⁻T_{con} cells. Figure 85C shows pSTAT5 responses in CD8⁺ T_{con} cells.

Figure 86 is a graph showing plasma hemoglobin A1C levels in aged mice following treatment with PBS or TGF α 15-TGFRs and/or 2t2.

Figures 87A-87C are a set of graphs showing levels of gene expression of senescence markers (IL-1 α , IL-6, and PAI-1, respectively) in tissues of aged mice following treatment with PBS; TGF α 15-TGFRs; 2t2; first dose TGF α 15-TGFRs at day 0 with second dose 2t2 at day 60; or first dose 2t2 at day 0 with second dose TGF α 15-TGFRs at day 60.

Figure 88A shows a schematic of the experimental design for feeding ApoE^{-/-} mice to induce NASH.

Figure 88B is a graph showing the treatment effects on hydroxyproline content related to collagen accumulation and fibrosis in the livers of the ApoE^{-/-} mice.

Figure 89A is a schematic of the experimental design for a high-fat diet-induced atherosclerosis animal model.

Figure 89B is a table showing the effect of 2t2 treatment on IL-1 β and MCP-1 plasma cytokine levels in ApoE^{-/-} mice where plasma samples were collected three days after the second injection.

Figure 90 is a graph showing the effect of 2t2 treatment on triglyceride plasma levels in high-fat diet-induced ApoE^{-/-} mice.

Figure 91 is a graph showing the effect of 2t2 treatment on LDL plasma levels in high-fat diet-induced ApoE^{-/-} mice.

Figure 92 is a graph showing the effect of administration of 2t2 on the body weight of high-fat diet-induced ApoE^{-/-} mice.

Figures 93A-93E show exemplary physical appearance of mice fed either a control or high fat diet and were either untreated or treated with TGF α 15-TGFRs, 2t2, or 21t15-TGFRs.

Figures 94A-94E are a set of images showing that treatment with an IL-2 based molecule (2t2) can induce formation of hair follicles following depilation in mouse model. Figure 94A is an image from a control mouse - only depilation done after hair was shaved, Figure 94B is an image from a mouse where depilation was followed by low dose IL-2 (1 mg/kg) administration, and Figures 94C-94E show images from mice where depilation was followed by 2t2 at 0.3 mg/kg, (Figure 94C), 1 mg/kg (Figure 94D), and (Figure 94E) 3 mg/kg. Black arrows indicate anagen-phase hair follicles that will later extend into dermis and facilitate hair growth.

Figure 95 shows the total number of anagen phase hair follicles counted per 10 fields for each treatment group.

Figure 96 is an exemplary schematic of the experimental design using a melanoma mouse model.

Figures 97A-97H are graphs showing the effect of administration of TGF α 15-TGFRs on NK/T cell proliferation, expansion, and activation in the blood of the melanoma mouse model.

Figures 98A-98C are graphs showing the effect of TGF α 15-TGFRs treatment on TGF- β 1, TGF- β 2, and TGF- β 3 levels in the plasma of the melanoma mouse model.

Figures 99A-99E are graphs showing the effect of treatment with dexamethasone or a combination of TGF α 15-TGFRs and dexamethasone on plasma levels of IL-2, IL-1 β , IL-6, and GM-CSF in the melanoma mouse model.

Figures 100A-100B are graphs showing the effect of treatment with dexamethasone or a combination of TGF α 15-TGFRs and dexamethasone on the levels of NK cells and CD8⁺ T-cells in the spleens of the melanoma mouse model.

Figures 101A-101C are a set of graphs showing the effect of treatment with saline (black line), dexamethasone (dark grey line), or a combination of dexamethasone, TGF α 15-TGFRs, and TA99 (light gray line) on the glycolytic activity of splenocytes.

Figures 102A-102L are a set of graphs the effect of treatment with saline, dexamethasone, or a combination of dexamethasone, TGF α 15-TGFRs, and TA99 on glycolytic activity (glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic acidification) of splenocytes from a melanoma mouse model.

Figures 103A-103C are a set of graphs showing the effect of treatment with PBS, dexamethasone, or a combination of dexamethasone, TGF α 15-TGFRs, and TA99 on mitochondrial respiration of splenocytes from a melanoma mouse model.

Figures 104A-104L are a set of graphs showing the effect of treatment with PBS, dexamethasone, or a combination of dexamethasone, TGF α 15-TGFRs, and TA99 on mitochondrial respiration of splenocytes (basal respiration, maximal respiration, spare respiratory capacity, and ATP production) from a mouse melanoma model.

Figures 105A-105H are a set of graphs showing the effect of treatment with PBS, dexamethasone, or a combination of dexamethasone, TGF α 15-TGFRs, and TA99 on the infiltration of NK/Ki67 cells, CD8/Ki67 cells, NK cells, CD8 cells, NK/CD25 cells, NK/Granzyme B cells, CD8/CD25 cells, and CD8/Granzyme B cells into melanoma tumors in a melanoma mouse model.

Figures 106A is a schematic of the experimental design for therapy-induced senescence in B16F10 tumors in a melanoma mouse model.

Figures 106B-106E are a set of graphs showing the effect of DTX treatment on senescence-associated gene expression (DPP4, IL-6, p16, and p21, respectively) in B16F10 tumor cells in the mice.

Figure 107A is a schematic of the experimental design for therapy-induced senescence in B16F10 tumors in a melanoma mouse model.

Figures 107B-107C are graphs showing the effect of treatment with saline, dexamethasone, or a combination of dexamethasone, TGF α 15-TGFRs, and TA99 on expression of p21 and IL-6, respectively in B16F10 tumors in a melanoma tumor model.

Figure 108A is a graph showing the effect of 2t2 or IL-2 on IL2R $\alpha\beta\gamma$ -containing or IL-2R $\beta\gamma$ -containing cell proliferation.

Figure 108B is a graph showing the effect of 2t2 of IL-2 on activation of human CD4⁺CD25⁺ Treg pSTAT5 and human CD8⁺ T_{con} pSTAT5.

Figure 108C is a graph showing the effect of 2t2 or IL-2 on activation of human CD4⁺CD25⁺T_{con} pSTAT5 or human CD56^{bright} NK pSTAT5.

Figure 108D is a graph showing the effect of 2t2 or IL-2 on activation of CD56^{dim} NK pSTAT5.

Figure 109A is a schematic of an experiment studying the effect of treatment with 2t2 in ApoE^{-/-} mice fed a Western diet.

Figure 109B is a set of graphs showing the effect of administration of 2t2 on the levels of CD25⁺Foxp3⁺Treg cells, CTLA4⁺Foxp3⁺ Treg cells, and CD39⁺Foxp3⁺ Treg cells in ApoE^{-/-} mice fed a Western diet.

Figure 109C is a set of graphs showing the effect of administration of 2t2 on the levels of CD4⁺ T-cells, CD8⁺ T-cells, and CD3⁺NK1.1⁺ NK cells in ApoE^{-/-} mice fed a Western diet.

Figure 109D is a set of graphs showing the effect of administration of 2t2 on the plasma levels of IL-1 β , MCP-1, and TNF- α in ApoE^{-/-} mice fed a Western diet.

Figure 109E is a set of graphs showing the effect of administration of 2t2 on plasma LDL cholesterol level, fasting glucose level, and HOMA-IR index in ApoE^{-/-} mice fed a Western diet.

Figures 110A-110D are a set of graphs showing levels of protein expression of senescence markers (PAI1, IL-1 α , CXCL1, and IL-2, respectively) in plasma of aged mice following treatment with PBS; TGFRt15-TGFRs; 2t2; first dose TGFRt15-TGFRs at day 0 with second dose 2t2 at day 60; or first dose 2t2 at day 0 with second dose TGFRt15-TGFRs at day 60.

DETAILED DESCRIPTION

Provided herein are methods of treating an aging-related or inflammatory disease in a subject that include: (i) a therapeutically effective amount of an NK cell activating agent and/or an NK cell and/or monoclonal antibody; and (ii) a therapeutically effective amount of a Treg cell activating agent and/or a Treg cell and/or a monoclonal antibody and/or an advanced glycation end product (AGE) inhibitor. In some embodiments, the aging-related disease is inflamm-aging related.

Methods of Treating Aging-Related and Inflammatory Disease in a Subject

In some embodiments of any of the methods described herein, (i) is administered to the subject at substantially the same time as (ii). In some embodiments of any of the methods described herein, (i) is administered to the subject prior to administration of (ii)

to the subject. In some embodiments of any of the methods described herein, (ii) is administered to the subject prior to administration of (i) to the subject.

In some embodiments of any of the methods described herein, the method includes administering a therapeutically effective amount of an NK cell to the subject. In some embodiments, the NK cell is an autologous NK cell. In some embodiments, the method can further include: isolating the NK cell from the subject; and culturing the isolated NK cell in a liquid culture medium under conditions sufficient to induce or increase proliferation of the NK cell, where following the isolating and culturing steps, the NK cell is administered to the subject. In some embodiments, the liquid culture medium includes one or more multi-chain chimeric polypeptide(s) (e.g., any of the exemplary multi-chain chimeric polypeptide(s) described herein).

In some embodiments, the NK cell includes a chimeric antigen receptor (e.g., a chimeric antigen receptor comprises an extracellular domain that binds specifically to tissue factor or CD26).

In some embodiments, the method can include administering a therapeutically effective amount of an NK cell activating agent to the subject. In some embodiments, the NK cell activating agent is one or more multi-chain chimeric polypeptide(s) (e.g., one or more of any of the multi-chain chimeric polypeptides described herein). In some embodiments, the NK cell activating agent is one or more of an anti-tissue factor antibody, an anti-CD26 antibody, and/or an anti-CD36 antibody. In some embodiments, the NK cell activating agent includes one or more multi-chain chimeric polypeptide(s) and one or more of an anti-tissue factor antibody, an anti-CD26 antibody, and/or an anti-CD36 antibody.

In some embodiments, the method includes administering a therapeutically effective amount of a Treg cell to the subject. In some embodiments, the Treg cell is an autologous Treg cell. In some embodiments, the method further includes: isolating the Treg cell from the subject; culturing the isolated Treg cell in a liquid culture medium under conditions sufficient to induce or increase proliferation of the Treg cell, where following the isolating and culturing steps, the Treg cell is administered to the subject. In some embodiments, the liquid culture medium includes one or more single-chain chimeric polypeptide(s).

In some embodiments, the Treg cell includes a chimeric antigen receptor (e.g., a chimeric antigen receptor including an extracellular domain that binds specifically to tissue factor or CD36).

In some embodiments, the method includes administering a therapeutically effective amount of a Treg cell activating agent to the subject. In some embodiments, the Treg cell activating agent is one or more single-chain chimeric polypeptide(s) (e.g., one or more of any of the single-chain chimeric polypeptides described herein). In some embodiments, the Treg cell activating agent is one or both of an anti-tissue factor antibody and an anti-CD36 antibody. In some embodiments, the Treg cell activating agent is a soluble RAGE trap.

In some embodiments, the Treg cell activating agent includes one or more single-chain chimeric polypeptide(s) and one or more of an anti-tissue factor antibody, an anti-CD36 antibody, and a soluble RAGE trap.

In some embodiments, the method includes administering a therapeutically effective amount of a monoclonal antibody to the subject. In some embodiments, a monoclonal antibody comprises one or more of an anti-tissue factor antibody, anti-CD36 antibody and/or anti-CD36 antibody that can directly or indirectly reduce inflammasome or senescent cell activity.

In some embodiments, the method includes administering a therapeutically effective amount of an advanced glycation end product (AGE) inhibitor to the subject. In some embodiments, an advanced glycation end product (AGE) inhibitor comprises one or more of soluble RAGE trap that can directly or indirectly reduce inflammasome or senescent cell activity.

In some embodiments of any of the methods described herein, the aging-related disease is inflamm-aging related. Non-limiting examples of 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, cancer, dementia, vascular disease, infection susceptibility, chronic inflammation, and renal dysfunction.

Non-limiting examples of inflammatory diseases include: rheumatoid arthritis, inflammatory bowel disease, lupus erythematosus, lupus nephritis, amyotrophic lateral sclerosis, diabetic nephropathy, CNS injury, Alzheimer's disease, Parkinson's disease, Crohn's disease, multiple sclerosis, Guillain-Barre syndrome, psoriasis, Grave's disease, ulcerative colitis, and non-alcoholic steatohepatitis.

In some embodiments, the subject can be a subject identified or diagnosed as having an age-related disease or having chronic inflammation.

In some embodiments, these methods can result in a reduction in the number, severity, or frequency of one or more symptoms of the aging-related disease in the subject (e.g., as compared to the number, severity, or frequency of the one or more symptoms of the cancer in the subject prior to treatment).

In some 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% 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,

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30 about 20% decrease to about 35% decrease, about 20% decrease to about 30% decrease,
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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, 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% 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% 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, 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, 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,

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, 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, 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% decrease to about 75% decrease, about 60% to about 70% decrease, about 60% decrease to about 65% decrease, about 65% decrease to about 99% decrease, 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, 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

disorder 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.

Treg Cells

In some embodiments, a Treg cell can be administered to the subject. In some embodiments, a Treg cell administered to the subject can be an autologous Treg cell, haploidentical Treg cell, or allogenic Treg cell isolated from peripheral blood or umbilical cord blood. In some embodiments, the methods described herein can further include isolating a Treg cell from a subject, culturing the isolated Treg cell in a liquid culture medium, and administering the Treg cell back to the subject. In some embodiments, isolating the Treg cell from the subject comprises obtaining a sample comprising Treg cells from the subject, and isolating the Treg cell from the sample using an antibody or ligand capable of binding CD39. In some embodiments, the step of isolating the Treg cell from the sample comprises: mixing the sample with the antibody or ligand capable of binding CD39 under conditions that allow binding of the antibody of ligand to Treg cells expressing CD39; and separating the Treg cell bound to the antibody or ligand from other components in the sample, thereby isolating the Treg cell. In some embodiments, 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, the separating comprises the use of flow cytometry, fluorescence-activated cell sorting (FACS), centrifugation, or column, plate, particle, or

bead-based methods. In some embodiments, 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, the Treg cell is a CD4⁺CD25⁺Foxp3⁺ cell. In some embodiments, the Treg cell is a CD4⁺CD25⁺CD127dim⁻ cell. In some embodiments, the Treg cell is immunosuppressive in vitro and in vivo.

In some embodiments, a Treg cell can be isolated using a commercially available kit (see, e.g., EasySep™ Human CD4⁺CD127^{low}CD25⁺ Regulatory T Cell Isolation Kit or Dynabeads Regulatory CD4⁺CD25⁺ T Cell Kit). In some embodiments, the liquid culture medium can include one or more of a single-chain chimeric polypeptide (e.g., any of the exemplary single-chain chimeric polypeptides described herein, e.g., 2t2 or 3t28). In some embodiments, the liquid culture medium can include the use of a bead having on its surface CD3 and CD28, and recombinant IL-2 or 2t2.

In some embodiments, the Treg cell can comprise a chimeric antigen receptor (e.g., a chimeric antigen receptor that includes an extracellular domain that binds specifically to tissue factor or CD36). Non-limiting examples of extracellular domains that can bind to tissue factor or CD36 are scFvs. Non-limiting examples of anti-CD36 antibodies are commercially available from Invitrogen, Abcam, GeneTex, Novus Biologicals, Proteintech, and EMD Millipore. Non-limiting examples of anti-tissue factor heavy chain variable domain and light chain variable domains are described in U.S. Patent No. 7,968,094 and U.S. Patent No. 8,007,795. Chimeric antigen receptors include a transmembrane domain, a costimulatory domain (e.g., an intracellular CD28 domain), and a CD3zeta signaling domain. For example, a transmembrane domain can include 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: 1 (FWVLVVVGGLACYSLLVTVAFIIFWV). For example, a costimulatory domain can include 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: 2 (RSKRSRLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS). For example, a CD3zeta signaling domain can include 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: 3

(RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPQRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR).

5 Treg Cell Activating Agents

In some embodiments, one or more Treg cell activating agents can be administered to the subject. In some embodiments, the Treg cell activating agent can be a single-chain chimeric polypeptide (e.g., any of the exemplary single-chain chimeric polypeptides described herein), an anti-tissue factor antibody (e.g., the anti-tissue factor antibodies described in U.S. Patent No. 7,968,094 and U.S. Patent No. 8,007,795), a soluble RAGE protein, or an anti-CD36 antibody.

A soluble RAGE protein can have 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: 4 or SEQ ID NO: 5.

Soluble Human RAGE Variant 1 (SEQ ID NO: 4)

maagtavgaw vlvslwgav vgaqnitari geplvlkckg apkkppqrle wklntgrtea
wkvlsppggg pwsvarvlp ngslflpavg iqdegifrcq amnrngketk snyrvrvyrk
nsrvfksasl lpkkkpstpa lahegl

Soluble Human RAGE Variant 2 (SEQ ID NO: 5)

maagtavgaw vlvslwgav vgaqnitari geplvlkckg apkkppqrle wklntgrtea
wkvlsppggg pwsvarvlp ngslflpavg iqdegifrcq amnrngketk snyrvrvyqi
pgkpeivdsa seltagvpnk vgtcvsegsy pagtlswld gkplvpnekg es

In some examples, a soluble RAGE protein is encoded by a nucleic acid having 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: 6 or SEQ ID NO: 7.

Soluble Human RAGE Variant 1 cDNA (SEQ ID NO: 6)

atggcagccg gaacagcagt tggagcctgg gtgctggtcc tcagtctgtg gggggcagta
gtaggtgctc aaaacatcac agcccgatt ggcgagccac tgggtgctgaa gtgtaagggg

gcccccaaga aaccacccca gcggtctggaa tggaaactga acacaggccg gacagaagct
 tggaaaggtcc tgtctcccca gggaggaggc ccctgggaca gtgtggctcg tgccttccc
 aacggctccc tcttccttcc ggctgtcggg atccaggatg aggggatttt ccggtgccag
 gcaatgaaca ggaatggaaa ggagaccaag tccaactacc gagtccgtgt ctaccgtaag
 5 aattccaggg tcttctccaa ggctccctc ttacctaaga aaaagccttc aaccccagcc
 ttggcccatg agggcctctg a

Mouse RAGE cDNA (SEQ ID NO: 7)

atggcagccg gaacagcagt tggagcctgg gtgctgggtcc tcagtctgtg gggggcagta
 10 gtaggtgctc aaaacatcac agcccggtt ggcgagccac tgggtgctgaa gtgtaagggg
 gcccccaaga aaccacccca gcggtctggaa tggaaactga acacaggccg gacagaagct
 tggaaaggtcc tgtctcccca gggaggaggc ccctgggaca gtgtggctcg tgccttccc
 aacggctccc tcttccttcc ggctgtcggg atccaggatg aggggatttt ccggtgccag
 gcaatgaaca ggaatggaaa ggagaccaag tccaactacc gagtccgtgt ctaccagatt
 15 cctgggaagc cagaaattgt agattctgcc tctgaactca cggctgggtg tccaataag
 gtggggacat gtgtgtcaga gggaagctac cctgcaggga ctcttagctg gcacttggat
 gggaagcccc tgggtgcctaa tgagaagggt gagtccctaa

As can be appreciated by those in the art, substitutions/mutations that are made at
 20 positions that are not conserved between different species are less likely to have a
 negative impact on the activity of the protein/nucleic acid, whereas
 substitutions/mutations that are made at positions that are conserved between species are
 more likely to have a negative impact on the activity of the protein/nucleic acid.

25 NK Cells

In some embodiments, a NK cell can be administered to the subject. In some
 embodiments, a NK cell administered to the subject can be an autologous NK cell,
 haploidentical NK cells, or allogeneic NK cells isolated from peripheral blood, umbilical
 cord blood, or isolated and differentiated from iPSC. In some embodiments, the methods
 30 described herein can further include isolating a NK cell from a subject, culturing the
 isolated NK cell in a liquid culture medium, and administering the NK cell back to the
 subject. In some embodiments, a NK cell can be isolated using a commercially available
 kit (see, e.g., EasySep™ Human NK Cell Isolation Kit, MojoSort Human NK Cell
 Isolation Kit, and Novus Biologicals Human NK Cell Isolation Kit). In some
 35 embodiments, the liquid culture medium can include one or more of a multi-chain

chimeric polypeptide (e.g., any of the exemplary multi-chain chimeric polypeptides described herein, e.g., 18t15-12s and/or 7t15-21s).

In some embodiments, the NK cell can comprise a chimeric antigen receptor (e.g., a chimeric antigen receptor that includes an extracellular domain that binds specifically to tissue factor or CD26). Non-limiting examples of extracellular domains that can bind to tissue factor or CD26 are scFvs. Non-limiting examples of anti-CD26 antibodies are commercially available from Abcam, Invitrogen, and GeneTex. Non-limiting examples of anti-tissue factor heavy chain variable domain and light chain variable domains are described in U.S. Patent No. 7,968,094 and U.S. Patent No. 8,007,795. Chimeric antigen receptors include a transmembrane domain, a costimulatory domain (e.g., an intracellular CD28 domain), and a CD3zeta signaling domain. For example, a transmembrane domain can include 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: 1. For example, a costimulatory domain can include 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: 2. For example, a CD3zeta signaling domain can include 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: 3.

NK Cell Activating Agents

In some embodiments, one or more NK cell activating agents can be administered to the subject. In some embodiments, the NK cell activating agent can be one or more multi-chain chimeric polypeptide (e.g., any of the exemplary multi-chain chimeric polypeptides described herein), an anti-tissue factor antibody (e.g., the anti-tissue factor antibodies described in U.S. Patent No. 7,968,094 and U.S. Patent No. 8,007,795), an anti-CD36 antibody (e.g., the anti-CD36 antibodies commercially available from Invitrogen, Abcam, GeneTex, Novus Biologicals, Proteintech, and EMD Millipore), and an anti-CD26 antibody (e.g., the anti-CD26 antibodies commercially available from Abcam, Invitrogen, and GeneTex). NK cell activating agents, such as cytokine-based agents, can act by directing activating NK cells or can enhance NK cell activity, such as antibodies mediating antibody-dependent cellular cytotoxicity (ADCC) of NK cells.

Multi-Chain Chimeric Polypeptides

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 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 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 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 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 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.

5

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 ~80Å above the membrane surface. Upon FVIIa binding to tissue factor, the FVa active site is repositioned ~6Å 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 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 80Å above the membrane surface greatly reduced the ability of the tissue factor-FVIIa complex to activate FX but did not diminish tissue factor-FVIIa amidolytic activity.

Alanine scanning mutagenesis has been used to assess the role of specific amino acid side chains in the tissue factor extracellular domain for interaction with FVIIa (Gibbs et al., *Biochemistry* 33(47): 14003-14010, 1994; Schullek et al., *J Biol Chem* 269(30): 19399-19403, 1994). Alanine substitution identified a limited number of residue positions at which alanine replacements cause 5- to 10-fold lower affinity for FVIIa binding. Most of these residue side chains were found to be well-exposed to solvent in the crystal structure, concordant with macromolecular ligand interaction. The FVIIa ligand-binding site is located over an extensive region at the boundary between the two modules. In the C-module, residues Arg¹³⁵ and Phe¹⁴⁰ located on the protruding B-C loop provide an independent contact with FVIIa. Leu¹³³ is located at the base of the fingerlike structure and packed into the cleft between the two modules. This provides continuity to a major cluster of important binding residues consisting of Lys²⁰, Thr⁶⁰, Asp⁵⁸, and Ile²². Thr⁶⁰ is only partially solvent-exposed and may play a local structural role rather than making a significant contact with ligand. The binding site extends onto the concave side of the intermodule angle involving Glu²⁴ and Gln¹¹⁰, and potentially the

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

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 Gla³⁵ 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.

Soluble Tissue Factor Domain

5 In some embodiments of any of the polypeptides described herein, the soluble tissue factor domain can be a wildtype tissue factor polypeptide lacking the signal sequence, the transmembrane domain, and the intracellular domain. In some examples, the soluble tissue factor domain can be a tissue factor mutant, wherein a wildtype tissue factor polypeptide lacking the signal sequence, the transmembrane domain, and the
10 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
15 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: 8)

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTD
20 TECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQ
PTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGK
KTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE

Exemplary Nucleic Acid Encoding Soluble Human Tissue Factor Domain (SEQ ID NO: 9)

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAGCACCA
ACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTTTACACC
GTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCTATACCAC
CGACACCGAGTGCGATCTACCGATGAGATCGTGAAAGATGTGAAACAGACC
30 TACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGCACTGGTTC
CGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTACCTCGAGA

CCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGCACAAAGGT
 GAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAACAACACCTTT
 CTCAGCCTCCGGGATGTGTTTCGGCAAAGATTTAATCTACACACTGTATTACTGG
 AAGTCCTCTTCCTCCGGCAAGAAGACAGCTAAAACCAACACAAACGAGTTTT
 5 TAATCGACGTGGATAAAGGCGAAAACTACTGTTTCAGCGTGCAAGCTGTGATC
 CCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGTTGAGTGCATGG
 GCCAAGAAAAGGGCGAGTTCCGGGAG

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

10 SGTNTNTVAAYNLTWKSTNFATALEWEPKPVNQVYTVQISTKSGDWKSKCFYTT
 DTECALTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNL
 GQPTIQSFEQVGTKVNVTVEDERTLVARNNTALSLRDVFGKDLIYTLYYWKSSSS
 GKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEF
 RE

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

15 SGTNTNTVAAYNLTWKSTNFATALEWEPKPVNQVYTVQISTKSGDAKSKCFYTTD
 TECALTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLAENSPEFTPYLETNLG
 QPTIQSFEQVGTKVNVTVEDERTLVARNNTALSLRDVFGKDLIYTLYYWKSSSSG
 KKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFR
 20 E

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

agipekafnltwistdfktilwqpkptnytytvqisdrsrnwknkcfstt
 dtecdltdeivkdvtwayeakvlsvprnrsvhgdgdqlvihgepppftnap
 25 kflpyrdtnlgqpviqqfeqdgrklrvvvkdsftlvrknegtftlvrqvfgk
 dlgyiityrkgsstgkknitntnefsidveegvsycffvqamifsrktnq
 nspgsstvcetqwkstflge

Exemplary Soluble Rat Tissue Factor Domain (SEQ ID NO: 13)

30 agtpppgkafnltwistdfktilwqpkptnytytvqisdrsrnwkykctgt
 tdtecdltdeivkdvnwtyearvlsvpwrnsthgketlfgthgepppftna
 rkflpyrdtkigqpviqkyeqggtklkvtkdsftlvrknegtftlvrqvfg

ndlgyltyrkdsstgrktnththneflidvekgvsycffaqavifsrktn
hkspesitkcteqwksvlge

In some embodiments, a soluble tissue factor domain can include a sequence that
 5 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: 8, 10, 11, 12, or 13. In some
 10 embodiments, a soluble tissue factor domain can include a sequence of SEQ ID NO: 8,
 10, 11, 12, or 13, 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.

15 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.

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

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

In some examples, the soluble tissue factor domain does not include one or more (e.g., two, three, four, five, six, or seven) of: a lysine at an amino acid position that corresponds to amino acid position 20 of mature wildtype human tissue factor protein; an isoleucine at an amino acid position that corresponds to amino acid position 22 of mature wildtype human tissue factor protein; a tryptophan at an amino acid position that corresponds to amino acid position 45 of mature wildtype human tissue factor protein; an aspartic acid at an amino acid position that corresponds to amino acid position 58 of mature wildtype human tissue factor protein; a tyrosine at an amino acid position that corresponds to amino acid position 94 of mature wildtype human tissue factor protein; an arginine at an amino acid position that corresponds to amino acid position 135 of mature wildtype human tissue factor protein; and a phenylalanine at an amino acid position that corresponds to amino acid position 140 of mature wildtype human tissue factor protein. In some embodiments, the mutant soluble tissue factor possesses the amino acid sequence of SEQ ID NO: 10 or SEQ ID NO: 11.

In some examples, the soluble tissue factor domain can be encoded by a nucleic acid including a sequence that is at least 70% identical, at least 72% identical, at least 74% identical, at least 76% identical, at least 78% identical, at least 80% identical, at least 82% identical, at least 84% identical, at least 86% identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical to SEQ ID NO: 9.

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* 27(10):325–330, 2014; Priyanka et al., *Protein Sci.* 22(2):153–167, 2013. In some examples, the linker sequence is a synthetic linker sequence.

In some embodiments of any of the multi-chain chimeric polypeptides described herein, the first chimeric polypeptide 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 multi-chain chimeric polypeptides described herein, the second chimeric polypeptide 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,
about 4 amino acids to about 20 amino acids, about 4 amino acids to about 18 amino
5 acids, about 4 amino acids to about 16 amino acids, about 4 amino acids to about 14
amino acids, about 4 amino acids to about 12 amino acids, about 4 amino acids to about
10 amino acids, about 4 amino acids to about 8 amino acids, about 4 amino acids to about
6 amino acids, about 6 amino acids to about 100 amino acids, about 6 amino acids to
about 90 amino acids, about 6 amino acids to about 80 amino acids, about 6 amino acids
10 to about 70 amino acids, about 6 amino acids to about 60 amino acids, about 6 amino
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acids, about 16 amino acids to about 22 amino acids, about 16 amino acids to about 20
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about 24 amino acids, about 18 amino acids to about 22 amino acids, about 18 amino
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amino acids to about 90 amino acids, about 20 amino acids to about 80 amino acids,
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15 about 35 amino acids, about 20 amino acids to about 30 amino acids, about 20 amino
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about 22 amino acids to about 90 amino acids, about 22 amino acids to about 80 amino
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about 50 amino acids, about 25 amino acids to about 45 amino acids, about 25 amino
acids to about 40 amino acids, about 25 amino acids to about 35 amino acids, about 25
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amino acids, about 30 amino acids to about 50 amino acids, about 30 amino acids to about 45 amino acids, about 30 amino acids to about 40 amino acids, about 30 amino acids to about 35 amino acids, about 35 amino acids to about 100 amino acids, about 35 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: 14). In some embodiments, the linker sequence
5 can be encoded by a nucleic acid comprising or consisting of:

GGCGGTGGAGGATCCGGAGGAGGTGGCTCCGGCGGCGGAGGATCT (SEQ ID NO: 15). In some embodiments, the linker sequence can comprise or consist of: GGGSGGGGS (SEQ ID NO: 16).

Target-Binding Domains

10 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
15 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, one or more of the first target-binding domain (e.g., any of the
20 exemplary first target binding domains described herein or known in the art), the second target-binding domain (e.g., any of the exemplary second target binding domains described herein or known in the art), and the one or more additional target binding domains can each, independently, bind specifically to a target selected from the group of: bind specifically to a target selected from the group consisting of: CD16a, CD28, CD3
25 (e.g., one or more of CD3 α , CD3 β , CD3 δ , CD3 ϵ , and CD3 γ), CD33, CD20, CD19, CD22, CD123, IL-1R, IL-1, VEGF, IL-6R, IL-4, IL-10, PDL-1, TIGIT, PD-1, TIM3, CTLA4, MICA, MICB, IL-6, IL-8, TNF α , CD26a, CD36, ULBP2, CD30, CD200, IGF-1R, MUC4AC, MUC5AC, Trop-2, CMET, EGFR, HER1, HER2, HER3, PSMA, CEA, B7H3, EPCAM, BCMA, P-cadherin, CEACAM5, a UL16-binding protein (e.g., ULBP1,
30 ULBP2, ULBP3, ULBP4, ULBP5, and ULBP6), HLA-DR, DLL4, TYRO3, AXL, MER,

CD122, CD155, PDGF-DD, a ligand of TGF- β receptor II (TGF- β RII), a ligand of TGF- β RIII, a ligand of 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,
 5 a receptor for IL-8, a receptor for IL-10, a receptor for IL-12, a receptor for IL-15, a receptor for IL-17, a receptor for IL-18, a receptor for IL-21, a receptor for PDGF-DD, a receptor for stem cell factor (SCF), a receptor for stem cell-like tyrosine kinase 3 ligand (FLT3L), a receptor for MICA, a receptor for MICB, a receptor for a ULP16-binding protein, a receptor for CD155, a receptor for CD122, and a receptor for CD28.

10 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,
 15 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,
 20 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,
 25 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

[illegible]

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

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

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

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about 900 amino acids, about 800 amino acids to about 850 amino acids, about 850 amino acids to about 1000 amino acids, about 850 amino acids to about 950 amino acids, about 850 amino acids to about 900 amino acids, about 900 amino acids to about 1000 amino acids, about 900 amino acids to about 950 amino acids, or about 950 amino acids to about 1000 amino acids.

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

Any of the target-binding domains described herein can bind to its target with a K_D of between about 1 pM to about 30 nM (e.g., about 1 pM to about 25 nM, about 1 pM to about 20 nM, about 1 pM to about 15 nM, about 1 pM to about 10 nM, about 1 pM to about 5 nM, about 1 pM to about 2 nM, about 1 pM to about 1 nM, about 1 pM to about 950 pM, about 1 pM to about 900 pM, about 1 pM to about 850 pM, about 1 pM to about 800 pM, about 1 pM to about 750 pM, about 1 pM to about 700 pM, about 1 pM to about 650 pM, about 1 pM to about 600 pM, about 1 pM to about 550 pM, about 1 pM to about 500 pM, about 1 pM to about 450 pM, about 1 pM to about 400 pM, about 1 pM to about 350 pM, about 1 pM to about 300 pM, about 1 pM to about 250 pM, about 1 pM to about 200 pM, about 1 pM to about 150 pM, about 1 pM to about 100 pM, about 1 pM to about 90 pM, about 1 pM to about 80 pM, about 1 pM to about 70 pM, about 1 pM to about 60 pM, about 1 pM to about 50 pM, about 1 pM to about 40 pM, about 1 pM to about 30 pM, about 1 pM to about 20 pM, about 1 pM to about 10 pM, about 1 pM to about 5 pM, about 1 pM to about 4 pM, about 1 pM to about 3 pM, about 1 pM to about 2 pM, about 2 pM to about 30 nM, about 2 pM to about 25 nM, about 2 pM to about 20 nM, about 2 pM to about 15 nM, about 2 pM to about 10 nM, about 2 pM to about 5 nM, about 2 pM to about 2 nM, about 2 pM to about 1 nM, about 2 pM to about 950 pM, about 2 pM to about 900 pM, about 2 pM to about 850 pM, about 2 pM to about 800 pM, about 2 pM to

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

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

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

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

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

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

pM to about 30 nM, about 900 pM to about 15 nM, about 900 pM to about 10 nM, about 900 pM to about 5 nM, about 900 pM to about 2 nM, about 900 pM to about 1 nM, about 900 pM to about 950 pM, about 1 nM to about 30 nM, about 1 nM to about 25 nM, about 1 nM to about 20 nM, about 1 nM to about 15 nM, about 1 nM to about 10 nM, about 1 nM to about 5 nM, about 2 nM to about 30 nM, about 2 nM to about 25 nM, about 2 nM to about 20 nM, about 2 nM to about 15 nM, about 2 nM to about 10 nM, about 2 nM to about 5 nM, about 4 nM to about 30 nM, about 4 nM to about 25 nM, about 4 nM to about 20 nM, about 4 nM to about 15 nM, about 4 nM to about 10 nM, about 4 nM to about 5 nM, about 5 nM to about 30 nM, about 5 nM to about 25 nM, about 5 nM to about 20 nM, about 5 nM to about 15 nM, about 5 nM to about 10 nM, about 10 nM to about 30 nM, about 10 nM to about 25 nM, about 10 nM to about 20 nM, about 10 nM to about 15 nM, about 15 nM to about 30 nM, about 15 nM to about 25 nM, about 15 nM to about 20 nM, about 20 nM to about 30 nM, and about 20 nM to about 25 nM).

Any of the target-binding domains described herein can bind to its target with a K_D of between about 1 nM to about 10 nM (e.g., about 1 nM to about 9 nM, about 1 nM to about 8 nM, about 1 nM to about 7 nM, about 1 nM to about 6 nM, about 1 nM to about 5 nM, about 1 nM to about 4 nM, about 1 nM to about 3 nM, about 1 nM to about 2 nM, about 2 nM to about 10 nM, about 2 nM to about 9 nM, about 2 nM to about 8 nM, about 2 nM to about 7 nM, about 2 nM to about 6 nM, about 2 nM to about 5 nM, about 2 nM to about 4 nM, about 2 nM to about 3 nM, about 3 nM to about 10 nM, about 3 nM to about 9 nM, about 3 nM to about 8 nM, about 3 nM to about 7 nM, about 3 nM to about 6 nM, about 3 nM to about 5 nM, about 3 nM to about 4 nM, about 4 nM to about 10 nM, about 4 nM to about 9 nM, about 4 nM to about 8 nM, about 4 nM to about 7 nM, about 4 nM to about 6 nM, about 4 nM to about 5 nM, about 5 nM to about 10 nM, about 5 nM to about 9 nM, about 5 nM to about 8 nM, about 5 nM to about 7 nM, about 5 nM to about 6 nM, about 6 nM to about 10 nM, about 6 nM to about 9 nM, about 6 nM to about 8 nM, about 6 nM to about 7 nM, about 7 nM to about 10 nM, about 7 nM to about 9 nM, about 7 nM to about 8 nM, about 8 nM to about 10 nM, about 8 nM to about 9 nM, and about 9 nM to about 10 nM).

A variety of different methods known in the art can be used to determine the K_D values of any of the polypeptides 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

5 In some embodiments of any of the single- 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 single- 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
10 single- or multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain include the same amino acid sequence.

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

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

20 In some embodiments of any of the single- or multi-chain chimeric polypeptides described herein, the antigen-binding domain includes or is a scFv or a single domain antibody (e.g., a VHH or a VNAR 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
25 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 (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
30 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

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), CD26a (see, e.g., those described in WO 2017/189526), CD36 (see, e.g., those described in U.S. Patent Application Publication No. 2015/0259429), ULBP2 (see, e.g., those described in U.S. Patent No. 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
 5 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).

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

15 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 that can be found in cartilaginous fish. Non-limiting aspects of VHH domains and VNAR 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
 20 Biotechnol.* 32:263-270, 2014; Kijanka et al., *Nanomedicine* 10:161-174, 2015; Kovaleva et al., *Expert. Opin. Biol. Ther.* 14:1527-1539, 2014; Krah et al., *Immunopharmacol. Immunotoxicol.* 38:21-28, 2016; Mujic-Delic et al., *Trends Pharmacol. Sci.* 35:247-255, 2014; Muyldermans, *J. Biotechnol.* 74:277-302, 2001; Muyldermans et al., *Trends Biochem. Sci.* 26:230-235, 2001; Muyldermans, *Ann. Rev. Biochem.* 82:775-797, 2013;
 25 Rahbarizadeh et al., *Immunol. Invest.* 40:299-338, 2011; Van Audenhove et al., *EBioMedicine* 8:40-48, 2016; Van Bockstaele et al., *Curr. Opin. Investig. Drugs* 10:1212-1224, 2009; Vincke et al., *Methods Mol. Biol.* 911:15-26, 2012; and Wesolowski et al., *Med. Microbiol. Immunol.* 198:157-174, 2009.

In some embodiments, each of the antigen-binding domains in the single- or
 30 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

antigen-binding domains in the single- 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- 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- 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, Zybody, 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 lmmTAC, 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.

5 A “Fab” fragment includes, the constant domain of the light chain and the first constant domain (C_{H1}) of the heavy chain, in addition to the heavy and light chain variable domains of the Fv fragment.

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

10 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. 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.

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

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

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

Soluble Interleukin or Cytokine Protein

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

30

Human Soluble IL-2 (SEQ ID NO: 17)

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

5

Human Soluble IL-3 (SEQ ID NO: 18)

apmtqttplkt swvnscnmid eiithlkqpp lplldfnnln gedqdilmen
nlrrpnleaf nravkslqna saiesilkn lpcplataa ptrhpihikd
gdwnefrrkl tfylktlena qaqqttlsia if

10

Human Soluble IL-7 (SEQ ID NO: 19)

dcdiegkdgkqyesv lmvsidqlld smkeigsnc l nnefnffkrh icdankegmf
lfraarklrq flkmnstgdf dlhllkvseg ttillnctgq vkgrkpaalg
eaqptkslee nkslkeqkkl ndlcflkrll qeiktcwnki lmgtkch

15

Human Soluble IL-8 (SEQ ID NO: 20)

egavlprsak elrcqciqty skpfhpkfik elrviesgph canteiivkl
sdgrelcldp kenwvqrvve kflkraens

Human Soluble IL-10 (SEQ ID NO: 21)

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

25

Human Soluble IL-15 (SEQ ID NO: 22)

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

30

Human Soluble IL-17 (SEQ ID NO: 23)

gitiprn pgcpnsedkn fprtvmvnlh ihnrtntnnp krssdyynrs
 tspwnlhrne dperypsviw eakcrhlhci nadgnvdyhm nsvpiqqeil
 vlrrepphpcp nsfrlekilv svgctcvtpi vhhva

5

Human Soluble IL-18 (SEQ ID NO: 24)

yfgklesklsvirn lndqvlfidq gnrplfedmt dsdcrdnapr tifiismykd
 sqprgmavti svkcekistl scenkiisfk emnppdnikd tksdiiffqr
 svpgghdnkmq fesssyegyf lacekerdlf klilkkedel gdrsimftvq ned

10

Human Soluble PDGF-DD (SEQ ID NO: 25)

rdtsatpqsasi kalrnanlrr desnhltdly rrdetiqvkg ngyvqsprfp
 nsyprnlllt wrlhsqentr iqlvfdnqfg leeaendicr ydfvevedis
 etstiirgrw cghkevppri ksrtngikit fksddyfvak pgfkiyysll
 edfqpaaase tnwesvtssi sgvsynspsv tdptliadal dkkiaefdtv
 edllkyfnpe swqedlenmy ldtpyrgrs yhdrkskvdL drlnddakry
 sctprnysvn ireelklanv vffprcllvq rcggncgcgt vnwrscctns
 gktvkkkyhev lqfepghikr rgraktmalv diqldhherc dcicssrppr

15

Human Soluble SCF (SEQ ID NO: 26)

egicrnrvtnnvkdv tkivanlpkd ymitlkyvpg mdvlpshcwi semvvqlsds
 ltdlldkfsn iseglsnysi idklvnivdd lvecvkenss kdlkksfksp
 eprlftpeef frifnrside fkdffvaset sdcvvsstls pekdsrvsvt
 kpfmlppvaa sslrndssss nrkaknppgd sslhwaamal palfsliigf
 afgalywkk rpsltraven iqineednei smlqekeref gev

25

Human Soluble FLT3L (SEQ ID NO: 27)

tqdcsfqhspissd favkirelsd yllqdyptv asnlqdealc gglwrlvlaq
 rwmerlktva gskmqgller vnteihfvtk cafqpppscl rfvqtnisrl
 lqetseqqlva lkpwithrnf srclclqcqp dsstlpppws prpleatapt
 apqpplllll llpvglllla aawclhwqrt rrrtprpgeq vppvpspql
 llveh

30

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

Human Soluble MICA (SEQ ID NO: 28)

5 ephslry nltvlswdgs vqsgfltevh ldgqpflrce rpkcrakpqq
 qwaedvlgk twdretldt gngkdlrmtl ahikdqkegl hslqeirvce
 ihednstrss qhfyydgelf lsqnletkew tmpqssraqt lamnvrnflk
 edamkthkthy hamhadclqe lrrylksgvv lrrtvppmvn vtrseasegn
 itvtcrasgf ypwnitlswr qdgvslshdt qqwgdvlpdg ngtyqtwvat
 10 ricqgeeqrq tcymehsgnh sthpvpqgkv lvlqshwqtf hvsavaaaai
 fviiifyvrc ckkktsaaeg pelvslqvld qhpvgtsdhr datqlgfqpl
 msdlgstgst ega

Human Soluble MICB (SEQ ID NO: 29)

15 aephslry nlmvlsqdes vqsgflaegh ldgqpflryd rpkrrakpqq
 qwaedvlgak twdtetedlt engqdlrrtl thikdqkggl hslqeirvce
 ihedsstrgs rhfyydgelf lsqnletqes tvpqssraqt lamnvtnfwk
 edamkthkthy ramqadclqk lqrylksgva irrtpppmvn vtcsevsegn
 itvtcrassf yprnitltwr qdgvslshnt qqwgdvlpdg ngtyqtwvat
 20 rirqgeeqrq tcymehsgnh gthpvpsgkv lvlqsqrtdf pyvsaampcf
 viiiilcvpc ckkktsaaeg pelvslqvld qhpvgtdghr
 daaqlgfqpl msatgstgst ega

Human Soluble ULBP1 (SEQ ID NO: 30)

25 wvdthclcydfiit pksrpepqwc evqglvderp flhydcvnhk akafaslgkk
 vnvtktweeq tetlrdvddf lkgqlldiqv enlipieplt lqarmscehe
 ahghgrgswq flfngqkflf fdsnnrkwtal lhpgakkmte kweknrdvtm
 ffqkislqdc kmwleeflmy weqmlcptkp pslapq

Human Soluble ULBP2 (SEQ ID NO: 31)

gradphslcyditvi pkfrpgprwc avqqqvdekt flhydcgnkt vtpvsplgkk
 lnvttawkaq npvlrevvdi lteqlrldiql enytpkeplt lqarmsceqk
 aeghssgswq fsfdgqifll fdsekrmwtt vhpqarkmke kwendkvvam
 5 sfhyfsmgdc igwledflmg mdstlepszag aplams

Human Soluble ULBP3 (SEQ ID NO: 32)

dahslwynfti ihlprhgqqw cevqsqvdk nflsydcgsd kvlsmghee
 qlyatdawgk qlemlrevgq rlrleladte ledftpsgpl tlqvrmscec
 10 eadgyirgsw qfsfdgrkfl lfdsnnrkwt vvhagarmk ekwekdsglt
 tffkmvsmrd ckswlrdflm hrkkrlpta pptmapg

Human Soluble ULBP4 (SEQ ID NO: 33)

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

Human Soluble ULBP5 (SEQ ID NO: 34)

gladp hslcyditvi pkfrpgprwc avqqqvdekt flhydcgskt
 vtpvsplgkk lnvttawkaq npvlrevvdi lteqlldiql enyipkeplt
 lqarmsceqk aeghgsgswq lsfdgqifll fdsenrmwtt vhpqarkmke
 kwendkdmtm sfhyismgdc tgwledflmg mdstlepszag apptmssg
 25

Human Soluble ULBP6 (SEQ ID NO: 35)

rrddp hslcyditvi pkfrpgprwc avqqqvdekt flhydcgnkt
 vtpvsplgkk lnvttawkaq npvlrevvdi lteqlldiql enytpkeplt
 lqarmsceqk aeghssgswq fsidgqtfll fdsekrmwtt vhpqarkmke
 30 kwendkdvam sfhyismgdc igwledflmg mdstlepszag aplamssg

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

Soluble Receptor

5 In some embodiments of any of the single- or 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 receptor, a soluble cytokine receptor or a ligand receptor. In some embodiments, the soluble receptor is a soluble TGF- β receptor II (TGF- β RII) (see, e.g., those described in Yung et al., *Am. J. Resp. Crit. Care Med.* 194(9):1140-1151, 2016), a soluble TGF- β RIII (see, e.g., those described in Heng et al., 10 *Placenta* 57:320, 2017), a soluble NKG2D (see, e.g., Cosman et al., *Immunity* 14(2):123-133, 2001; Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150), a soluble NKp30 (see, e.g., Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150), a soluble NKp44 15 (see, e.g., those described in Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150), a soluble NKp46 (see, e.g., Mandelboim et al., *Nature* 409:1055-1060, 2001; Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150), a soluble DNAM-1 (see, e.g., those described in Costa et al., *Front. Immunol.*, Vol. 9, Article 1150, May 29, 2018; doi: 10.3389/fimmu.2018.01150), a scMHCI (see, e.g., those described in Washburn et al., 20 *PLoS One* 6(3):e18439, 2011), a scMHCII (see, e.g., those described in Bishwajit et al., *Cellular Immunol.* 170(1):25-33, 1996), a scTCR (see, e.g., those described in Weber et al., *Nature* 356(6372):793-796, 1992), a soluble CD155 (see, e.g., those described in Tahara-Hanaoka et al., *Int. Immunol.* 16(4):533-538, 2004), or a soluble CD28 (see, e.g., 25 Hebbbar et al., *Clin. Exp. Immunol.* 136:388-392, 2004).

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

Additional Antigen-Binding Domains

30 In some embodiments of any of the single- or 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). In some embodiments of any of the multi-chain chimeric polypeptides, at least one of the one or more additional antigen-binding domain(s) can be 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).

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 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 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 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 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 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 or any of the exemplary pairs of affinity domains described herein) in the first chimeric polypeptide. In some

5 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

10 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

15 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 or any of the exemplary pairs of affinity domains described herein) in the first chimeric polypeptide. In some embodiments, the at least one of the one or more

20 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 any of the exemplary pairs of affinity domains described herein), directly abuts the

25 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 tissue factor domains described herein) and the at least one of the one or more additional

30 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 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 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 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
5 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
10 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
15 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
20 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
25 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 target-binding domains is an antigen-binding domain. In some embodiments, the first target-binding domain, the second
30 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).

Pairs of Affinity Domains

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 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 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 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 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).

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 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} 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 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 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 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
ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAH

WTTPSLKCIR (SEQ ID NO: 36). In some embodiments, a sushi domain from an alpha chain of IL15R α can be encoded by a nucleic acid including

ATTACATGCCCCCTCCCATGAGCGTGGAGCACGCCGACATCTGGGTGAAGAG
CTATAGCCTCTACAGCCGGGAGAGGTATATCTGTAACAGCGGCTTCAAGAGGA
5 AGGCCGGCACCAGCAGCCTCACCGAGTGCGTGCTGAATAAGGCTACCAACGT
GGCTCACTGGACAACACCCTCTTTAAAGTGCATCCGG (SEQ ID NO: 37).

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

10 NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLLELQVISLESGD
ASIHDTVENLILANNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINT
S (SEQ ID NO: 22). In some embodiments, a soluble IL-15 can be encoded by a nucleic acid including the sequence of

AACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATTTAATTCAGTC
15 CATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTCTTGTA
GGTGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAGAG
CGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTTAGCCAATA
ACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAGGAGTGCGA
AGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTGTGCACATTG
20 TCCAGATGTTTCATCAATACCTCC (SEQ ID NO: 38).

Signal Sequence

In some embodiments, a multi-chain chimeric polypeptide includes a first chimeric polypeptide that includes a signal sequence at its N-terminal end. In some
25 embodiments, a multi-chain chimeric polypeptide includes a second chimeric polypeptide that includes a signal sequence at its N-terminal end. In some embodiments, both the first chimeric polypeptide of a multi-chain chimeric polypeptide and a second chimeric polypeptide of the multi-chain chimeric polypeptide include a signal sequence. As will be understood by those of ordinary skill in the art, a signal sequence is an amino acid
30 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 first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both includes a signal sequence having an amino acid sequence

MKWVTFISLLFLFSSAYS (SEQ ID NO: 39). In some embodiments, a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both includes a signal sequence encoded by the nucleic acid sequence:

ATGAAATGGGTGACCTTTATTTCTTTACTGTTTCCTCTTTAGCAGCGCCTACTCC

(SEQ ID NO: 40),

ATGAAGTGGGTCACATTTATCTCTTTACTGTTTCCTCTTCTCCAGCGCCTACAGC (SEQ ID NO: 41), or

ATGAAATGGGTGACCTTTATTTCTTTACTGTTTCCTCTTTAGCAGCGCCTACTCC (SEQ ID NO: 42).

In some embodiments, a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both includes a signal sequence having an amino acid sequence

MKCLLYLAFLFLGVNC (SEQ ID NO: 43). In some embodiments, a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both includes a signal sequence having an amino acid sequence

MGQIVTMFEALPHIIDEVINIVIIIVLIITSIKAVYNFATCGILALVSFLFLAGRSCG (SEQ ID NO: 44). In some embodiments, a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric

polypeptide, or both includes a signal sequence having an amino acid sequence

MPNHQSGSPTGSSDLLLSGKKQRPHLALRRKRRREMRKINRKVRRMNLAPIKEK

TAWQHLQALISEAEEVLKTSQTPQNSLTLFLALLSVLGPPVTG (SEQ ID NO: 45).

In some embodiments, a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both includes a signal sequence having an amino acid sequence

- 5 MDSKGSSQKGSRLLLLLLVSNLLLCQGVVS (SEQ ID NO: 46). Those of ordinary skill in the art will be aware of other appropriate signal sequences for use in a first chimeric polypeptide and/or a second chimeric polypeptide of multi-chain chimeric polypeptides described herein.

In some embodiments, a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both includes a signal sequence that is about 10 to 100 amino acids in length. For example, a signal sequence can be about 10 to 100 amino acids in length, about 15 to 100 amino acids in length, about 20 to 100 amino acids in length, about 25 to 100 amino acids in length, about 30 to 100 amino acids in length, about 35 to 100 amino acids in length, about 40 to 100 amino 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.

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

MKCLLYLAFLFLGVNC (SEQ ID NO: 43) can include one or more additional amino acids at the N-terminus or C-terminus, while still retaining the ability to direct a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both to the secretory pathway.

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

Peptide Tags

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

Exemplary peptide tags that can be included in a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both include, without limitation, AviTag

(GLNDIFEAQKIEWHE; SEQ ID NO: 47), a calmodulin-tag (KRRWKKNFIAVSAANRFKKISSSGAL; SEQ ID NO: 48), a polyglutamate tag (EEEEEE; SEQ ID NO: 49), an E-tag (GAPVPYPDPLEPR; SEQ ID NO: 50), a FLAG-tag (DYKDDDDDK; SEQ ID NO: 51), an HA-tag, a peptide from hemagglutinin (YPYDVPDYA; SEQ ID NO: 52), a his-tag (HHHHH (SEQ ID NO: 53); HHHHHH (SEQ ID NO: 54); HHHHHHH (SEQ ID NO: 55); HHHHHHHH (SEQ ID NO: 56); HHHHHHHHH (SEQ ID NO: 57); or HHHHHHHHHH (SEQ ID NO: 58)), a myc-tag (EQKLISEEDL; SEQ ID NO: 59), NE-tag (TKENPRSNQEESYDDNES; SEQ ID NO: 60), S-tag, (KETAAAKFERQHMDS; SEQ ID NO: 61), SBP-tag (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP; SEQ ID NO: 62), Softag 1 (SLAELLNAGLGGS; SEQ ID NO: 63), Softag 3 (TQDPSRVG; SEQ ID NO: 64), Spot-tag (PDRVRAVSHWSS; SEQ ID NO: 65), Strep-tag (WSHPQFEK; SEQ ID NO: 66), TC tag (CCPGCC; SEQ ID NO: 67), Ty tag (EVHTNQDPLD; SEQ ID NO: 68), V5 tag (GKPIPNPLLGLDST; SEQ ID NO: 69), VSV-tag (YTDIEMNRLGK; SEQ ID NO: 70), and Xpress tag (DLYDDDDDK; SEQ ID NO: 71). In some embodiments, tissue factor protein is a peptide tag.

Peptide tags that can be included in a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both can be used in any of a variety of applications related to the multi-chain chimeric polypeptide. For example, a peptide tag can be used in the purification of a multi-chain chimeric polypeptide. As one non-limiting example, a first chimeric polypeptide of a multi-chain chimeric polypeptide (e.g., a recombinantly expressed first chimeric polypeptide), a second chimeric polypeptide of the multi-chain chimeric polypeptide (e.g., a recombinantly expressed second chimeric polypeptide), or both can include a myc tag; the multi-chain chimeric polypeptide that includes the myc-tagged first chimeric polypeptide, the myc-tagged second chimeric polypeptide, or both 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 first chimeric polypeptide of a multi-chain chimeric polypeptide (e.g., a recombinantly expressed first chimeric polypeptide), a second chimeric polypeptide of the multi-chain

chimeric polypeptide (e.g., a recombinantly expressed second chimeric polypeptide), or both can include a histidine tag; the multi-chain chimeric polypeptide that includes the histidine-tagged first chimeric polypeptide, the histidine-tagged second chimeric polypeptide, or both 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 multi-chain chimeric polypeptide. In some embodiments, a peptide tag is removed from the first chimeric polypeptide and/or the second chimeric polypeptide of the multi-chain chimeric polypeptide after purification. In some embodiments, a peptide tag is not removed from the first chimeric polypeptide and/or the second chimeric polypeptide of the multi-chain chimeric polypeptide after purification.

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

In some embodiments, a first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both includes a peptide tag that is about 10 to 100 amino acids in length. For example, a peptide tag can be about 10 to 100 amino acids in length, about 15 to 100 amino acids in length, about 20 to 100 amino acids in length, about 25 to 100 amino acids in length, about 30 to 100 amino acids 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 first chimeric polypeptide of a multi-chain chimeric polypeptide, a second chimeric polypeptide of the multi-chain chimeric polypeptide, or both 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 multi-chain 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: 59) 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).

Exemplary Multi-Chain Chimeric Polypeptides- Type A

In some embodiments of any of the multi-chain chimeric polypeptides described herein, the first target-binding domain and the second targeting-binding domain each independently bind specifically to a receptor of IL-18 or a receptor of IL-12. In some examples of these multi-chain chimeric polypeptides, the first target-binding domain and the soluble tissue factor domain directly abut each other in the first chimeric polypeptide. In some examples of these multi-chain chimeric polypeptides, the first chimeric

polypeptide further comprises a linker sequence (e.g., any of the exemplary linkers described herein) between the first target-binding domain and the soluble tissue factor domain in the first chimeric polypeptide.

In some embodiments of these multi-chain chimeric polypeptides, the second
5 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 these multi-chain chimeric polypeptides, the second chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linkers described herein) between the second domain of the pair of affinity domains and the second target-binding domain in the
10 second chimeric polypeptide.

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

15 In some embodiments of these multi-chain chimeric polypeptides, one or both of the first target-binding domain and the second target-binding domain is an agonistic antigen-binding domain. In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain are each agonistic antigen-binding domains. In some embodiments of these multi-chain
20 chimeric polypeptides, the antigen-binding domain includes a scFv or single-domain antibody.

In some embodiments of these multi-chain chimeric polypeptides, one or both of the first target-binding domain and the second target-binding domain is a soluble IL-15 or a soluble IL-18. In some embodiments of these multi-chain chimeric polypeptides, the
25 first target-binding domain and the second target-binding domain are each independently a soluble IL-15 or a soluble IL-18. In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain both bind specifically to a receptor of IL-18 or a receptor of IL-12. In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain and the second
30 target-binding domain bind specifically to the same epitope. In some embodiments of

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

In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain binds specifically to a receptor for IL-12, and the second target-binding domain binds specifically to a receptor for IL-18. In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain binds specifically to a receptor for IL-18, and the second target-binding domain bind specifically to a receptor for IL-12.

In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain includes a soluble IL-18 (e.g., a soluble human IL-18).

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-18 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:

YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISMYKDSQ
PRGMAVTISVKCEKISTLSCENKIISFKEMNPPDNIKDTKSDIIFQRSVPGHDNKM
QFESSYEGYFLACEKERDLFKLILKKEDELGDRSIMFTVQNE (SEQ ID NO: 72).

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-18 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:

TACTTCGGCAAACCTGGAATCCAAGCTGAGCGTGATCCGGAATTTAAACGACC
AAGTTCTGTTTATCGATCAAGGTAACCGGCCTCTGTTCGAGGACATGACCGAC
TCCGATTGCCGGGACAATGCCCCCGGACCATCTTCATTATCTCCATGTACAA
GGACAGCCAGCCCCGGGGCATGGCTGTGACAATTAGCGTGAAGTGTGAGAA
AATCAGCACTTTATCTTGTGAGAACAAGATCATCTCCTTTAAGGAAATGAACC
CCCCGATAACATCAAGGACACCAAGTCCGATATCATCTTCTTCCAGCGGTCC
GTGCCCGGTCACGATAACAAGATGCAGTTCGAATCCTCCTCCTACGAGGGCT
ACTTTTATAGCTTGTGAAAAGGAGAGGGATTTATTCAAGCTGATCCTCAAGAA
GGAGGACGAGCTGGGCGATCGTTCATCATGTTACCGTCCAAAACGAGGAT
(SEQ ID NO: 73).

In some embodiments of these multi-chain chimeric polypeptides, the second target-binding domain includes a soluble IL-12 (e.g., a soluble human IL-12). In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-15 includes a sequence of soluble human IL-12 β (p40) and a sequence of soluble human IL-12 α (p35). In some embodiments of these multi-chain chimeric polypeptides, the soluble IL-15 human IL-15 further includes a linker sequence (e.g., any of the exemplary linker sequences described herein) between the sequence of soluble IL-12 β (p40) and the sequence of soluble human IL-12 α (p35). In some examples of these multi-chain chimeric polypeptides, the linker sequence comprises GGGSGGGSGGGGS (SEQ ID NO: 14).

In some embodiments of these multi-chain chimeric polypeptides, the sequence of soluble human IL-12 β (p40) 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:

IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLT
IQVKEFGDAGQYTCHKGGVLSHSLLLHKKEDGIWSTDILKDQKEPKNKTFRL
CEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERVGRDN
KEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIIKPDPPKN
LQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKT
SATVICRKNASISVRAQDRYSSSWSEWASVPCS (SEQ ID NO: 74).

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-12 β (p40) 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:

ATTTGGGAAGTGAAGAAGGACGTCTACGTGGTCGAACTGGACTGGTATCCCG
ATGCTCCCGGCGAAATGGTGGTGCTCACTTGTGACACCCCGAAGAAGACGG
CATCACTTGGACCTCGATCAGAGCAGCGAGGTGCTGGGCTCCGGAAAGACC
CTCACAATCCAAGTTAAGGAGTTCGGAGACGCTGGCCAATACACATGCCACA
AGGGAGGCGAGGTGCTCAGCCATTCTTATTATTATTACACAAGAAGGAAGA
CGGAATCTGGTCCACCGACATTTTAAAAGATCAGAAGGAGCCCAAGAATAAG
ACCTTTTAAAGGTGTGAGGCCAAAACTACAGCGGTCGTTTCACTTGTGTTGGTG
GCTGACCACCATTTCCACCGATTTAACCTTCTCCGTGAAAAGCAGCCGGGGA
AGCTCCGACCTCAAGGTGTGACATGTGGAGCCGCTACCTCAGCGCTGAGA

GGGTTCGTGGCGATAACAAGGAATACGAGTACAGCGTGGAGTGCCAAGAAG
 ATAGCGCTTGTCCCGCTGCCGAAGAATCTTTACCCATTGAGGTGATGGTGGAC
 GCCGTGCACAACTCAAGTACGAGAACTACACCTCCTCCTTCTTTATCCGGGA
 CATCATTAAGCCCGATCCTCCTAAGAATTTACAGCTGAAGCCTCTCAAAAATA
 5 GCCGGCAAGTTGAGGTCTCTTGGGAATATCCCGACACTTGGAGCACACCCCA
 CAGCTACTTCTCTTTAACCTTTTGTGTGCAAGTTCAAGGTAAAAGCAAGCGGG
 AGAAGAAAGACCGGGTGTTCACCGACAAAACCAGCGCCACCGTCATCTGTCTG
 GAAGAACGCCTCCATCAGCGTGAGGGCTCAAGATCGTTATTACTCCAGCAGC
 TGGTCCGAGTGGGCCAGCGTGCCTTGTTC (SEQ ID NO: 75).

10 In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-12 α (p35) 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:

15 RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPTSEEIDHEDITKD
 KTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSYEDLKM
 YQVEFKTMNAKLLMDPKRQIFLDQNMMLAVIDELMQALNFNSETVPQKSSLEEPD
 FYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS (SEQ ID NO: 76).

In some embodiments of these multi-chain chimeric polypeptides, the soluble
 20 human IL-12 α (p35) 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:

CGTAACCTCCCCGTGGCTACCCCCGATCCCGGAATGTTCCCTTGTTTACACCA
 25 CAGCCAGAATTTACTGAGGGCCGTGAGCAACATGCTGCAGAAAGCTAGGCAG
 ACTTTAGAATTTTACCCTTGCACCAGCGAGGAGATCGACCATGAAGATATCA
 CCAAGGACAAGACATCCACCGTGGAGGCTTGTTTACCTCTGGAGCTGACAAA
 GAACGAGTCTTGTCTCAACTCTCGTGAAACCAGCTTCATCACAATGGCTCTT
 GTTTAGCTTCCCGGAAGACCTCCTTTATGATGGCTTTATGCCTCAGCTCCATCT
 30 ACGAGGATTTAAAGATGTACCAAGTGGAGTTCAAGACCATGAACGCCAAGCT
 GCTCATGGACCCTAAACGGCAGATCTTTTACAGCAGAACATGCTGGCTGTG
 ATTGATGAGCTGATGCAAGCTTTAACTTCAACTCCGAGACCGTCCCTCAGA
 AGTCCCTCCCTCGAGGAGCCCGATTTTACAAGACAAAGATCAAACCTGTGCAT
 TTTACTCCACGCCTTTAGGATCCGGGCCGTGACCATTGACCGGGTCATGAGCT
 35 ATTTAAACGCCAGC (SEQ ID NO: 77).

In some embodiments, the first chimeric polypeptide can include a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86%

identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

5 YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISMYKDSQ
 PRGMAVTISVKCEKISTLSCENKIISFKEMNPPDNIKDTKSDIIFQRSVPGHDKM
 QFESSYEGYFLACEKERDLFKLILKKEDELGDRSIMFTVQNEDSGTTNTVAAYN
 LTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKD
 VKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGT
 KVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEF
 10 LIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRENWVNVISDLK
 KIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLESGDASIHDVTENLI
 ILANNSLSSNGNVTESGCKECELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO:
 78).

In some embodiments, a first chimeric polypeptide is encoded by a sequence that
 15 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:

TACTTCGGCAAACCTGGAATCCAAGCTGAGCGTGATCCGGAATTTAAACGACC
 20 AAGTTCTGTTTATCGATCAAGGTAACCGGCCTCTGTTCGAGGACATGACCGAC
 TCCGATTGCCGGGACAATGCCCCCGGACCATCTTCATTATCTCCATGTACAA
 GGACAGCCAGCCCCGGGGCATGGCTGTGACAATTAGCGTGAAGTGTGAGAA
 AATCAGCACTTTATCTTGTGAGAACAAGATCATCTCCTTTAAGGAAATGAACC
 CCCCCGATAACATCAAGGACACCAAGTCCGATATCATCTTCTTCCAGCGGTCC
 25 GTGCCCCGTACGATAACAAGATGCAGTTCGAATCCTCCTCCTACGAGGGCT
 ACTTTTATAGCTTGTGAAAAGGAGAGGGATTTATTCAAGCTGATCCTCAAGAA
 GGAGGACGAGCTGGGCGATCGTTCCATCATGTTACCGTCCAAAACGAGGAT
 AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAGCACCA
 ACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTTTACAC
 30 CGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCTATACC
 ACCGACACCGAGTGCGATCTCACCGATGAGATCGTGAAAGATGTGAAACAGA
 CCTACCTCGCCCGGGTGTGTTAGCTACCCCGCCGGCAATGTGGAGAGCACTGG
 TTCCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTACCTCG
 AGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGCACAAA
 35 GGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAACAACAC
 CTTTCTCAGCCTCCGGGATGTGTTCTGGCAAAGATTTAATCTACACACTGTATT
 ACTGGAAGTCCTCTTCTCCGGCAAGAAGACAGCTAAAACCAACACAAACGA
 GTTTTTAATCGACGTGGATAAAGGCGAAACTACTGTTTCAGCGTGCAAGCT
 GTGATCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGTTGAGT

GCATGGGCCAAGAAAAGGGCGAGTTCCGGGAGAACTGGGTGAACGTCATCA
 GCGATTTAAAGAAGATCGAAGATTTAATTCAGTCCATGCATATCGACGCCAC
 TTTATACACAGAATCCGACGTGCACCCCTCTTGTAAGGTGACCGCCATGAAAT
 GTTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAGAGCGGAGACGCTAGCATC
 5 CACGACACCGTGGAGAATTTAATCATTTTAGCCAATAACTCTTTATCCAGCAA
 CGGCAACGTGACAGAGTCCGGCTGCAAGGAGTGCGAAGAGCTGGAGGAGAA
 GAACATCAAGGAGTTTCTGCAATCCTTTGTGCACATTGTCCAGATGTTTCATCA
 ATACCTCC (SEQ ID NO: 79).

In some embodiments, a first chimeric polypeptide can include a sequence that is
 10 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:

MKWVTFISLLFLFSSAYSIFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSD
 15 CRDNAPRTIFIISMYKDSQPRGMAVTISVKCEKISTLSCENKIISFKEMNPPDNIKD
 TKSDIIFQRSVPGHDNKMQFESSYEGYFLACEKERDLFKLILKKEDELGDRSIM
 FTVQNEDSGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKS
 KCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTP
 YLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYY
 20 WKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRVTNRKSTDSPVECMG
 QEKGEFRENWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLLEL
 QVISLESGDASIHTVENLIILANNSLSSNGNVTESGCKECELEEKNIKEFLQSFV
 HIVQMFINTS (SEQ ID NO: 80).

In some embodiments, a first chimeric polypeptide is encoded by a sequence that
 25 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:

ATGAAGTGGGTCACATTTATCTCTTTACTGTTCTCTTCTCCAGCGCCTACAGC
 30 TACTTCGGCAAACCTGGAATCCAAGCTGAGCGTGATCCGGAATTTAAACGACC
 AAGTTCTGTTTATCGATCAAGGTAACCGGCCTCTGTTCGAGGACATGACCGAC
 TCCGATTGCCGGGACAATGCCCCCGGACCATCTTCATTATCTCCATGTACAA
 GGACAGCCAGCCCCGGGGCATGGCTGTGACAATTAGCGTGAAGTGTGAGAA
 AATCAGCACTTTATCTTGTGAGAACAAAGATCATCTCCTTTAAGGAAATGAACC
 35 CCCCCGATAACATCAAGGACACCAAGTCCGATATCATCTTCTTCCAGCGGTCC
 GTGCCCCGGTCACGATAACAAGATGCAGTTCGAATCCTCCTCCTACGAGGGCT
 ACTTTTTAGCTTGTGAAAAGGAGAGGGATTTATTCAAGCTGATCCTCAAGAA
 GGAGGACGAGCTGGGCGATCGTTCCATCATGTTACCGTCCAAAACGAGGAT

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAGCACCA
 ACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTTTACAC
 CGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCTATACC
 ACCGACACCGAGTGCGATCTCACCGATGAGATCGTGAAAGATGTGAAACAGA
 5 CCTACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGCACTGG
 TTCCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTACCTCG
 AGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGCACAAA
 GGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAACAACAC
 CTTTCTCAGCCTCCGGGATGTGTTCCGGCAAAGATTTAATCTACACACTGTATT
 10 ACTGGAAGTCCTCTTCCTCCGGCAAGAAGACAGCTAAAACCAACACAAACGA
 GTTTTTAATCGACGTGGATAAAGGCGAAAACACTACTGTTTCAGCGTGCAAGCT
 GTGATCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGTTGAGT
 GCATGGGCCAAGAAAAGGGCGAGTTCCGGGAGAACTGGGTGAACGTCATCA
 GCGATTTAAAGAAGATCGAAGATTTAATTCAGTCCATGCATATCGACGCCAC
 15 TTTATACACAGAATCCGACGTGCACCCCTCTTGTAAGGTGACCGCCATGAAAT
 GTTTTTTACTGGAGCTGCAAGTTATCTCTTTAGAGAGCGGAGACGCTAGCATC
 CACGACACCGTGGAGAATTTAATCATTTTAGCCAATAACTCTTTATCCAGCAA
 CGGCAACGTGACAGAGTCCGGCTGCAAGGAGTGCGAAGAGCTGGAGGAGAA
 GAACATCAAGGAGTTTCTGCAATCCTTTGTGCACATTGTCCAGATGTTTCATCA
 20 ATACCTCC (SEQ ID NO: 81).

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

IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLT
 IQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNKTFRLR
 CEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERVGRDN
 KEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIKPDPPKN
 30 LQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKT
 SATVICRKNASISVRAQDRYYSSSWSEWASVPCSGGGGSGGGGSGGGGSRNLPV
 ATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPTSEEIDHEDITKDKTSTV
 EACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEF
 KTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTK
 35 IKLCILLHAFRIRAVTIDRVMSYLNASITCPPPMSVEHADIWVKSYSLYSRERYICN
 SGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCIR (SEQ ID NO: 82).

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

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

ATTTGGGAACTGAAGAAGGACGTCTACGTGGTCGAACTGGACTGGTATCCCCG
 ATGCTCCCGGCGAAATGGTGGTGCTCACTTGTGACACCCCCGAAGAAGACGG
 5 CATCACTTGGACCCTCGATCAGAGCAGCGAGGTGCTGGGCTCCGGAAAGACC
 CTCACAATCCAAGTTAAGGAGTTCGGAGACGCTGGCCAATACACATGCCACA
 AGGGAGGCGAGGTGCTCAGCCATTCTTATTATTATTACACAAGAAGGAAGA
 CGGAATCTGGTCCACCGACATTTTAAAAGATCAGAAGGAGCCCAAGAATAAG
 ACCTTTTAAAGGTGTGAGGCCAAAACTACAGCGGTCGTTTCACTTGTGGTG
 10 GCTGACCACCATTTCACCGATTAACTTCTCCGTGAAAAGCAGCCGGGGA
 AGCTCCGACCCTCAAGGTGTGACATGTGGAGCCGCTACCTCAGCGCTGAGA
 GGGTTCGTGGCGATAACAAGGAATACGAGTACAGCGTGGAGTGCCAAGAAG
 ATAGCGCTTGTCGCTGCCGAAGAATCTTTACCCATTGAGGTGATGGTGGAC
 GCCGTGCACAACTCAAGTACGAGAACTACACCTCCTCCTTCTTTATCCGGGA
 15 CATCATTAAGCCCGATCCTCCTAAGAATTTACAGCTGAAGCCTCTCAAAAATA
 GCCGGCAAGTTGAGGTCTCTTGGAATATCCCGACACTTGGAGCACACCCCA
 CAGCTACTTCTCTTTAACCTTTTGTGTGCAAGTTCAAGGTAAAAGCAAGCGGG
 AGAAGAAAGACCGGGTGTTTACCGACAAAACCAGCGCCACCGTCATCTGTCG
 GAAGAACGCCTCCATCAGCGTGAGGGCTCAAGATCGTTATTACTCCAGCAGC
 20 TGGTCCGAGTGGGCCAGCGTGCCTTGTTCCGGCGGTGGAGGATCCGGAGGAG
 GTGGCTCCGGCGGCGGAGGATCTCGTAACCTCCCCGTGGCTACCCCGATCC
 CGGAATGTTCCCTTGTTTACACCACAGCCAGAATTTACTGAGGGCCGTGAGC
 AACATGCTGCAGAAAGCTAGGCAGACTTTAGAATTTTACCCTTGCACCAGCG
 AGGAGATCGACCATGAAGATATCACCAGGACAAGACATCCACCGTGGAGG
 25 CTTGTTTACCTCTGGAGCTGACAAAGAACGAGTCTTGTCTCAACTCTCGTGAA
 ACCAGCTTCATCACAAATGGCTCTTGTTTAGCTTCCCGGAAGACCTCCTTTAT
 GATGGCTTTATGCCTCAGCTCCATCTACGAGGATTTAAAGATGTACCAAGTGG
 AGTTCAAGACCATGAACGCCAAGCTGCTCATGGACCCTAAACGGCAGATCTT
 TTAGACCAGAACATGCTGGCTGTGATTGATGAGCTGATGCAAGCTTTAAACT
 30 TCAACTCCGAGACCGTCCCTCAGAAGTCCTCCCTCGAGGAGCCCGATTTTTAC
 AAGACAAAGATCAAACCTGTGCATTTTACTCCACGCCTTTAGGATCCGGGCGG
 TGACCATTGACCGGGTCATGAGCTATTTAAACGCCAGCATTACATGCCCCCT
 CCCATGAGCGTGGAGCACGCCGACATCTGGGTGAAGAGCTATAGCCTCTACA
 GCCGGGAGAGGTATATCTGTAACAGCGGCTTCAAGAGGAAGGCCGGCACCA
 35 GCAGCCTCACCGAGTGCCTGCTGAATAAGGCTACCAACGTGGCTCACTGGAC
 AACACCCTCTTTAAAGTGCATCCGG (SEQ ID NO: 83).

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

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

MKWVTFISLLFLFSSAYSIWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGI
 TWTLDDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIW
 5 STDILKDQKEPKNKTFLRCEAKNYSGRFTCWWTITSTDLTFSVKSSRGSSDPQG
 VTCGAATLSAERVVRGDNKEYEYSVEQCEDSACPAAEESLPIEVMVDAVHKLKYE
 NYTSSFFIRDIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQV
 QGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCSGGG
 GSGGGGSGGGGSRNLPVATPDPGMFPCLLHHSQNLLRAVSNMLQKARQTLEFYP
 10 CTSEEIDHEDITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMM
 ALCLSSIIYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNS
 ETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNASITCPPPMSVEHA
 DIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCIR
 (SEQ ID NO: 84).

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

20 ATGAAATGGGTGACCTTTATTTCTTTACTGTTCTCTTTAGCAGCGCCTACTCC
 ATTTGGGAAGTGAAGAAGGACGTCTACGTGGTCGAACTGGACTGGTATCCCCG
 ATGCTCCCGGCGAAATGGTGGTGCTCACTTGTGACACCCCCGAAGAAGACGG
 CATCACTTGGACCTCGATCAGAGCAGCGAGGTGCTGGGCTCCGGAAAGACC
 CTCACAATCCAAGTTAAGGAGTTCGGAGACGCTGGCCAATACACATGCCACA
 25 AGGGAGGCGAGGTGCTCAGCCATTCTTATTATTATTACACAAGAAGGAAGA
 CGGAATCTGGTCCACCGACATTTTAAAAGATCAGAAGGAGCCCAAGAATAAG
 ACCTTTTAAAGGTGTGAGGCCAAAACCTACAGCGGTCGTTTCACTTGTGTTGGT
 GCTGACCACCATTTCCACCGATTAACTTCTCCGTGAAAAGCAGCCGGGGA
 AGCTCCGACCCTCAAGGTGTGACATGTGGAGCCGCTACCCTCAGCGCTGAGA
 30 GGGTTCGTGGCGATAACAAGGAATACGAGTACAGCGTGGAGTGCCAAGAAG
 ATAGCGCTTGTCCCGCTGCCGAAGAATCTTTACCCATTGAGGTGATGGTGGAC
 GCCGTGCACAACTCAAGTACGAGAACTACACCTCCTCCTTCTTTATCCGGGA
 CATCATTAAGCCCGATCCTCCTAAGAATTTACAGCTGAAGCCTCTCAAAAATA
 GCCGGAAGTTGAGGTCTCTTGGGAATATCCCGACACTTGGAGCACACCCCA
 35 CAGCTACTTCTCTTTAACCTTTTGTGTGCAAGTTCAAGGTAAAAGCAAGCGGG
 AGAAGAAAGACCGGGTGTTTACCGACAAAACCAGCGCCACCGTCATCTGTGCG
 GAAGAACGCCTCCATCAGCGTGAGGGCTCAAGATCGTTATTACTCCAGCAGC
 TGGTCCGAGTGGGCCAGCGTGCCTTGTTCGGCGGTGGAGGATCCGGAGGAG
 GTGGCTCCGGCGGCGGAGGATCTCGTAACCTCCCCGTGGCTACCCCCGATCC
 40 CGGAATGTTCCCTTGTTTACACCACAGCCAGAATTTACTGAGGGCCGTGAGC

AACATGCTGCAGAAAGCTAGGCAGACTTTAGAATTTTACCCTTGCACCAGCG
 AGGAGATCGACCATGAAGATATCACCAAGGACAAGACATCCACCGTGGAGG
 CTTGTTTACCTCTGGAGCTGACAAAGAACGAGTCTTGTCTCAACTCTCGTGAA
 ACCAGCTTCATCACAAATGGCTCTTGTGTTAGCTTCCCGGAAGACCTCCTTTAT
 5 GATGGCTTTATGCCTCAGCTCCATCTACGAGGATTTAAAGATGTACCAAGTGG
 AGTTCAAGACCATGAACGCCAAGCTGCTCATGGACCCTAAACGGCAGATCTT
 TTTAGACCAGAACATGCTGGCTGTGATTGATGAGCTGATGCAAGCTTTAAACT
 TCAACTCCGAGACCGTCCCTCAGAAGTCCTCCCTCGAGGAGCCCGATTTTTAC
 AAGACAAAGATCAAACGTGTGCATTTTACTCCACGCCTTTAGGATCCGGGCGG
 10 TGACCATTGACCGGGTCATGAGCTATTTAAACGCCAGCATTACATGCCCCCT
 CCCATGAGCGTGGAGCACGCCGACATCTGGGTGAAGAGCTATAGCCTCTACA
 GCCGGGAGAGGTATATCTGTAACAGCGGCTTCAAGAGGAAGGCCGGCACCA
 GCAGCCTCACCGAGTGCCTGCTGAATAAGGCTACCAACGTGGCTCACTGGAC
 AACACCCTCTTTAAAGTGCATCCGG (SEQ ID NO: 85).

Exemplary Multi-Chain Chimeric Polypeptides- Type B

In some embodiments of any of the multi-chain chimeric polypeptides described
 herein, the first target-binding domain and the second targeting-binding domain each
 independently bind specifically to a receptor of IL-7 or a receptor of IL-21. In some
 20 examples of these multi-chain chimeric polypeptides, the first target-binding domain and
 the soluble tissue factor domain directly abut each other in the first chimeric polypeptide.
 In some examples of these multi-chain chimeric polypeptides, the first chimeric
 polypeptide further comprises a linker sequence (e.g., any of the exemplary linkers
 described herein) between the first target-binding domain and the soluble tissue factor
 25 domain in the first chimeric polypeptide.

In some embodiments of these multi-chain chimeric polypeptides, 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 these multi-chain
 chimeric polypeptides, the first chimeric polypeptide further includes a linker sequence
 30 (e.g., any of the exemplary linkers described herein) 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 these multi-chain chimeric polypeptides, the second
 domain of the pair of affinity domains and the second target-binding domain directly abut
 35 each other in the second chimeric polypeptide. In some embodiments of these multi-

chain chimeric polypeptides, the second chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linkers described herein) between the second domain of the pair of affinity domains and the second target-binding domain in the second chimeric polypeptide.

5 In some embodiments of these multi-chain chimeric polypeptides, the soluble tissue factor domain can be any of the exemplary soluble tissue factor domains described herein. In some embodiments of these multi-chain chimeric polypeptides, the pair of affinity domains can be any of the exemplary pairs of affinity domains described herein.

10 In some embodiments of these multi-chain chimeric polypeptides, one or both of the first target-binding domain and the second target-binding domain is a soluble IL-21 (e.g., a soluble human IL-21 polypeptide) or a soluble IL-7 (e.g., a soluble human IL-7 polypeptide). In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain are each independently a soluble IL-21 or a soluble IL-7. In some embodiments of these multi-chain chimeric
15 polypeptides, the first target-binding domain and the second target-binding domain both bind specifically to a receptor of IL-21 or a receptor of IL-7. In some embodiments of these 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 multi-chain chimeric polypeptides, the first target-binding domain and the second
20 target-binding domain include the same amino acid sequence.

 In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain binds specifically to a receptor for IL-21, and the second target-binding domain binds specifically to a receptor for IL-7. In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain binds specifically to a
25 receptor for IL-7, and the second target-binding domain binds specifically to a receptor for IL-21.

 In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain includes a soluble IL-21 (e.g., a soluble human IL-21).

30 In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-21 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:

QGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWSAFSCFQKAQ
LKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLTCPSCDSYEKKPPKEFLER
5 FKSLLQKMIHQHLSSRTHGSEDS (SEQ ID NO: 86).

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-21 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:

CAAGGTCAAGATCGCCACATGATTAGAATGCGTCAACTTATAGATATTGTTG
ATCAGCTGAAAAATTATGTGAATGACTTGGTCCCTGAATTTCTGCCAGCTCCA
GAAGATGTAGAGACAACTGTGAGTGGTCAGCTTTTCTCTGTTTTCAGAAGG
CCCAACTAAAGTCAGCAAATACAGGAAACAATGAAAGGATAATCAATGTATC
15 AATTAAGAGCTGAAGAGGAAACCACCTTCCACAAATGCAGGGAGAAGACA
GAAACACAGACTAACATGCCCTTCATGTGATTCTTATGAGAAAAAACACCC
AAAGAATTCTAGAAAGATTCAAATCACTTCTCCAAAAGATGATTCATCAGC
ATCTGTCCTCTAGAACACACGGAAGTGAAGATTCC (SEQ ID NO: 87).

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-21 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:

CAGGGCCAGGACAGGCACATGATCCGGATGAGGCAGCTCATCGACATCGTTCG
25 ACCAGCTGAAGAACTACGTGAACGACCTGGTGCCCGAGTTTCTGCCTGCCCC
CGAGGACGTGGAGACCAACTGCGAGTGGTCCGCCTTCTCCTGCTTTCAGAAG
GCCAGCTGAAGTCCGCCAACACCGGCAACAACGAGCGGATCATCAACGTG
AGCATCAAGAAGCTGAAGCGGAAGCCTCCCTCCACAAACGCCGGCAGGAGG
CAGAAGCACAGGCTGACCTGCCCCAGCTGTGACTCCTACGAGAAGAAGCCCC
30 CCAAGGAGTTCCTGGAGAGGTTCAAGTCCCTGCTGCAGAAGATGATCCATCA
GCACCTGTCCTCCAGGACCCACGGCTCCGAGGACTCC (SEQ ID NO: 88).

In some embodiments of these multi-chain chimeric polypeptides, the sequence of soluble human IL-7 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:

DCDIEGKDGKQYESVLMVSIQQLDSMKEIGSNCLNNEFNFFKRHICDANKEGM
FLFRAARKLRQFLKMNSTGDFDLHLLKVSEGTILLNCTGQVKGRKPAALGEAQ
5 PTKSLEENKSLKEQKKLNDLCFLKRLLQEIKTCWNKILMGTKEH (SEQ ID NO:
19).

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-7 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
10 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:

GATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTCTAATGG
TCAGCATCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCT
GAATAATGAATTTAACTTTTTTAAAAGACATATCTGTGATGCTAATAAGGAA
15 GGTATGTTTTTATCCGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAATGAA
TAGCACTGGTGATTTTGATCTCCACTTATTAAGTTTCAGAAGGCACAACAA
TACTGTTGAACTGCACTGGCCAGGTTAAAGGAAGAAAACCAGCTGCCCTGGG
TGAAGCCCAACCAACAAAGAGTTTGAAGAAAATAAATCTTTAAAGGAACA
GAAAAAACTGAATGACTTGTGTTTCCTAAAGAGACTATTACAAGAGATAAAA
20 ACTTGTGGAATAAAATTTTGATGGGCACTAAAGAACAC (SEQ ID NO: 89).

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

QGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWSAFSCFQKAQ
LKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLTCPSCDSYEKKPPKEFLER
FKSLLQKMIHQHLSSRTHGSEDSSGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQ
VYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVEST
30 GSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLS
LRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRT
VNRKSTDSPVECMGQEKGEFRENWVNVISDLKKIEDLIQSMHIDATLYTESDVHP
SCKVTAMKCFLELQVISLESGDASIHDVTENLIILANNSLSSNGNVTESGCKECE
ELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO: 90).

In some embodiments, a first chimeric polypeptide is encoded by a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86%

identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

5 CAAGGTCAAGATCGCCACATGATTAGAATGCGTCAACTTATAGATATTGTTG
 ATCAGCTGAAAAATTATGTGAATGACTTGGTCCCTGAATTTCTGCCAGCTCCA
 GAAGATGTAGAGACAAACTGTGAGTGGTCAGCTTTTTCTGTTTTCAGAAGG
 CCCAACTAAAGTCAGCAAATACAGGAAACAATGAAAGGATAATCAATGTATC
 AATTAAAAAGCTGAAGAGGAAACCACCTTCCACAAATGCAGGGAGAAGACA
 GAAACACAGACTAACATGCCCTTCATGTGATTCTTATGAGAAAAAACACCC
 10 AAAGAATTCCTAGAAAGATTCAAATCACTTCTCCAAAAGATGATTCATCAGC
 ATCTGTCCTCTAGAACACACGGAAGTGAAGATTCCTCAGGCACTACAAATAC
 TGTGGCAGCATATAATTTAACTTGGAAATCAACTAATTTCAAGACAATTTTGG
 AGTGGGAACCCAAACCCGTCAATCAAGTCTACACTGTTCAAATAAGCACTAA
 GTCAGGAGATTGGAAAAGCAAATGCTTTTACACAACAGACACAGAGTGTGAC
 15 CTCACCGACGAGATTGTGAAGGATGTGAAGCAGACGTAAGTGGCACGGGTCT
 TCTCCTACCCGGCAGGGAATGTGGAGAGCACCGGTTCTGCTGGGGAGCCTCT
 GTATGAGAACTCCCCAGAGTTCACACCTTACCTGGAGACAAACCTCGGACAG
 CCAACAATTCAGAGTTTTGAACAGGTGGGAACAAAAGTGAATGTGACCGTAG
 AAGATGAACGGACTTTAGTCAGAAGGAACAACACTTTCCTAAGCCTCCGGGA
 20 TGTTTTTGGCAAGGACTTAATTTATACACTTTATTATTGGAAATCTTCAAGTTC
 AGGAAAGAAAACAGCCAAAACAAACACTAATGAGTTTTTGATTGATGTGGAT
 AAAGGAGAAAACACTACTGTTTCAGTGTTCAGCAGTGATTCCCTCCCGAACAG
 TTAACCGGAAGAGTACAGACAGCCCGGTAGAGTGTATGGGCCAGGAGAAAG
 GGGAATTCAGAGAAAACACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCG
 25 AAGATTTAATTCAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGAC
 GTGCACCCCTCTTGTAAGGTGACCGCCATGAAATGTTTTTTACTGGAGCTGCA
 AGTTATCTCTTTAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAAT
 TTAATCATTTTAGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTC
 CGGCTGCAAGGAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCT
 30 GCAATCCTTTGTGCACATTGTCCAGATGTTTCATCAATACCTCC (SEQ ID NO:
 91).

In some embodiments, a first chimeric polypeptide can include a sequence that is
 at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86%
 identical, at least 88% identical, at least 90% identical, at least 92% identical, at least
 35 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or
 100% identical) to:

MGVKVLFAICIAVAEAQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPED
 VETNCEWSAFSCFQKAQLKSANTGNNERIINVSIIKKLKRKPPSTNAGRRQKHRLT
 CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSSGTTNTVAAYNLTW

KSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQ
 TYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVN
 VTVEDERTLVRRNNTFLSLRDVFGKDLIYTYWKSSSSGKKTAKTNTNEFLIDV
 DKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRENWVNVISDLKKIED
 5 LIQSMHIDATLYTESDVHPSCKVTAMKCFLLLELQVISLESGDASIHDTVENLIILAN
 NSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO: 92).

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

ATGGGAGTGAAAGTTCTTTTTGCCCTTATTTGTATTGCTGTGGCCGAGGCCCA
 AGGTCAAGATCGCCACATGATTAGAATGCGTCAACTTATAGATATTGTTGATC
 AGCTGAAAAATTATGTGAATGACTTGGTCCCTGAATTTCTGCCAGCTCCAGAA
 15 GATGTAGAGACAACTGTGAGTGGTCAGCTTTTTCCTGTTTTTCAGAAGGCCCA
 ACTAAAGTCAGCAAATACAGGAAACAATGAAAGGATAATCAATGTATCAATT
 AAAAAGCTGAAGAGGAAACCACCTTCCACAAATGCAGGGAGAAGACAGAAA
 CACAGACTAACATGCCCTTCATGTGATTCTTATGAGAAAAAACACCCAAAG
 AATTCCTAGAAAGATTCAAATCACTTCTCCAAAAGATGATTCATCAGCATCTG
 20 TCCTCTAGAACACACGGAAGTGAAGATTCCTCAGGCACTACAAATACTGTGG
 CAGCATATAATTTAACTTGGAAATCAACTAATTTCAAGACAATTTTGGAGTGG
 GAACCCAAACCCGTCATCAAGTCTACACTGTTCAAATAAGCACTAAGTCAG
 GAGATTGGAAAAGCAAATGCTTTTACACAACAGACACAGAGTGTGACCTCAC
 CGACGAGATTGTGAAGGATGTGAAGCAGACGTACTTGGCACGGGTCTTCTCC
 25 TACCCGGCAGGGAATGTGGAGAGCACCGGTTCTGCTGGGGAGCCTCTGTATG
 AGAACTCCCCAGAGTTCACACCTTACCTGGAGACAAACCTCGGACAGCCAAC
 AATTCAGAGTTTTGAACAGGTGGGAACAAAAGTGAATGTGACCGTAGAAGAT
 GAACGGACTTTAGTCAGAAGGAACAACACTTTCCTAAGCCTCCGGGATGTTT
 TTGGCAAGGACTTAATTTATACACTTTATTATTGGAAATCTTCAAGTTCAGGA
 30 AAGAAAACAGCCAAAACAAACACTAATGAGTTTTTGATTGATGTGGATAAAG
 GAGAAAACACTGTTTCAGTGTTCAAGCAGTGATTCCCTCCCGAACAGTTAAC
 CGGAAGAGTACAGACAGCCCGGTAGAGTGTATGGGCCAGGAGAAAGGGGAA
 TTCAGAGAAAACCTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATT
 TAATTCAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCAC
 35 CCCTCTTGTAAGGTGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTTAT
 CTCTTTAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATC
 ATTTTAGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCT
 GCAAGGAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAAT
 CCTTTGTGCACATTGTCCAGATGTTTCATCAATACCTCC (SEQ ID NO: 93)

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

DCDIEGKDGKQYESVLMVSIQQLDSMKEIGSNCLNNEFNFFKRHICDANKEGM
FLFRAARKLRQFLKMNSTGDFDLHLLKVSEGTILLNCTGQVKGRKPAALGEAQ
PTKSLEENKSLKEQKKLNDLCFLKRLLQEIKTCWNKILMGTKEHITCPPPMSVEH
ADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCIR
(SEQ ID NO: 94).

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

GATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTTCTAATGG
TCAGCATCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCT
GAATAATGAATTTAACTTTTTTAAAAGACATATCTGTGATGCTAATAAGGAA
GGTATGTTTTTATTCCGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAATGAA
TAGCACTGGTGATTTTGATCTCCACTTATTAAGTTTCAGAAGGCACAACAA
TACTGTTGAACTGCACTGGCCAGGTAAAGGAAGAAAACCAGCTGCCCTGGG
TGAAGCCCAACCAACAAAGAGTTTGAAGAAAATAAATCTTTAAAGGAACA
GAAAAAACTGAATGACTTGTGTTTCCTAAAGAGACTATTACAAGAGATAAAA
ACTTGTTGGAATAAAATTTTGATGGGCACTAAAGAACACATCACGTGCCCTC
CCCCCATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTACAGCTTGTA
CTCCAGGGAGCGGTACATTTGTAACCTCTGGTTTCAAGCGTAAAGCCGGCACG
TCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCCACTGGA
CAACCCCCAGTCTCAAATGCATTAGA (SEQ ID NO: 95).

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

MGVKVLFALICIAVAEADCDIEGKDGKQYESVLMVSIQQLDSMKEIGSNCLN
EFNFFKRHICDANKEGMFLFRAARKLRQFLKMNSTGDFDLHLLKVSEGTILLNC
TGQVKGRKPAALGEAQPTKSLEENKSLKEQKKLNDLCFLKRLLQEIKTCWNKIL

MGTKEHITCPPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLTECVLNK
ATNVAHWTTPSLKCIR (SEQ ID NO: 96).

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

ATGGGAGTGAAAGTTCTTTTTGCCCTTATTTGTATTGCTGTGGCCGAGGCCGA
TTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTTCTAATGGTC
10 AGCATCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCTGA
ATAATGAATTTAACTTTTTTAAAAGACATATCTGTGATGCTAATAAGGAAGGT
ATGTTTTTATTCCTGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAAATGAATAG
CACTGGTGATTTTGATCTCCACTTATTAAGTTTCAGAAGGCACAACAATAC
TGTTGAACTGCACTGGCCAGGTTAAAGGAAGAAAACCAGCTGCCCTGGGTGA
15 AGCCCAACCAACAAAGAGTTTGGAAGAAAATAAATCTTTAAAGGAACAGAA
AAAAGTGAATGACTTGTGTTTCCTAAAGAGACTATTACAAGAGATAAAAAGT
TGTTGGAATAAAATTTTGATGGGCACTAAAGAACACATCACGTGCCCTCCCC
CCATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTACAGCTTGTACTC
CAGGGAGCGGTACATTTGTAAGTCTGGTTTCAAGCGTAAAGCCGGCACGTCC
20 AGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCCACTGGACAA
CCCCCAGTCTCAAATGCATTAGA (SEQ ID NO: 97).

Exemplary Multi-Chain Chimeric Polypeptides- Type C

In some embodiments of any of the multi-chain chimeric polypeptides described herein, the first target-binding domain and the second targeting-binding domain each independently bind specifically to a receptor of IL-7 or a receptor of IL-21. In some examples of these multi-chain chimeric polypeptides, the first target-binding domain and the soluble tissue factor domain directly abut each other in the first chimeric polypeptide. In some examples of these multi-chain chimeric polypeptides, the first chimeric polypeptide further comprises a linker sequence (e.g., any of the exemplary linkers described herein) between the first target-binding domain and the soluble tissue factor domain in the first chimeric polypeptide.

In some embodiments of these multi-chain chimeric polypeptides, 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 these multi-chain chimeric polypeptides, the first chimeric polypeptide further includes a linker sequence

(e.g., any of the exemplary linkers described herein) 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 these multi-chain chimeric polypeptides, 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 these multi-chain chimeric polypeptides, the second chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linkers described herein) 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 these multi-chain chimeric polypeptides, the soluble tissue factor domain can be any of the exemplary soluble tissue factor domains described herein. In some embodiments of these multi-chain chimeric polypeptides, the pair of affinity domains can be any of the exemplary pairs of affinity domains described herein.

In some embodiments of these multi-chain chimeric polypeptides, one or both of the first target-binding domain and the second target-binding domain is a soluble IL-21 (e.g., a soluble human IL-21 polypeptide) or a soluble IL-7 (e.g., a soluble human IL-7 polypeptide). In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain are each independently a soluble IL-21 or a soluble IL-7. In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain both bind specifically to a receptor of IL-21 or a receptor of IL-7. In some embodiments of these 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 multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain include the same amino acid sequence.

In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain binds specifically to a receptor for IL-21, and the second target-binding domain binds specifically to a receptor for IL-7. In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain binds specifically to a

receptor for IL-7, and the second target-binding domain binds specifically to a receptor for IL-21.

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-21 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:

QGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWSAFSCFQKAQ
LKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLTCPSCDSYEKKPPKEFLER
FKSLLQKMIHQHLSSRTHGSEDS (SEQ ID NO: 86).

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-21 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:

CAAGGTCAAGATCGCCACATGATTAGAATGCGTCAACTTATAGATATTGTTG
ATCAGCTGAAAAATTATGTGAATGACTTGGTCCCTGAATTTCTGCCAGCTCCA
GAAGATGTAGAGACAACTGTGAGTGGTCAGCTTTTTCCTGTTTTCAGAAGG
CCCAACTAAAGTCAGCAAATACAGGAAACAATGAAAGGATAATCAATGTATC
AATTAAAAAGCTGAAGAGGAAACCACCTTCCACAAATGCAGGGAGAAGACA
GAAACACAGACTAACATGCCCTTCATGTGATTCTTATGAGAAAAAACACCC
AAAGAATTCTTAGAAAGATTCAAATCACTTCTCCAAAAGATGATTCATCAGC
ATCTGTCCTCTAGAACACACGGAAGTGAAGATTCC (SEQ ID NO: 87).

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-21 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:

CAGGGCCAGGACAGGCACATGATCCGGATGAGGCAGCTCATCGACATCGTCG
ACCAGCTGAAGAACTACGTGAACGACCTGGTGCCCGAGTTTCTGCCTGCCCC
CGAGGACGTGGAGACCAACTGCGAGTGGTCCGCCTTCTCCTGCTTTCAGAAG
GCCCAGCTGAAGTCCGCCAACACCGGCAACAACGAGCGGATCATCAACGTG
AGCATCAAGAAGCTGAAGCGGAAGCCTCCCTCCACAAACGCCGGCAGGAGG
CAGAAGCACAGGCTGACCTGCCCCAGCTGTGACTCCTACGAGAAGAAGCCCC

CCAAGGAGTTCCTGGAGAGGTTCAAGTCCCTGCTGCAGAAGATGATCCATCA
GCACCTGTCCTCCAGGACCCACGGCTCCGAGGACTCC (SEQ ID NO: 88).

In some embodiments of these multi-chain chimeric polypeptides, the sequence of soluble human IL-7 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:

DCDIEGKDGKQYESVLMVSIQQLDLSMKEIGSNCLNNEFNFFKRHICDANKEGM
FLFRAARKLRQFLKMNSTGDFDLHLLKVSEGTTILLNCTGQVKGRKPAALGEAQ
PTKSLEENKSLKEQKKLNDLCFLKRLLQEIKTCWNKILMGTKEH (SEQ ID NO: 19).

In some embodiments of these multi-chain chimeric polypeptides, the soluble human IL-7 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:

GATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTCTAATGG
TCAGCATCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCT
GAATAATGAATTTAACTTTTTTAAAAGACATATCTGTGATGCTAATAAGGAA
GGTATGTTTTTATTCCGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAAATGAA
TAGCACTGGTGATTTTGATCTCCACTTATTAAGTTTCAGAAGGCACAACAA
TACTGTTGAACTGCACTGGCCAGGTTAAAGGAAGAAAACCAGCTGCCCTGGG
TGAAGCCCAACCAACAAAGAGTTTGGAAGAAAATAAATCTTTAAAGGAACA
GAAAAAACTGAATGACTTGTGTTTCCTAAAGAGACTATTACAAGAGATAAAA
ACTTGTTGGAATAAAATTTTGATGGGCACTAAAGAACAC (SEQ ID NO: 89).

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

DCDIEGKDGKQYESVLMVSIQQLDLSMKEIGSNCLNNEFNFFKRHICDANKEGM
FLFRAARKLRQFLKMNSTGDFDLHLLKVSEGTTILLNCTGQVKGRKPAALGEAQ
PTKSLEENKSLKEQKKLNDLCFLKRLLQEIKTCWNKILMGTKEHSGTTNTVAAY
NLWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVK
DVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVG
TKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNE

FLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRENWVNVISDL
KKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIHTVEN
LIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID
NO: 98).

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

10 GATTGCGACATCGAGGGCAAGGACGGCAAGCAGTACGAGAGCGTGCTGATG
GTGTCCATCGACCAGCTGCTGGACAGCATGAAGGAGATCGGCTCCAACTGCC
TCAACAACGAGTTCAACTTCTTCAAGCGGCACATCTGCGACGCCAACAAGGA
GGGCATGTTCTGTTTCAAGGGCCGCCAGGAACTGCGGCAGTTCCTGAAGATG
AACTCCACCGGCGACTTCGACCTGCACCTGCTGAAGGTGTCCGAGGGCACCA
15 CCATCCTGCTGAACTGCACCGGACAGGTGAAGGGCCGAAACCTGCTGCTCT
GGGAGAGGGCCCAACCCACCAAGAGCCTGGAGGAGAACAAGTCCCTGAAGGA
GCAGAAGAAGCTGAACGACCTGTGCTTCTGAAGAGGCTGCTGCAGGAGATC
AAGACCTGCTGGAACAAGATCCTGATGGGCACCAAGGAGCATAGCGGCACA
ACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAGCACCAACTTCAAAA
20 CCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTTTACACCGTGCAGAT
CAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCTATAACCACCGACACC
GAGTGCGATCTCACCGATGAGATCGTGAAAGATGTGAAACAGACCTACCTCG
CCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGCACTGGTTCGCTGG
CGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTACCTCGAGACCAATT
25 TAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGCACAACAGGTGAATGT
GACAGTGAGGACGAGCGGACTTTAGTGCGGCGGAACAACACCTTTCTCAGC
CTCCGGGATGTGTTTCGGCAAAGATTTAATCTACACACTGTATTACTGGAAGTC
CTCTTCCTCCGGCAAGAAGACAGCTAAAACCAACACAAACGAGTTTTTAATC
GACGTGGATAAAGGCGAAAACCTACTGTTTCAGCGTGCAAGCTGTGATCCCCT
30 CCCGGACCGTGAAATAGGAAAAGCACCGATAGCCCCGTTGAGTGCATGGGCCA
AGAAAAGGGCGAGTTCCGGGAGAACTGGGTGAACGTCATCAGCGATTTAAA
GAAGATCGAAGATTTAATTCAGTCCATGCATATCGACGCCACTTTATACACA
GAATCCGACGTGCACCCCTCTTGTAAGGTGACCGCCATGAAATGTTTTTTACT
GGAGCTGCAAGTTATCTCTTTAGAGAGCGGAGACGCTAGCATCCACGACACC
35 GTGGAGAATTTAATCATTTTAGCCAATAACTCTTTATCCAGCAACGGCAACGT
GACAGAGTCCGGCTGCAAGGAGTGCGAAGAGCTGGAGGAGAAGAACATCAA
GGAGTTTCTGCAATCCTTTGTGCACATTGTCCAGATGTTTCATCAATACCTCC
(SEQ ID NO: 99).

In some embodiments, a first chimeric polypeptide can include a sequence that is
40 at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86%

identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

5 MKWVTFISLLFLFSSAYSDCDIEGKD GKQYESVLMV SIDQLLD SMKEIGSNCLNN
EFNFFKRHICDANKEGMFLFRAARKLRQFLKMNSTGDFDLHLLKVSEGT TILLNC
TGQVKGRKPAALGEAQPTKSLEENKSLKEQKKLNDLCFLKRL LQEIKTCWNKIL
MGTKEHSGTTNTVAAYNL TWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKS
KCFYTTDTECDLTDEIVKDV KQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTP
10 YLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYY
WKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSR TVNRKSTDSPVECMG
QEKGEFRENWVNVISDLKKIEDLIQSMHIDATLYTESDVHP SCKVTAMKCFLEL
QVISLESGDASIHD TVENLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFV
HIVQMFINTS (SEQ ID NO: 100).

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

20 ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCCTGTTCTCCAGCGCCTACTC
CGATTGCGACATCGAGGGCAAGGACGGCAAGCAGTACGAGAGCGTGCTGAT
GGTGTCCATCGACCAGCTGCTGGACAGCATGAAGGAGATCGGCTCCA ACTGC
CTCAACAACGAGTTCAACTTCTTCAAGCGGCACATCTGCGACGCCAACAAGG
AGGGCATGTTCTGTTCTAGGGCCGCCAGGAACTGCGGCAGTTCTCTGAAGAT
GAACTCCACCGGCGACTTCGACCTGCACCTGCTGAAGGTGTCCGAGGGCACC
25 ACCATCCTGCTGAACTGCACCGGACAGGTGAAGGGCCGGAAACCTGCTGCTC
TGGGAGAGGCCCAACCCACCAAGAGCCTGGAGGAGAACAAGTCCCTGAAGG
AGCAGAAGAAGCTGAACGACCTGTGCTTCTCTGAAGAGGCTGCTGCAGGAGAT
CAAGACCTGCTGGAACAAGATCCTGATGGGCACCAAGGAGCATAGCGGCAC
AACCAACACAGTCGCTGCCTATAACCTCACTTGAAGAGCACCAACTTCAAAA
30 ACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTTTACACCGTGCAGA
TCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCTATACACCGACAC
CGAGTGCGATCTCACCGATGAGATCGTGAAAGATGTGAAACAGACCTACCTC
GCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGCACTGGTTCCGCTG
GCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTACCTCGAGACCAA
35 TTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGCACAAAGGTGAAT
GTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAACAACACCTTTCTCA
GCCTCCGGGATGTGTTCCGGCAAAGATTTAATCTACACACTGTATTACTGGAAG
TCCTCTTCTCCGGCAAGAAGACAGCTAAAACCAACACAAACGAGTTTTTAA
TCGACGTGGATAAAGGCGAAAAC TACTGTTTCAGCGTGCAAGCTGTGATCCC

CTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGTTGAGTGCATGGGC
 CAAGAAAAGGGCGAGTTCCGGGAGAACTGGGTGAACGTCATCAGCGATTTA
 AAGAAGATCGAAGATTTAATTCAGTCCATGCATATCGACGCCACTTTATACA
 CAGAATCCGACGTGCACCCCTCTTGTAAGGTGACCGCCATGAAATGTTTTTTA
 5 CTGGAGCTGCAAGTTATCTCTTTAGAGAGCGGAGACGCTAGCATCCACGACA
 CCGTGGAGAATTTAATCATTTTAGCCAATAACTCTTTATCCAGCAACGGCAAC
 GTGACAGAGTCCGGCTGCAAGGAGTGCGAAGAGCTGGAGGAGAAGAACATC
 AAGGAGTTTCTGCAATCCTTTGTGCACATTGTCCAGATGTTTCATCAATACCTC
 C (SEQ ID NO: 101).

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

15 QGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWSAFSCFQKAQ
 LKSANTGNNERIINVSIKKLKRKPPSTNAGRRQKHRLTCPSCDSYEKKPPKEFLER
 FKSLQKMIHQHLSSRTHGSEDSITCPPMSVEHADIWVKSYSLYSRERYICNSGF
 KRKAGTSSLTECVLNKATNVAHWTPSLKCIR (SEQ ID NO: 102).

In some embodiments, a second chimeric polypeptide is encoded by a sequence
 20 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:

CAGGGCCAGGACAGGCACATGATCCGGATGAGGCAGCTCATCGACATCGTCCG
 25 ACCAGCTGAAGAACTACGTGAACGACCTGGTGCCCGAGTTTCTGCCTGCCCC
 CGAGGACGTGGAGACCAACTGCGAGTGGTCCGCCTTCTCCTGCTTTCAGAAG
 GCCCAGCTGAAGTCCGCCAACACCGGCAACAACGAGCGGATCATCAACGTG
 AGCATCAAGAAGCTGAAGCGGAAGCCTCCCTCCACAAACGCCGGCAGGAGG
 CAGAAGCACAGGCTGACCTGCCCCAGCTGTGACTCCTACGAGAAGAAGCCCC
 30 CCAAGGAGTTCCTGGAGAGGTTCAAGTCCCTGCTGCAGAAGATGATCCATCA
 GCACCTGTCCTCCAGGACCCACGGCTCCGAGGACTCCATTACATGCCCCCCTC
 CCATGAGCGTGGAGCACGCCGACATCTGGGTGAAGAGCTATAGCCTCTACAG
 CCGGGAGAGGTATATCTGTAACAGCGGCTTCAAGAGGAAGGCCGGCACCAG
 CAGCCTCACCGAGTGCGTGCTGAATAAGGCTACCAACGTGGCTCACTGGACA
 35 ACACCCTCTTTAAAGTGCATCCGG (SEQ ID NO: 103).

In some embodiments, a second chimeric polypeptide can include a sequence that
 is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86%

identical, at least 88% identical, at least 90% identical, at least 92% identical, at least 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or 100% identical) to:

MKWVTFISLLFLFSSAYSQGDHRMIRMRLIDIVDQLKNYVNDLVPEFLPAPED
 5 VETNCEWSAFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRRQKHRLT
 CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSITCPPPMSVEHADIW
 VKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCIR (SEQ
 ID NO: 104).

In some embodiments, a second chimeric polypeptide is encoded by a sequence
 10 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:

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCCAGCGCCTACTC
 15 CCAGGGCCAGGACAGGCACATGATCCGGATGAGGCAGCTCATCGACATCGTC
 GACCAGCTGAAGAACTACGTGAACGACCTGGTGCCCGAGTTTCTGCCTGCCC
 CCGAGGACGTGGAGACCAACTGCGAGTGGTCCGCCTTCTCCTGCTTTCAGAA
 GGCCCAGCTGAAGTCCGCCAACACCGGCAACAACGAGCGGATCATCAACGT
 GAGCATCAAGAAGCTGAAGCGGAAGCCTCCCTCCACAAACGCCGGCAGGAG
 20 GCAGAAGCACAGGCTGACCTGCCCCAGCTGTGACTCCTACGAGAAGAAGCCC
 CCCAAGGAGTTCCTGGAGAGGTTCAAGTCCCTGCTGCAGAAGATGATCCATC
 AGCACCTGTCCTCCAGGACCCACGGCTCCGAGGACTCCATTACATGCCCCCCT
 CCCATGAGCGTGGAGCACGCCGACATCTGGGTGAAGAGCTATAGCCTCTACA
 GCCGGGAGAGGTATATCTGTAACAGCGGCTTCAAGAGGAAGGCCGGCACCA
 25 GCAGCCTCACCGAGTGCGTGCTGAATAAGGCTACCAACGTGGCTCACTGGAC
 AACACCCTCTTTAAAGTGCATCCGG (SEQ ID NO: 105).

Exemplary Multi-Chain Chimeric Polypeptides- Type D

In some embodiments of any of the multi-chain chimeric polypeptides described
 30 herein, the first target-binding domain and the second targeting-binding domain each
 independently bind specifically to TGF- β . In some examples of these multi-chain
 chimeric polypeptides, the first target-binding domain and the soluble tissue factor
 domain directly abut each other in the first chimeric polypeptide. In some examples of
 these multi-chain chimeric polypeptides, the first chimeric polypeptide further comprises
 35 a linker sequence (e.g., any of the exemplary linkers described herein) between the first

target-binding domain and the soluble tissue factor domain in the first chimeric polypeptide.

In some embodiments of these multi-chain chimeric polypeptides, 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 these multi-chain chimeric polypeptides, the first chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linkers described herein) 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 these multi-chain chimeric polypeptides, 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 these multi-chain chimeric polypeptides, the second chimeric polypeptide further includes a linker sequence (e.g., any of the exemplary linkers described herein) 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 these multi-chain chimeric polypeptides, the soluble tissue factor domain can be any of the exemplary soluble tissue factor domains described herein. In some embodiments of these multi-chain chimeric polypeptides, the pair of affinity domains can be any of the exemplary pairs of affinity domains described herein.

In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain each independently bind specifically to TGF- β . In some embodiments of these 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 multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain include the same amino acid sequence.

In some embodiments of these multi-chain chimeric polypeptides, the first target-binding domain and the second target-binding domain is a soluble TGF- β receptor (e.g., a soluble TGF β RII receptor, e.g., a soluble human TGF β RII). In some embodiments of these multi-chain chimeric polypeptides, the soluble human TGF β RII

includes a first sequence of soluble human TGFR β RII and a second sequence of soluble human TGFR β RII. In some embodiments of these multi-chain chimeric polypeptides, the soluble human TGFR β RII includes a linker disposed between the first sequence of soluble human TGFR β RII and the second sequence of soluble human TGFR β RII. In
 5 some examples of these multi-chain chimeric polypeptides, the linker includes the sequence GGGGSGGGGSGGGGS (SEQ ID NO: 14).

In some embodiments of these multi-chain chimeric polypeptides, the first sequence of soluble human TGFR β RII receptor comprises a sequence that is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86% identical, at
 10 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:
 IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICE
 KPQEVCVAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGET
 FFMCS CSSDECNDNIIFSEEYNTSNPD (SEQ ID NO: 106).

In some embodiments of these multi-chain chimeric polypeptides, the second sequence of soluble human TGFR β RII receptor 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%
 15 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
 20 ID NO: 106.

In some embodiments of these multi-chain chimeric polypeptides, the first sequence of soluble human TGFR β RII receptor 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,
 25 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:

ATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACCGACAA
 CAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCAGGTTCA
 30 GCACCTGCGATAATCAGAAGTCCTGCATGTCCAACCTGCAGCATCACCTCCATCT
 GCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAATGACGAGA
 ACATCACCTGGAGACCGTGTGTCACGACCCCAAGCTCCCTTATCACGACTTC
 ATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAAGAAGAAGC

CCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGTAACGACAAC
ATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGAT (SEQ ID NO: 107).

In some embodiments of these multi-chain chimeric polypeptides, the second
sequence of soluble human TGF β RII receptor is encoded by a sequence that is at least
5 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:

ATTCCTCCCCACGTGCAGAAGAGCGTGAATAATGACATGATCGTGACCGATA
10 ACAATGGCGCCGTGAAATTTCCCCAGCTGTGCAAATTCTGCGATGTGAGGTTT
TCCACCTGCGACAACCAGAAGTCCTGTATGAGCAACTGCTCCATCACCTCCAT
CTGTGAGAAGCCTCAGGAGGTGTGCGTGGCTGTCTGGCGGAAGAATGACGAG
AATATCACCTGGAAACCGTCTGCCACGATCCCAAGCTGCCCTACCACGATTT
CATCCTGGAAGACGCCGCCAGCCCTAAGTGCATCATGAAAGAGAAAAAGAA
15 GCCTGGCGAGACCTTTTTTCATGTGCTCCTGCAGCAGCGACGAATGCAACGAC
AATATCATCTTTAGCGAGGAATAACAATACCAGCAACCCCGAC (SEQ ID NO:
108).

In some embodiments of these multi-chain chimeric polypeptides, the soluble
TGF- β receptor includes a sequence that is at least 80% identical (e.g., at least 82%
20 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:

IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICE
KPQEVCVAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGET
25 FFMCS CSSDECNDNIIFSEYNTSNPDGGGGSGGGSGGGGSIPPHVQKSVNNDM
IVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRK
NDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCS CSSDECND
NIIFSEYNTSNPD (SEQ ID NO: 109).

In some embodiments of these multi-chain chimeric polypeptides, the soluble
30 TGF- β receptor 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:

ATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACCGACAA
35 CAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCAGGTTCA

GCACCTGCGATAATCAGAAGTCCTGCATGTCCAACCTGCAGCATCACCTCCATCT
 GCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAATGACGAGA
 ACATCACCCCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATCACGACTTC
 ATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAAGAAGAAGC
 5 CCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGTAAACGACAAC
 ATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGATGGAGGTGGCGGATC
 CGGAGGTGGAGGTTCTGGTGGAGGTGGGAGTATTCCTCCCCACGTGCAGAAG
 AGCGTGAATAATGACATGATCGTGACCGATAACAATGGCGCCGTGAAATTTCC
 CCAGCTGTGCAAATTCTGCGATGTGAGGTTTTCCACCTGCGACAACCAGAAGT
 10 CCTGTATGAGCAACTGCTCCATCACCTCCATCTGTGAGAAGCCTCAGGAGGTG
 TGGTGGCTGTCTGGCGGAAGAATGACGAGAATATCACCCCTGGAAACCGTCTG
 CCACGATCCCAAGCTGCCCTACCACGATTTCATCCTGGAAGACGCCGCCAGCC
 CTAAGTGCATCATGAAAGAGAAAAAGAAGCCTGGCGAGACCTTTTTTCATGTGC
 TCCTGCAGCAGCGACGAATGCAACGACAATATCATCTTTAGCGAGGAATACAA
 15 TACCAGCAACCCCGAC (SEQ ID NO: 110).

In some embodiments, the first chimeric polypeptide can include a sequence that
 is at least 80% identical (e.g., at least 82% identical, at least 84% identical, at least 86%
 identical, at least 88% identical, at least 90% identical, at least 92% identical, at least
 20 94% identical, at least 96% identical, at least 98% identical, at least 99% identical, or
 100% identical) to:

IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICE
 KPQEVCAVAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGET
 FFMCSOSSDECNDNIIFSEYNTSNPDGGGGSGGGSGGGGSIPPHVQKSVNNDM
 25 IVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCAVAVWRK
 NDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCSOSSDECND
 NIIFSEYNTSNPDSGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKS
 GDWKSCKFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYEN
 SPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLI
 30 YTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFVQAVIPSRTVNRKSTDSPV
 ECMGQEKGEFRENWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKC
 FLLELQVISLESGDASIHTVENLILANNSLSSNGNVTESGCKECEELEEKNKEFL
 QSFVHIVQMFINTS (SEQ ID NO: 111).

In some embodiments, a first chimeric polypeptide is encoded by a sequence that
 35 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:

ATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACCGACA
 40 ACAACGGCGCCGTGAAGTTTCCCAGCTCTGCAAGTTCTGCGATGTCAGGTTCT

AGCACCTGCGATAATCAGAAGTCCTGCATGTCCAACTGCAGCATCACCTCCA
 TCTGCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAATGACG
 AGAACATCACCCCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATCACGA
 CTTCAATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAAGAAG
 5 AAGCCCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGTAACG
 ACAACATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGATGGAGGTGG
 CGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGTATTCCTCCCCACGTG
 CAGAAGAGCGTGAATAATGACATGATCGTGACCGATAACAATGGCGCCGTGA
 AATTTCCCCAGCTGTGCAAATTCTGCGATGTGAGGTTTTCCACCTGCGACAAC
 10 CAGAAGTCCTGTATGAGCAACTGCTCCATCACCTCCATCTGTGAGAAGCCTC
 AGGAGGTGTGCGTGGCTGTCTGGCGGAAGAATGACGAGAATATCACCCCTGGA
 AACCGTCTGCCACGATCCCAAGCTGCCCTACCACGATTTTCATCCTGGAAGAC
 GCCGCCAGCCCTAAGTGCATCATGAAAGAGAAAAAGAAGCCTGGCGAGACC
 TTTTTCATGTGCTCCTGCAGCAGCGACGAATGCAACGACAATATCATCTTTAG
 15 CGAGGAATACAATACCAGCAACCCCGACAGCGGCACAACCAACACAGTCGC
 TGCCTATAACCTCACTTGGAAGAGCACCAACTTCAAAACCATCCTCGAATGG
 GAACCCAAACCCGTTAACCAAGTTTACACCGTGCAGATCAGCACCAAGTCCG
 GCGACTGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTCAC
 CGATGAGATCGTGAAAGATGTGAAACAGACCTACCTCGCCCGGGTGTTTAGC
 20 TACCCCGCCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACG
 AGAACAGCCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCCAC
 CATCCAAAGCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGAGGAGAC
 GAGCGGACTTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGT
 TCGGCAAAGATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGC
 25 AAGAAGACAGCTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAA
 GGCGAAACTACTGTTTCAGCGTGCAAGCTGTGATCCCCTCCCGGACCGTGA
 ATAGGAAAAGCACCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCG
 AGTTCCGGGAGAACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGA
 TTTAATTCAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGC
 30 ACCCTCTTGTAAGGTGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTT
 ATCTCTTTAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAA
 TCATTTTAGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGC
 TGCAAGGAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAA
 TCCTTTGTGCACATTGTCCAGATGTTTCATCAATACCTCC (SEQ ID NO: 112).

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

40 MKWVTFISLLFLFSSAYSIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFST
 CDNQKSCMSNCSITSICEKPQEVCAVVRKNDENITLETVCHDPKLPYHDFILED

AASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEYNTSNPDGGGGSGGGGSG
 GGGSIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSI
 TSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAAASPKCIMKEKKK
 PGETFFMCSCSSDECNDNIIFSEYNTSNPDSGTTNTVAAYNLTWKSTNFKTILEW
 5 EPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYP
 AGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLV
 RRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFSV
 QAVIPSRTVNRKSTDSPVECMGQEKGEFRENWVNVISDLKKIEDLIQSMHIDATL
 YTESDVHPSCKVTAMKCFLELQVISLES GDASIHDTVENLIILANNSLSSNGNVT
 10 ESGCKECEEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO: 113).

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

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCTCCAGCGCCTACTC
 CATCCCCCCCCATGTGCAAAGAGCGTGAACAACGATATGATCGTGACCGAC
 AACAAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCAGGTT
 CAGCACCTGCGATAATCAGAAGTCCTGCATGTCCAACCTGCAGCATCACCTCC
 20 ATCTGCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAATGAC
 GAGAACATCACCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATCACG
 ACTTCATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAAGAA
 GAAGCCCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGTAAC
 GACAACATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGATGGAGGTG
 25 GCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGTATTCCTCCCCACGT
 GCAGAAGAGCGTGAATAATGACATGATCGTGACCGATAACAATGGCGCCGTG
 AAATTTCCCCAGCTGTGCAAATTCTGCGATGTGAGGTTTTCCACCTGCGACAA
 CCAGAAGTCCTGTATGAGCAACTGCTCCATCACCTCCATCTGTGAGAAGCCTC
 AGGAGGTGTGCGTGGCTGTCTGGCGGAAGAATGACGAGAATATCACCTGGA
 30 AACCGTCTGCCACGATCCCAAGCTGCCCTACCACGATTTTCATCCTGGAAGAC
 GCCGCCAGCCCTAAGTGCATCATGAAAGAGAAAAAGAAGCCTGGCGAGACC
 TTTTTCATGTGCTCCTGCAGCAGCGACGAATGCAACGACAATATCATCTTTAG
 CGAGGAATACAATACCAGCAACCCCGACAGCGGCACAACCAACACAGTCGC
 TGCCTATAACCTCACTTGGAAGAGCACCAACTTCAAAACCATCCTCGAATGG
 35 GAACCCAAACCCGTTAACCAAGTTTACACCGTGCAGATCAGCACCAAGTCCG
 GCGACTGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTCAC
 CGATGAGATCGTGAAAGATGTGAAACAGACCTACCTCGCCCGGGTGTTTAGC
 TACCCCGCCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACG
 AGAACAGCCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCAC
 40 CATCCAAAGCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGAGGAGAC
 GAGCGGACTTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGT

TCGGCAAAGATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGC
 AAGAAGACAGCTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAA
 GGCGAAAACACTACTGTTTCAGCGTGCAAGCTGTGATCCCCTCCCGGACCGTGA
 ATAGGAAAAGCACCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCG
 5 AGTTCCGGGAGAAGTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGA
 TTTAATTCAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGC
 ACCCTCTTGTAAGGTGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTT
 ATCTCTTTAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAA
 TCATTTTAGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGC
 10 TGCAAGGAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAA
 TCCTTTGTGCACATTGTCCAGATGTTTCATCAATACCTCC (SEQ ID NO: 114).

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

IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICE
 KPQEVCVAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGET
 FFMCSRSSDECNDNIIFSEYNTSNPDGGGGSGGGGSGGGGSIPPHVQKSVNNDM
 20 IVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRK
 NDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCSSSDECND
 NIIFSEYNTSNPDITCPPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLT
 ECVLNKATNVAHWTTPSLKCIR (SEQ ID NO: 115).

In some embodiments, a second chimeric polypeptide is encoded by a sequence
 25 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:

ATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACCGACA
 30 ACAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCAGGTTC
 AGCACCTGCGATAATCAGAAGTCCTGCATGTCCAACCTGCAGCATCACCTCCA
 TCTGCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAATGACG
 AGAACATCACCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATCACGA
 CTTCATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAAGAAG
 35 AAGCCCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGTAACG
 ACAACATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGATGGAGGTGG
 CGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGTATTCTCCCCACGTG
 CAGAAGAGCGTGAATAATGACATGATCGTGACCGATAACAATGGCGCCGTGA

AATTTCCCCAGCTGTGCAAATTCTGCGATGTGAGGTTTTCCACCTGCGACAAC
 CAGAAGTCCTGTATGAGCAACTGCTCCATCACCTCCATCTGTGAGAAGCCTC
 AGGAGGTGTGCGTGGCTGTCTGGCGGAAGAATGACGAGAATATCACCTGGA
 AACCGTCTGCCACGATCCCAAGCTGCCCTACCACGATTTTCATCCTGGAAGAC
 5 GCCGCCAGCCCTAAGTGCATCATGAAAGAGAAAAAGAAGCCTGGCGAGACC
 TTTTTCATGTGCTCCTGCAGCAGCGACGAATGCAACGACAATATCATCTTTAG
 CGAGGAATACAATACCAGCAACCCCGACATTACATGCCCCCCTCCCATGAGC
 GTGGAGCACGCCGACATCTGGGTGAAGAGCTATAGCCTCTACAGCCGGGAGA
 GGTATATCTGTAACAGCGGCTTCAAGAGGAAGGCCGGCACCAGCAGCCTCAC
 10 CGAGTGCCTGCTGAATAAGGCTACCAACGTGGCTCACTGGACAACACCCTCT
 TTAAAGTGCATCCGG (SEQ ID NO: 116).

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

MKWVTFISLLFLFSSAYSIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFST
 CDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILED
 AASPKCIMKEKKKPGETFFMCSSDECNDNIIFSEEYNTSNPDGGGGSGGGGSG
 20 GGGSSIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSI
 TSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILED AASPKCIMKEKKK
 PGETFFMCSSDECNDNIIFSEEYNTSNPDITCPPPMSVEHADIWVKSYSLSYRER
 YICNSGFKRKAGTSSLTECVLNKATNVAHWTPSLKCIR (SEQ ID NO: 117).

In some embodiments, a second chimeric polypeptide is encoded by a sequence
 25 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:

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCCTGTTCTCCAGCGCCTACTC
 30 CATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACCGAC
 AACAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCAGGTT
 CAGCACCTGCGATAATCAGAAGTCCTGCATGTCCAACCTGCAGCATCACCTCC
 ATCTGCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAATGAC
 GAGAACATCACCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATCAG
 35 ACTTCATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAAGAA
 GAAGCCCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGTAAC
 GACAACATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGATGGAGGTG
 GCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGTATTCTCCCCACGT

GCAGAAGAGCGTGAATAATGACATGATCGTGACCGATAACAATGGCGCCGTG
 AAATTTCCCCAGCTGTGCAAATTCTGCGATGTGAGGTTTTCCACCTGCGACAA
 CCAGAAGTCCTGTATGAGCAACTGCTCCATCACCTCCATCTGTGAGAAGCCTC
 AGGAGGTGTGCGTGGCTGTCTGGCGGAAGAATGACGAGAATATCACCTGGA
 5 AACCGTCTGCCACGATCCCAAGCTGCCCTACCACGATTTTCATCCTGGAAGAC
 GCCGCCAGCCCTAAGTGCATCATGAAAGAGAAAAAGAAGCCTGGCGAGACC
 TTTTTCATGTGCTCCTGCAGCAGCGACGAATGCAACGACAATATCATCTTTAG
 CGAGGAATACAATACCAGCAACCCCGACATTACATGCCCCCCTCCCATGAGC
 GTGGAGCACGCCGACATCTGGGTGAAGAGCTATAGCCTCTACAGCCGGGAGA
 10 GGTATATCTGTAACAGCGGCTTCAAGAGGAAGGCCGGCACCAGCAGCCTCAC
 CGAGTGCGTGCTGAATAAGGCTACCAACGTGGCTCACTGGACAACACCCTCT
 TTAAAGTGCATCCGG (SEQ ID NO: 118).

15 **Single-Chain Chimeric Polypeptides**

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

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).

5 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

10 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).

15 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

20 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).

Exemplary Embodiments of Single-Chain Chimeric Polypeptides- Type A

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

30 In some embodiments, the first target-binding domain binds specifically to CD3 (e.g., human CD3) and the second target-binding domain binds specifically to CD28 (e.g.,

human CD28). In some embodiments, the first target-binding domain binds specifically to CD28 (e.g., human CD28) and the second target-binding domain binds specifically to CD3 (e.g., human CD3).

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

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

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

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

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLAS
 GVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRGGG
 30 GSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQR

PGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYY
CARYYDDHYCLDYWGQGTTLTVSS (SEQ ID NO: 119).

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

CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAAGTGGTATC
AGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAGC
TGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCACCAGCTAC
TCTTTAACCATTTCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCCAG
CAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAATCAA
TCGTGGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAGCCA
AGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCGGCGCCTCCGTC
AAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCATTGG
GTCAAGCAGAGGCCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAACCCTTC
CCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTTTAACCA
CTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACCAGCGAG
GACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTGTTTAGAC
TATTGGGGACAAGGTACCACTTTAACCGTCAGCAGC (SEQ ID NO: 120).

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

VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYN
DYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRGT
TLTVSSGGGGSGGGGSGGGGSDIEMTQSPAIMSASLGERVTMTCTASSSVSSSYFH
WYQQKPGSSPKLCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQY
HRSPTFGGGTKLETKR (SEQ ID NO: 121).

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

GTCCAGCTGCAGCAGAGCGGACCCGAACTCGTGAAACCCGGTGCTTCCGTGA
AAATGTCTTGTAAAGGCCAGCGGATACACCTTCACCTCCTATGTGATCCAGTGGG
TCAAACAGAAGCCCGGACAAGGTCTCGAGTGGATCGGCAGCATCAACCCTTA
CAACGACTATACCAAATACAACGAGAAGTTTAAGGGAAAGGCTACTTTAACCT
CCGACAAAAGCTCCATCACAGCCTACATGGAGTTCAGCTCTTTAACATCCGAG
GACAGCGCTCTGTACTATTGCGCCCGGTGGGGCGACGGCAATTACTGGGGACG
GGGCACAACACTGACCGTGAGCAGCGGAGGCGGAGGCTCCGGCGGAGGCGG
ATCTGGCGGTGGCGGCTCCGACATCGAGATGACCCAGTCCCCCGCTATCATGT
CCGCCTCTTTAGGCGAGCGGGTCACAATGACTTGTACAGCCTCCTCCAGCGTC
TCCTCCTCCTACTTCCATTGGTACCAACAGAAACCCGGAAGCTCCCCTAAACT
GTGCATCTACAGCACCAGCAATCTCGCCAGCGGCGTGCCCCCTAGGTTTTCCG
GAAGCGGAAGCACCAGCTACTCTTTAACCATCTCCTCCATGGAGGCTGAGGAT
GCCGCCACCTACTTTTGTACACCAGTACCACCGGTCCCCCACCTTCGGAGGCGG
CACCAAACCTGGAGACAAAGAGG (SEQ ID NO: 122).

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

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

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLAS
GVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRGGG

GSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQR
 PGQGLEWIGYINPSRGYTNYNQKFQDKATLTDDKSSSTAYMQLSSLTSEDSAVYY
 CARYYDDHYCLDYWGQGTTTLTVSSSGTTNTVAAYNLWKSTNFKTILEWEPKPV
 5 NQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVE
 STGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTF
 LSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFQSVQAVIPSR
 TVNRKSTDSPVECMGQEKGEFREVQLQQSGPELVKPGASVKMSCKASGYTFTSY
 VIQWVKQKPGQGLEWIGSINPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTS
 EDSALYYCARWGDGNYWGRGTTTLTVSSGGGGSGGGGSGGGGSDIEMTQSPAIM
 10 SASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYSTSNLASGVPPRFSGSG
 STSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR (SEQ ID NO: 123).

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

CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGA
 AGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACTGGTATC
 AGCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAGC
 20 TGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCACCAGCTAC
 TCTTTAACCATTTCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCCAG
 CAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAATCAA
 TCGTGGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAGCCA
 AGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCGGCGCCTCCGTC
 25 AAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCATTGG
 GTCAAGCAGAGGCCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAACCCTTC
 CCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTTTAACCA
 CTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACCAGCGAG
 GACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTGTTTAGAC
 30 TATTGGGGACAAGGTACCACTTTAACCGTCAGCAGCTCCGGCACCACCAATAC
 CGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGACAATTCTGG

AATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGATCTCCACCAAA
 TCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACCGAGTGTGATT
 TAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGGCTCGGGTCTTT
 TCCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGCGAGCCTCTCTA
 5 CGAGAATTCCCCCGAATTCACCCCTTATTTAGAGACCAATTTAGGCCAGCCTAC
 CATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTCACCGTCGAGGAT
 GAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCCTCCGGGATGTGTTC
 GGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCCAGCTCCTCCGGCAA
 AAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTGACGTGGACAAAGGC
 10 GAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTCTCGTACCGTCAACCG
 GAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTC
 CGGGAGGTCCAGCTGCAGCAGAGCGGACCCGAACCTCGTGAAACCCGGTGCTT
 CCGTGAAAATGTCTTGTAAGGCCAGCGGATACACCTTCACCTCCTATGTGATCC
 AGTGGGTCAAACAGAAGCCCGGACAAGGTCTCGAGTGGATCGGCAGCATCAA
 15 CCCTTACAACGACTATACCAAATACAACGAGAAGTTTAAGGGAAAGGCTACTT
 TAACCTCCGACAAAAGCTCCATCACAGCCTACATGGAGTTCAGCTCTTTAACA
 TCCGAGGACAGCGCTCTGTACTATTGCGCCCGGTGGGGCGACGGCAATTACTG
 GGGACGGGGCACAACACTGACCGTGAGCAGCGGAGGCGGAGGCTCCGGCGG
 AGGCGGATCTGGCGGTGGCGGCTCCGACATCGAGATGACCCAGTCCCCCGCTA
 20 TCATGTCCGCCTCTTTAGGCGAGCGGGTCACAATGACTTGTACAGCCTCCTCC
 AGCGTCTCCTCCTCCTACTTCCATTGGTACCAACAGAAACCCGGAAGCTCCCC
 TAAACTGTGCATCTACAGCACCAGCAATCTCGCCAGCGGCGTGCCCCCTAGGT
 TTTCGGAAGCGGAAGCACCAGCTACTCTTTAACCATCTCCTCCATGGAGGCT
 GAGGATGCCGCCACCTACTTTTGTACCAGTACCACCGGTCCCCCACCTTCGG
 25 AGGCGGCACCAAACCTGGAGACAAAGAGG (SEQ ID NO: 124).

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

MKWVTFISLLFLFSSAYSQIVLTQSPAIMSASPGKVTMTCSASSSVSYMNWYQQ
 KSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSS
 NPFTFGSGTKLEINRGGGGSGGGGSGGGGSQVQLQQSGAELARPGASVKMSCK
 ASGYTFTRYTMHWVKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSS
 5 TAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSSGTTNTVAAYNL
 TWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTDDTECDLTDEIVKD
 VKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTK
 VNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLI
 DVDKGENYCFVSQAVIPSRTVNRKSTDSPVECMGQEKGEFREVQLQQSGPELVK
 10 PGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGSINPYNDYTKYNEKFKG
 KATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNYWGRGTTTLTVSSGGGGSG
 GGGSGGGGSDIEMTQSPAIMSASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPK
 LCIYSTSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKL
 ETKR (SEQ ID NO: 125).

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

20 ATGAAGTGGGTGACCTTCATCAGCTTATTATTTTATTCAGCTCCGCCTATTCCC
 AGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGTGAGAA
 GGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAACTGGTATCA
 GCAGAAAAGCGGAACCAGCCCCAAAAGGTGGATCTACGACACCAGCAAGCT
 GGCCTCCGGAGTGCCCGCTCATTTCGGGGGCTCTGGATCCGGCACCAGCTACT
 25 CTTTAACCATTTCCGGCATGGAAGCTGAAGACGCTGCCACCTACTATTGCCAG
 CAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCTCGAAATCAA
 TCGTGGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAGCCA
 AGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCGGCCCGGCGCCTCCGTC
 AAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAATGCATTGG
 30 GTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATATCAACCCTTC
 CCGGGGCTACACCAACTATAACCAAAAAGTTCAAGGATAAAGCCACTTTAACCA

CTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTTAACCAGCGAG
GACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCACTACTGTTTAGAC
TATTGGGGACAAGGTACCACTTTAACCGTCAGCAGCTCCGGCACCACCAATAC
CGTGGCCGCTTATAACCTCACATGGAAGAGCACCAACTTCAAGACAATTCTGG
5 AATGGGAACCCAAGCCCGTCAATCAAGTTTACACCGTGCAGATCTCCACCAAA
TCCGGAGACTGGAAGAGCAAGTGCTTCTACACAACAGACACCGAGTGTGATT
TAACCGACGAAATCGTCAAGGACGTCAAGCAAACCTATCTGGCTCGGGTCTTT
TCCTACCCCGCTGGCAATGTCGAGTCCACCGGCTCCGCTGGCGAGCCTCTCTA
CGAGAATTCCCCGAATTCACCCCTTATTTAGAGACCAATTTAGGCCAGCCTAC
10 CATCCAGAGCTTCGAGCAAGTTGGCACCAAGGTGAACGTCACCGTCGAGGAT
GAAAGGACTTTAGTGCGGCGGAATAACACATTTTTATCCCTCCGGGATGTGTTC
GGCAAAGACCTCATCTACACACTGTACTATTGGAAGTCCAGCTCCTCCGGCAA
AAAGACCGCTAAGACCAACACCAACGAGTTTTTAATTGACGTGGACAAAGGC
GAGAACTACTGCTTCAGCGTGCAAGCCGTGATCCCTTCTCGTACCGTCAACCG
15 GAAGAGCACAGATTCCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTC
CGGGAGGTCCAGCTGCAGCAGAGCGGACCCGAACCTCGTGAAACCCGGTGCTT
CCGTGAAAATGTCTTGTAAGGCCAGCGGATACACCTTCACCTCCTATGTGATCC
AGTGGGTCAAACAGAAGCCCGGACAAGGTCTCGAGTGGATCGGCAGCATCAA
CCCTTACAACGACTATACCAAATACAACGAGAAGTTTAAGGGAAAGGCTACTT
20 TAACCTCCGACAAAAGCTCCATCACAGCCTACATGGAGTTCAGCTCTTTAACA
TCCGAGGACAGCGCTCTGTACTATTGCGCCCGGTGGGGCGACGGCAATTACTG
GGGACGGGGCACAACACTGACCGTGAGCAGCGGAGGCGGAGGCTCCGGCGG
AGGCGGATCTGGCGGTGGCGGCTCCGACATCGAGATGACCCAGTCCCCCGCTA
TCATGTCCGCCTCTTTAGGCGAGCGGGTCACAATGACTTGTACAGCCTCCTCC
25 AGCGTCTCCTCCTCCTACTTCCATTGGTACCAACAGAAACCCGGAAGCTCCCC
TAAACTGTGCATCTACAGCACCAGCAATCTCGCCAGCGGCGTGCCCCCTAGGT
TTTCCGGAAGCGGAAGCACCAGCTACTCTTTAACCATCTCCTCCATGGAGGCT
GAGGATGCCGCCACCTACTTTTGTACCAGTACCACCGGTCCCCCACCTTCGG
AGGCGGCACCAAACCTGGAGACAAAGAGG (SEQ ID NO: 126).

Exemplary Embodiments of Single-Chain Chimeric Polypeptides- Type B

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

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

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

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

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKH
LQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADET
ATIVEFLNRWITFCQSIISTLT (SEQ ID NO: 17).

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

GCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCATTACT
 GCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCCAACT
 CACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAACTGA
 AACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAA
 5 TTTAGCTCAAAGCAAAAACCTTTCACTTAAGACCCAGGGACTTAATCAGCAATA
 TCAACGTAATAGTTCTGGAACTAAAGGGATCTGAAACAACATTCATGTGTGAA
 TATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGATGGATTACCTTT
 TGTCAAAGCATCATCTCAACACTAACT (SEQ ID NO: 127).

In some embodiments, an IL-2 protein that binds specifically to an IL-2 receptor
 10 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
 15 GCTGGATTTACAGATGATTTTAAACGGCATCAACAACCTACAAGAACCCCAAGC
 TGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCACCGAGCTG
 AAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAGGTGCTGA
 ATTTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAATCAGCAACA
 TCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTCATGTGCGAG
 20 TACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTTGGATCACCTT
 CTGCCAGTCCATCATCTCCACTTTAACC (SEQ ID NO: 128).

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

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

30 APTSSSTKKTKQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKH
 LQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADET

ATIVEFLNRWITFCQSIISTLTSGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYT
 VQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG
 EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDV
 FGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRK
 5 STDSPVECMGQEKGEFREAPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLT
 RMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIV
 LELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT (SEQ ID NO: 129).

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

GCCCCACCTCCTCCTCCACCAAGAAGACCCAGCTGCAGCTGGAGCATTACT
 GCTGGATTACAGATGATTTTAAACGGCATCAACAACACTACAAGAACCCCAAGC
 15 TGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCACCGAGCTG
 AAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAGGTGCTGA
 ATTTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAATCAGCAACA
 TCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTCATGTGCGAG
 TACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTTGGATCACCTT
 20 CTGCCAGTCCATCATCTCCACTTTAACCAGCGGCACAACCAACACAGTCGCTG
 CCTATAACCTCACTTGGAAGAGCACCAACTTCAAAACCATCCTCGAATGGGAA
 CCCAAACCCGTTAACCAAGTTTACACCGTGCAGATCAGCACCAAGTCCGGCG
 ACTGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTACCGAT
 GAGATCGTGAAAGATGTGAAACAGACCTACCTCGCCCGGGTGTTTAGCTACCC
 25 CGCCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACGAGAAC
 AGCCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCCACCATCCA
 AAGCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGAGGACGAGCG
 GACTTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCA
 AAGATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGA
 30 CAGCTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAAGGCGAAAA
 CTACTGTTTCAGCGTGCAAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAA

GCACCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGA
 GGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCATTTAC
 TGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCAAAC
 TCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAACTG
 5 AAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAA
 ATTTAGCTCAAAGCAAAAACCTTTCCTTAAGACCCAGGGACTTAATCAGCAAT
 ATCAACGTAATAGTTCTGGAACTAAAGGGATCTGAAACAACATTCATGTGTGA
 ATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGATGGATTACCTT
 TTGTCAAAGCATCATCTCAACACTAACT (SEQ ID NO: 130).

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

15 MKWVTFISLLFLFSSAYSAPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTR
 MLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVL
 ELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTSGTTNTVAAYNLTWKSTN
 FKILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTDTECDLTDEIVKDVKQTYLA
 RVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVED
 20 ERTLVRNRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGEN
 YCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFREAPTSSSTKKTQLQLEHLLLDL
 QMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSK
 NFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT
 (SEQ ID NO: 131).

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

30 ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCTCCAGCGCCTACTCC
 GCCCCACCTCCTCCTCCACCAAGAAGACCCAGCTGCAGCTGGAGCATTTACT

GCTGGATTTACAGATGATTTTAAACGGCATCAACAACACTACAAGAACCCCAAGC
TGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCACCGAGCTG
AAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCTCGAGGAGGTGCTGA
ATTTAGCCCAGTCCAAGAATTTCCATTTAAGGCCCCGGGATTTAATCAGCAACA
5 TCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTCATGTGCGAG
TACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTTGGATCACCTT
CTGCCAGTCCATCATCTCCACTTTAACCAGCGGCACAACCAACACAGTCGCTG
CCTATAACCTCACTTGGAAGAGCACCAACTTCAAAACCATCCTCGAATGGGAA
CCCAAACCCGTTAACCAAGTTTACACCGTGCAGATCAGCACCAAGTCCGGCG
10 ACTGGAAGTCCAAATGTTTCTATACCACCGACACCGAGTGCGATCTCACCGAT
GAGATCGTGAAAGATGTGAAACAGACCTACCTCGCCCGGGTGTTTAGCTACCC
CGCCGGCAATGTGGAGAGCACTGGTTCCGCTGGCGAGCCTTTATACGAGAAC
AGCCCCGAATTTACCCCTTACCTCGAGACCAATTTAGGACAGCCCACCATCCA
AAGCTTTGAGCAAGTTGGCACAAAGGTGAATGTGACAGTGGAGGACGAGCG
15 GACTTTAGTGCGGCGGAACAACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCA
AAGATTTAATCTACACACTGTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGA
CAGCTAAAACCAACACAAACGAGTTTTTAATCGACGTGGATAAAGGCGAAAA
CTACTGTTTCAGCGTGCAAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAA
GCACCGATAGCCCCGTTGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGA
20 GGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCATTTAC
TGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCAAAC
TCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAACTG
AAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAA
ATTTAGCTCAAAGCAAAAACCTTCACTTAAGACCCAGGGACTTAATCAGCAAT
25 ATCAACGTAATAGTTCTGGAATAAAGGGATCTGAAACAACATTCATGTGTGA
ATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGATGGATTACCTT
TTGTCAAAGCATCATCTCAACACTAACT (SEQ ID NO: 132).

Additional Therapeutic Agents

30 Some embodiments of any of the methods described herein can further include administering to a subject (e.g., any of the subjects described herein) a therapeutically

effective amount of one or more additional therapeutic agents. The one or more additional therapeutic agents can be administered to the subject at substantially the same time as the multi-chain chimeric polypeptide (e.g., any of the multi-chain chimeric polypeptides described herein) or immune cell (e.g., administered as a single formulation or two or more formulations to the subject). In some embodiments, one or more additional therapeutic agents can be administered to the subject prior to administration of the multi-chain chimeric polypeptide (e.g., any of the multi-chain chimeric polypeptides described herein) or immune cell. In some embodiments, one or more additional therapeutic agents can be administered to the subject after administration of the multi-chain chimeric polypeptide (e.g., any of the multi-chain chimeric polypeptides described herein) or immune cell to the subject.

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

Non-limiting examples of anticancer drugs include antimetabolic drugs (e.g., 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, hydroxycarbamide, methotrexate, 6-thioguanine, cladribine, nelarabine, pentostatin, or pemetrexed), plant alkaloids (e.g., vinblastine, vincristine, vindesine, camptothecin, 9-methoxycamptothecin, coronaridine, taxol, naucleorals, diprenylated indole alkaloid, montamine, schischkiniin, protoberberine, berberine, sanguinarine, chelerythrine, chelidonine, liriiodenine, 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, 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
5 (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,
10 temozolomide, carmustine, lumustine, streptozocin, carboplatin, cisplatin, and oxaliplatin.

Non-limiting examples of activating receptor agonists include any agonists for activating receptors which activate and enhance the cytotoxicity of NK cells, including anti-CD16 antibodies (e.g., anti-CD16/CD30 bispecific monoclonal antibody (BiMAb))
15 and Fc-based fusion proteins. Non-limiting examples of checkpoint inhibitors include anti-PD-1 antibodies (e.g., MEDI0680), anti-PD-L1 antibodies (e.g., BCD-135, BGB-A333, CBT-502, CK-301, CS1001, FAZ053, KN035, MDX-1105, MSB2311, SHR-1316, anti-PD-L1/CTLA-4 bispecific antibody KN046, anti-PD-L1/TGF β R2 fusion protein M7824, anti-PD-L1/TIM-3 bispecific antibody LY3415244, atezolizumab, or
20 avelumab), anti-TIM3 antibodies (e.g., TSR-022, Sym023, or MBG453) and anti-CTLA-4 antibodies (e.g., AGEN1884, MK-1308, or an anti-CTLA-4/OX40 bispecific antibody ATOR-1015). Non-limiting examples of agents for blocking HLA-specific inhibitory receptors include monalizumab (e.g., an anti-HLA-E NKG2A inhibitory receptor monoclonal antibody). Non-limiting examples of GSK3 inhibitor include tideglusib or
25 CHIR99021. Non-limiting examples of antibodies that can be used as additional therapeutic agents include anti-CD26 antibodies (e.g., YS110), anti-CD36 antibodies, and any other antibody or antibody construct that can bind to and activate an Fc receptor (e.g., CD16) on a NK cell. In some embodiments, an additional therapeutic agent can be insulin or metformin.

EXAMPLES

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

5 **Example 1. Construction of exemplary multi-chain chimeric polypeptides and evaluation of properties thereof**

Two multi-chain chimeric polypeptides were generated and their properties were evaluated. Each of the two multi-chain chimeric polypeptides includes a first chimeric polypeptide that includes a soluble tissue factor domain covalently linked a first target-
10 binding domain and a first domain of an affinity pair of domains. The second chimeric polypeptide in each of the two multi-chain chimeric polypeptides includes a second domain of the affinity pair of domains, and a second target-binding domain.

Description of logic underlying construction of multi-chain chimeric polypeptides

15 Tissue factor (TF) is a stable, transmembrane protein containing 236 amino acid residues. The truncated, recombinant 219-amino-acid extracellular domain of tissue factor is soluble and is known to be expressed at high levels in bacteria or mammalian cells. Without wishing to be bound to a particular theory, the applicants speculated that the 219-aa tissue factor could be used as a connector linker for creation of unique multi-
20 chain chimeric polypeptides.

First chimeric polypeptides including soluble tissue factor domain were produced at high levels by CHO cells grown in fermentation broth. These first chimeric polypeptides were purified by an anti-tissue factor monoclonal antibody (mAb) coupled on a solid matrix. Notably, tissue factor contains binding sites for FVIIa and FX. The
25 catalytic activity of the tissue factor-FVIIa complex for FX is approximately 1 million-fold lower when tissue factor is not anchored to a phospholipid bilayer. Thus, without wishing to be bound to a particular theory, applicants speculated that using the 219-aa extracellular domain of tissue factor without the transmembrane in construction of the first chimeric polypeptides may eliminate the pro-coagulation activity of tissue factor in
30 the first chimeric polypeptides. In an effort to further reduce or eliminate the pro-coagulation activity of the 219-aa tissue factor, select mutations in tissue factor can be

made, specifically at seven amino acid residues that are known to contribute to binding energy of the FVIIa binding site.

Characterization of binding interactions for described chimeric polypeptides

5 To determine if the first and second chimeric polypeptides bind to each other to form multi-chain chimeric polypeptides, *in vitro* binding assays were performed. To determine if the first chimeric polypeptide comprising soluble tissue factor domain are recognized and bound by anti-TF mAb, *in vitro* binding assays were performed. Notably, the data indicated that the mutated tissue factor proteins are still recognized and
10 selectively bound by the anti-TF mAb which is known to bind to the FX binding site on tissue factor. To determine if the first chimeric polypeptides comprising soluble tissue factor domain covalently linked to scFvs or cytokines (see Figure 1 and Figure 2) possess functional scFvs or cytokines, *in vitro* binding assays were performed. The data from the aforementioned assays were consistent with the purified first chimeric polypeptides
15 having the expected biological activities (e.g. scFvs selectively bind expected target antigens or cytokines selectively bind expected receptors or binding proteins).

In addition, experiments performed using the two multi-chain chimeric polypeptides including a first and second chimeric polypeptide bound to each other demonstrate the expected target binding activity (e.g., the multi-chain chimeric
20 polypeptide binds specifically to the target specifically recognized by the first target-binding domain and the target specifically recognized by the second target-binding domain).

Based on the aforementioned results, applicants concluded that the soluble tissue factor connector linker provided or enabled appropriate display of the polypeptides
25 encoding either scFvs, interleukins, cytokines, interleukin receptors, or cytokine receptors in three-dimensional space relative to soluble tissue factor domain and relative to one another such that each retained expected biological properties and activities.

When both the first and second chimeric polypeptides were co-expressed, the heterodimeric complexes were secreted into the fermentation broths at high levels. The
30 complexes were captured and readily purified by anti-TF mAb conjugated to a solid matrix using affinity chromatography. The first and second target-binding domains of

these multi-chain chimeric polypeptides retained their expected biological activities as assayed by *in vitro* binding assays. Thus, the assembly of the multi-chain chimeric polypeptides provides the appropriate spatial display and folding of the domains for biological activities. Importantly, the spatial arrangement of the multi-chain chimeric polypeptides does not interfere with the FX binding site on tissue factor which enables the use of anti-TF mAb for affinity purification.

Characterization of stability for described chimeric polypeptides

Both purified multi-chain chimeric polypeptides are stable. These multi-chain chimeric polypeptides are structurally intact and fully biologically active when they are incubated in human serum at 37 °C for 72 hours.

Characterization of propensity of described chimeric polypeptides to aggregate

Both purified multi-chain chimeric polypeptides developed do not form aggregates when stored at 4 °C in PBS.

Characterization of viscosity of described chimeric polypeptides

There is no viscosity issue when the multi-chain chimeric polypeptides are formulated at a concentration as high as 50 mg/mL in PBS.

Additional applications of the multi-chain chimeric polypeptide platform

The data from these studies show that the platform technologies described herein can be utilized to create molecules that could be fused to target-binding domains derived from antibodies, in any of the formats as described herein including, without limitation, adhesion molecules, receptors, cytokines, ligands, and chemokines. With the appropriate target-binding domain, the resulting multi-chain chimeric polypeptides could promote conjugation of various immune effector cells and mediate destruction of target cells, including cancer cells, virally-infected cells, or senescent cells. Other domains in the multi-chain chimeric polypeptides stimulate, activate, and attract the immune system for enhancing cytotoxicity of effector cells for the targeted cells.

Example 2: Creation of an IL-12/IL-15R α Su DNA construct

In a non-limiting example, an IL-12/IL-15R α Su DNA construct was created (Figure 3). The human IL-12 subunit sequences, human IL-15R α Su sequence, human IL-15 sequence, human tissue factor 219 sequence, and human IL-18 sequence were obtained from the UniProt website and DNA for these sequences was synthesized by Genewiz. A DNA construct was made linking the IL-12 subunit beta (p40) to IL-12 subunit alpha (p35) with a GS (3) linker to generate a single chain version of IL-12 and then directly linking the IL-12 sequence to the IL-15R α Su sequence. The final IL-12/IL-15R α Su DNA construct sequence was synthesized by Genewiz.

The nucleic acid sequence of the IL12/IL-15R α Su construct (including signal peptide sequence) is as follows (SEQ ID NO: 85):

(Signal peptide)

ATGAAATGGGTGACCTTTATTTCTTTACTGTTCTCTTTAGCAGCGCCT
ACTCC

(Human IL-12 subunit beta (p40))

ATTTGGGAACTGAAGAAGGACGTCTACGTGGTCGAACTGGACTGGTAT
CCCGATGCTCCCGGCGAAATGGTGGTGCTCACTTGTGACACCCCGAAGAAG
ACGGCATCACTTGGACCCTCGATCAGAGCAGCGAGGTGCTGGGCTCCGGAAA
GACCCTCACAATCCAAGTTAAGGAGTTCGGAGACGCTGGCCAATACACATGC
CACAAGGGAGGCGAGGTGCTCAGCCATTCTTATTATTATTACACAAGAAGG
AAGACGGAATCTGGTCCACCGACATTTTAAAAGATCAGAAGGAGCCCAAGA
ATAAGACCTTTTTAAGGTGTGAGGCCAAAACTACAGCGGTCGTTTCACTTGT
TGGTGGCTGACCACCATTTCCACCGATTTAACCTTCTCCGTGAAAAGCAGCCG
GGGAAGCTCCGACCCTCAAGGTGTGACATGTGGAGCCGCTACCTCAGCGCT
GAGAGGGTTCGTGGCGATAACAAGGAATACGAGTACAGCGTGGAGTGCCAA
GAAGATAGCGCTTGTCCTCGCTGCCGAAGAATCTTTACCCATTGAGGTGATGG
TGGACGCCGTGCACAACTCAAGTACGAGAACTACACCTCCTCCTTCTTTATC
CGGGACATCATTAAGCCCGATCCTCCTAAGAATTTACAGCTGAAGCCTCTCA
AAAATAGCCGGCAAGTTGAGGTCTCTTGGGAATATCCCGACACTTGGAGCAC
ACCCACAGCTACTTCTCTTTAACCTTTTGTGTGCAAGTTCAAGGTAAAAGCA
AGCGGGAGAAGAAAGACCGGGTGTTTACCGACAAAACCAGCGCCACCGTCA

TCTGTCGGAAGAACGCCTCCATCAGCGTGAGGGCTCAAGATCGTTATTACTCC
AGCAGCTGGTCCGAGTGGGCCAGCGTGCCTTGTTCC

(Linker)

GGCGGTGGAGGATCCGGAGGAGGTGGCTCCGGCGGGCGGAGGATCT

5 *(Human IL-12 subunit alpha (p35))*

CGTAACCTCCCCGTGGCTACCCCCGATCCCGGAATGTTCCCTTGTTTAC
ACCACAGCCAGAATTTACTGAGGGCCGTGAGCAACATGCTGCAGAAAGCTAG
GCAGACTTTAGAATTTTACCCTTGACACGAGGAGATCGACCATGAAGAT
ATCACCAAGGACAAGACATCCACCGTGGAGGCTTGTTTACCTCTGGAGCTGA
10 CAAAGAACGAGTCTTGTCTCAACTCTCGTGAAACCAGCTTCATCACAAATGG
CTCTTGTTTAGCTTCCCGGAAGACCTCCTTTATGATGGCTTTATGCCTCAGCTC
CATCTACGAGGATTTAAAGATGTACCAAGTGGAGTTCAAGACCATGAACGCC
AAGCTGCTCATGGACCCTAAACGGCAGATCTTTTACAGACAGAACATGCTGG
CTGTGATTGATGAGCTGATGCAAGCTTTAAACTTCAACTCCGAGACCGTCCCT
15 CAGAAGTCCTCCCTCGAGGAGCCCGATTTTACAAGACAAAGATCAAACCTGT
GCATTTTACTCCACGCCTTTAGGATCCGGGCCGTGACCATTGACCGGGTCATG
AGCTATTTAAACGCCAGC

(Human IL-15R α sushi domain)

ATTACATGCCCCCTCCCATGAGCGTGGAGCACGCCGACATCTGGGTG
20 AAGAGCTATAGCCTCTACAGCCGGGAGAGGTATATCTGTAACAGCGGCTTCA
AGAGGAAGGCCGGCACCAGCAGCCTCACCGAGTGCGTGCTGAATAAGGCTA
CCAACGTGGCTCACTGGACAACACCCTCTTTAAAGTGCATCCGG

Example 3: Creation of an IL-18/TF/IL-15 DNA construct

25 In a non-limiting example, an IL-18/TF/IL-15 construct was made (Figure 4) linking the IL-18 sequence to the N-terminus coding region of tissue factor 219, and further linking the IL-18/TF construct with the N-terminus coding region of IL-15. The nucleic acid sequence of the IL-18/TF/IL-15 construct (including leader sequence), synthesized by Genewiz, is as follows (SEQ ID NO: 81):

30 *(Signal peptide)*

ATGAAGTGGGTCACATTTATCTCTTTACTGTTCCCTCTTCTCCAGCGCCT
ACAGC

(Human IL-18)

TACTTCGGCAAACCTGGAATCCAAGCTGAGCGTGATCCGGAATTTAAAC
5 GACCAAGTTCTGTTTATCGATCAAGGTAACCGGCCTCTGTTTCGAGGACATGAC
CGACTCCGATTGCCGGGACAATGCCCCCGGACCATCTTCATTATCTCCATGT
ACAAGGACAGCCAGCCCCGGGGCATGGCTGTGACAATTAGCGTGAAGTGTGA
GAAAATCAGCACTTTATCTTGTGAGAACAAGATCATCTCCTTTAAGGAAATG
AACCCCCCGATAACATCAAGGACACCAAGTCCGATATCATCTTCTTCCAGC
10 GGTCCGTGCCCGGTCACGATAACAAGATGCAGTTCGAATCCTCCTCCTACGA
GGGCTACTTTTTAGCTTGTGAAAAGGAGAGGGATTTATTCAAGCTGATCCTCA
AGAAGGAGGACGAGCTGGGCGATCGTTCCATCATGTTACCGTCCAAAACGA
GGAT

(Human Tissue Factor 219)

15 AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAG
CACCAACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTT
TACACCGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCT
ATACCACCGACACCGAGTGCGATCTCACCGATGAGATCGTGAAAGATGTGAA
ACAGACCTACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGC
20 ACTGGTTCCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTA
CCTCGAGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGC
ACAAAGGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAAC
AACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCAAAGATTTAATCTACACACT
GTATTACTGGAAGTCCTCTTCTCCTCCGGCAAGAAGACAGCTAAAACCAACACA
25 AACGAGTTTTTAATCGACGTGGATAAAGGCGAAAACACTACTGTTTCAGCGTGC
AAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGT
TGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(Human IL-15)

AACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATTTAATT
30 CAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTC
TTGTAAGGTGACCGCCATGAAATGTTTTTTTACTGGAGCTGCAAGTTATCTCTT

TAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTT
 AGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAG
 GAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTG
 TGCACATTGTCCAGATGTTTCATCAATACCTCC

5

Example 4: Secretion of IL-12/IL-15R α Su and IL-18/TF/IL-15 fusion proteins

The IL-12/IL-15R α Su and IL-18/TF/IL-15 DNA constructs were cloned into a pMSGV-1 modified retrovirus expression vector (as described by Hughes, *Hum Gene Ther* 16:457–72, 2005, hereby incorporated by reference), and the expression vector was
 10 transfected into CHO-K1 cells. Co-expression of the two constructs in CHO-K1 cells allowed for formation and secretion of a soluble IL-18/TF/IL-15:IL-12/IL-15R α Su protein complex (referred to as 18t15-12s; Figure 5 and Figure 6). The 18t15-12s protein was purified from CHO-K1 cell culture supernatant using anti-TF antibody affinity chromatography and size exclusion chromatography resulting in soluble (non-aggregated)
 15 protein complexes consisting of IL-12/IL-15R α Su and IL-18/TF/IL-15 fusion proteins.

The amino acid sequence of the IL12/IL-15R α Su fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 84):

(Signal peptide)

MKWVTFISLLFLFSSAYS

20

(Human IL-12 subunit beta (p40))

IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGS
 GKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLHKKEDGIWSTDILKDQKEPKN
 KTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERV
 RGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIKPD
 25 PPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVF
 TDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCS

(Linker)

GGGSGGGSGGGGS

(Human IL-12 subunit alpha (p35))

30

RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPTSEEIDHE
 DITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYE

DLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSS
LEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKAT

5 NVAHWTTPSLKCIR

The amino acid sequence of the IL-18/TF/IL-15 fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 80):

(Signal peptide)

MKWVTFISLLFLFSSAYS

10 *(Human IL-18)*

YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISM
YKDSQPRGMAVTISVKCEKISTLSCENKIISFKEMNPPDNIKDTKSDIIFQRSVPG
HDNKMQFESSYEGYFLACEKERDLFKLILKKEDELGDRSIMFTVQNED

(Human Tissue Factor 219)

15 SGTNTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLYTLYYW
KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
KGEFRE

20 *(Human IL-15)*

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCCKVTAMKCFLELQVIS
LESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQ
MFINTS

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

Example 5: Purification of 18t15-12s by immunoaffinity chromatography

30 An anti-TF antibody affinity column was connected to a GE Healthcare™ AKTA Avant protein purification system. The flow rate was 4 mL/min for all steps except the elution step, which was 2 mL/min.

Cell culture harvest of 18t15-12s was adjusted to pH 7.4 with 1M Tris base and loaded onto the anti-TF antibody affinity column equilibrated with 5 column volumes of PBS. After loading the sample, the column was washed with 5 column volumes PBS, followed by elution with 6 column volumes 0.1M acetic acid, pH 2.9. Absorbance at 280 nm was collected and then the sample was neutralized 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. Figure 7 shows that the 18t15-12s complex binds the anti-TF antibody affinity column, wherein TF is an 18t15-12s binding partner. The buffer-exchanged protein sample is stored at 2-8°C for further biochemical analysis and biological activity testing.

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% sodium azide and stored at 2-8°C.

Example 6: Size exclusion chromatography of 18t15-12s

A GE Healthcare Superdex® 200 Increase 10/300 GL gel filtration column was connected to a GE Healthcare AKTA™ Avant protein purification system. The column was equilibrated with 2 column volumes of PBS. The flow rate was 0.8 mL/min. A capillary loop was used to inject 200µL of 1 mg/mL of 18t15-12s complex onto the column. The injection was chased with 1.25 column volumes of PBS. The SEC chromatograph is shown in Figure 8. There is a main 18t15-12s protein peak with a minor high molecular weight peak, likely due to differing degrees of glycosylation of 18t15-12s dimers or aggregates.

Example 7: SDS-PAGE of 18t15-12s

To determine the purity and protein molecular weight, the purified 18t15-12s protein sample was analyzed using 4-12% NuPage Bis-Tris protein gel SDS-PAGE. The gel was stained with InstantBlue™ for about 30 min, followed by destaining overnight in purified water. Figure 9 shows an example SDS gel of anti-TF antibody affinity purified 18t15-12s, with bands at the expected molecular weights (66 kDa and 56 kDa).

Example 8: Glycosylation of 18t15-12s in CHO-K1 cells

Glycosylation of 18t15-12s in CHO-K1 cells was confirmed using the Protein Deglycosylation Mix II kit (New England Biolabs), according to the manufacturer's instructions. Figure 10 shows an example SDS PAGE of deglycosylated and non-
5 deglycosylated 18t15-12s. Deglycosylation reduces the molecular weight of 18t15-12s as seen in Figure 10, lane 4.

Example 9: Recombinant protein quantitation of 18t15-12s complexes

The 18t15-12s complex was detected and quantified using standard sandwich
10 ELISA methods (Figures 11-14). Anti-human tissue factor antibody served as the capture antibody and biotinylated anti-human IL-12, IL-15, or IL-18 antibody (BAF 219, BAM 247, D045-6, all R&D Systems) served as the detection antibody. Tissue factor in purified 18t15-12s protein complexes was also detected using an anti-human tissue factor capture antibody (I43), and anti-human tissue factor antibody detection antibody. The
15 I43/ anti-TF antibody ELISA was compared to purified tissue factor at similar concentrations.

Example 10: Immunostimulatory capacity of the 18t15-12s complex

To assess the IL-15 immunostimulatory activity of the 18t15-12s complex,
20 increasing concentrations of 18t15-12s was added to 32D β cells (104 cell/well) in 200 μ L IMDM:10% FBS media. The 32D β cells were incubated for 3 days at 37°C. On the fourth day, WST-1 proliferation reagent (10 μ L/well) was added and after 4 hours, absorbance was measured at 450 nm to determine cell proliferation based on cleavage of WST-1 to a soluble formazan dye. Bioactivity of human recombinant IL-15 was assessed
25 as a positive control. As shown in Figure 15, 18t15-12s demonstrated IL-15-dependent cell proliferation of 32D β cells. The 18t15-12s complex demonstrated reduced activity compared to human recombinant IL-15, possibly due to the linkage of IL-18 and tissue factor to the IL-15 domain.

In order to assess the individual activities of IL-12 and IL-18 in the 18t15-12s
30 complex, 18t15-12s was added to HEK-Blue IL-12 and HEK-Blue IL-18 reporter cells (5×10^4 cell/well; hkb-il12 and hkb-hmil18, InvivoGen) in 200 μ L IMDM:10% heat-

inactivated FBS media. Cells were incubated for overnight at 37°C. 20 µl of induced HEK-Blue IL-12 and HEK-Blue IL-18 reporter cell supernatant was added to 180 µl of QUANTI-Blue (InvivoGen), and incubated for 1-3 hours at 37°C. IL-12 or IL-18 activity was assessed by measuring absorbance at 620 nm. Human recombinant IL-12 or IL-18 was assessed as a positive or negative control. As shown in Figure 16 and Figure 17, each of the cytokine domains of the 18t15-12s complex retain specific biological activity. The activity of 18t15-12s was reduced compared to that of human recombinant IL-18 or IL-12, possibly due to linkage of IL-15 and tissue factor to the IL-18 domain and linkage of IL-12 to the IL-15R α sushi domain.

Example 11: Induction of cytokine-induced memory-like NK cells by the 18t15-12s complex

Cytokine-induced memory-like NK cells can be induced *ex vivo* following overnight stimulation of purified NK cells with saturating amounts of IL-12 (10 ng/mL), IL-15 (50 ng/mL), and IL-18 (50 ng/mL). These memory-like properties have been measured through expression of IL-2 receptor α (IL-2R α , CD25), CD69 (and other activation markers), and increased IFN- γ production. To evaluate the ability of 18t15-12s complexes to promote generation of cytokine-induced memory-like NK cells, purified human NK cells (>95% CD56+) were stimulated for 14-18 hours with 0.01nM to 10000 nM of the 18t15-12s complex or a combination of individual cytokines (recombinant IL-12 (10 ng/mL), IL-18 (50 ng/mL), and IL-15 (50 ng/mL)). Cell-surface CD25 and CD 69 expression and intracellular IFN- γ levels were assessed by antibody-staining and flow cytometry.

Fresh human leukocytes were obtained from a blood bank and CD56+ NK cells were isolated with the RosetteSep/human NK cell reagent (StemCell Technologies). The purity of NK cells was >70% and confirmed by staining with antibodies specific to CD56-BV421, CD16-BV510, CD25-PE, CD69-APCFire750 (BioLegend). Cells were counted and resuspended in 0.2×10^6 /mL in a 96 well flat bottom plate in 0.2 mL of complete media (RPMI 1640 (Gibco), supplemented with 2 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone)). Cells were stimulated with either a mixture of

cytokines hIL-12 (10 ng/mL) (Biolegend), hIL-18 (50 ng/mL) (R&D Systems) and hIL-15 (50 ng/mL) (NCI) or with 0.01 nM to 10000 nM of 18t15-12s at 37°C, 5% CO₂ for 14-18 hrs. The cells were then harvested and surface stained with antibodies specific to CD56-BV421, CD16-BV510, CD25-PE, CD69-APCFire750 (BioLegend) for 30 minutes. After staining, 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, cells were analyzed using a BD FACSCelesta™ flow cytometer (Plotted Data-Mean Fluorescence Intensity; Figure. 18A and Figure 18B).

Fresh human leukocytes were obtained from a blood bank and CD56+ NK cells were isolated with the RosetteSep/human NK cell reagent (StemCell Technologies). The purity of NK cells was >70% and confirmed by staining with CD56-BV421, CD16-BV510, CD25-PE, CD69-APCFire750 specific antibodies (BioLegend). Cells were counted and resuspended in 0.2×10^6 /mL in a 96 well flat bottom plate in 0.2 mL of complete media (RPMI 1640 (Gibco), supplemented with 2 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone)). Cells were stimulated with either a cytokine mix of hIL-12 (10 ng/mL) (Biolegend), hIL-18 (50 ng/mL) (R&D), and hIL-15 (50 ng/mL) (NCI), or 0.01 nM to 10000 nM of the 18t15-12s complex at 37°C, 5% CO₂ for 14-18 hrs. The cells were then treated with 10 µg/mL of Brefeldin A (Sigma) and 1X of Monensin (eBioscience) for 4 hrs before harvesting and staining with antibodies specific to CD56-BV421, CD16-BV510, CD25-PE, CD69-APCFire750 for 30 minutes. After staining, cells were washed (1500 RPM for 5 minutes in room temperature) in FACS buffer (1X PBS (Hyclone), with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)) and fixed for 10 minutes at room temperature. After fixation, cells were washed (1500 RPM for 5 minutes in room temperature) in 1x permeabilized buffer (eBioscience) and stained with IFN-γ- PE Ab (Biolegend) for 30 minutes at room temperature. Cells were washed once again with 1x permeabilized buffer and then washed with FACS buffer. Cell pellets were resuspended in 300 µl of FACS buffer and analyzed using a BD FACSCelesta™ flow cytometer (Plotted % of IFN-γ Positive Cells; Figure 19).

Example 12: In vitro cytotoxicity of NK cells against human tumor cells

Human myelogenous leukemia cells, K562 (CellTrace violet labelled), were incubated with purified human NK cells in the presence of increasing concentrations of the 18t15-12s complex or a mixture of cytokines as a control. After 20 hours, the cultures were harvested, stained with propidium iodide (PI), and assessed by flow cytometry. As shown in Figure 20, the 18t15-12s complex induced human NK cytotoxicity against K562, at levels similar or greater than the cytokine mixture, wherein both the 18t15-12s complex and the cytokine mixture induced greater cytotoxicity than the medium control.

Example 13: Creation of IL-18/IL-15R α Su and IL-12/TF/IL-15 DNA constructs

In a non-limiting example, IL-18/IL-15R α Su and IL-12/TF/IL-15 DNA constructs were created. The human IL-18 subunit sequences, human IL-15R α Su sequence, human IL-12 sequence, human tissue factor 219 sequence, and human IL-15 sequence were synthesized by Genewiz. A DNA construct was made linking IL-18 directly to IL-15R α Su. An additional construct was also made linking IL-12 sequence to the N-terminus coding region of human tissue factor 219 form, and further linking the IL-12/TF construct to the N-terminus coding region of IL-15. As described above, a single-chain version of IL-12 (p40-linker-p35) was used.

The nucleic acid sequence of the IL-18/IL-15R α Su construct (including signal peptide sequence) is as follows (SEQ ID NO: 133):

(Signal peptide)

ATGAAGTGGGTCACATTTATCTCTTTACTGTTCCCTCTTCTCCAGCGCCT
ACAGC

(Human IL-18)

TACTTCGGCAAACCTGGAATCCAAGCTGAGCGTGATCCGGAATTTAAAC
GACCAAGTTCTGTTTATCGATCAAGGTAACCGGCCTCTGTTTCGAGGACATGAC
CGACTCCGATTGCCGGGACAATGCCCCCGGACCATCTTCATTATCTCCATGT
ACAAGGACAGCCAGCCCCGGGGCATGGCTGTGACAATTAGCGTGAAGTGTGA
GAAAATCAGCACTTTATCTTGTGAGAACAAGATCATCTCCTTTAAGGAAATG
AACCCCCCGATAACATCAAGGACACCAAGTCCGATATCATCTTCTTCCAGC

GGTCCGTGCCCCGGTCACGATAACAAGATGCAGTTCGAATCCTCCTCCTACGA
GGGCTACTTTTTAGCTTGTGAAAAGGAGAGGGATTTATTCAAGCTGATCCTCA
AGAAGGAGGACGAGCTGGGCGATCGTTCCATCATGTTACCGTCCAAAACGA
GGAT

5 (*Human IL-15R α sushi domain*)

ATTACATGCCCCCTCCCATGAGCGTGGAGCACGCCGACATCTGGGTG
AAGAGCTATAGCCTCTACAGCCGGGAGAGGTATATCTGTAACAGCGGCTTCA
AGAGGAAGGCCGGCACCAGCAGCCTCACCGAGTGCGTGCTGAATAAGGCTA
CCAACGTGGCTCACTGGACAACACCCTCTTTAAAGTGCATCCGG

10 The nucleic acid sequence of the IL-12/TF/IL-15 construct (including leader
sequence) is as follows (SEQ ID NO: 134):

 (*Signal peptide*)

ATGAAATGGGTGACCTTTATTTCTTTACTGTTCTCTTTAGCAGCGCCT
ACTCC

15 (*Human IL-12 subunit beta (p40)*)

ATTTGGGAAGTGAAGAAGGACGTCTACGTGGTTCGAACTGGACTGGTAT
CCCGATGCTCCCGGCGAAATGGTGGTGCTCACTTGTGACACCCCCGAAGAAG
ACGGCATCACTTGGACCCTCGATCAGAGCAGCGAGGTGCTGGGCTCCGGAAA
GACCCTCACAATCCAAGTTAAGGAGTTCGGAGACGCTGGCCAATACACATGC
20 CACAAGGGAGGCGAGGTGCTCAGCCATTCTTATTATTATTACACAAGAAGG
AAGACGGAATCTGGTCCACCGACATTTTAAAAGATCAGAAGGAGCCCAAGA
ATAAGACCTTTTTAAGGTGTGAGGCCAAAACTACAGCGGTCGTTTCACTTGT
TGGTGGCTGACCACCATTTCCACCGATTTAACCTTCTCCGTGAAAAGCAGCCG
GGGAAGCTCCGACCCTCAAGGTGTGACATGTGGAGCCGCTACCCTCAGCGCT
25 GAGAGGGTTCGTGGCGATAACAAGGAATACGAGTACAGCGTGGAGTGCCAA
GAAGATAGCGCTTGTCCCGCTGCCGAAGAATCTTTACCCATTGAGGTGATGG
TGGACGCCGTGCACAACTCAAGTACGAGAACTACACCTCCTCCTTCTTTATC
CGGGACATCATTAAGCCCGATCCTCCTAAGAATTTACAGCTGAAGCCTCTCA
AAAATAGCCGGCAAGTTGAGGTCTCTTGGGAATATCCCGACACTTGGAGCAC
30 ACCCCACAGCTACTTCTCTTTAACCTTTTGTGTGCAAGTTCAAGGTAAAAGCA
AGCGGGAGAAGAAAGACCGGGTGTTTACCGACAAAACCAGCGCCACCGTCA

TCTGTCGGAAGAACGCCTCCATCAGCGTGAGGGCTCAAGATCGTTATTACTCC
AGCAGCTGGTCCGAGTGGGCCAGCGTGCCTTGTTCC

(Linker)

GGCGGTGGAGGATCCGGAGGAGGTGGCTCCGGCGGCGGAGGATCT

5 *(Human IL-12 subunit alpha (p35))*

CGTAACCTCCCCGTGGCTACCCCCGATCCCGGAATGTTCCCTTGTTTAC
ACCACAGCCAGAATTTACTGAGGGCCGTGAGCAACATGCTGCAGAAAGCTAG
GCAGACTTTAGAATTTTACCCTTGCACCAGCGAGGAGATCGACCATGAAGAT
ATCACCAAGGACAAGACATCCACCGTGGAGGCTTGTTTACCTCTGGAGCTGA
10 CAAAGAACGAGTCTTGTCTCAACTCTCGTGAAACCAGCTTCATCACAAATGG
CTCTTGTTTAGCTTCCCGGAAGACCTCCTTTATGATGGCTTTATGCCTCAGCTC
CATCTACGAGGATTTAAAGATGTACCAAGTGGAGTTCAAGACCATGAACGCC
AAGCTGCTCATGGACCCTAAACGGCAGATCTTTTTAGACCAGAACATGCTGG
CTGTGATTGATGAGCTGATGCAAGCTTTAAACTTCAACTCCGAGACCGTCCCT
15 CAGAAGTCCTCCCTCGAGGAGCCCGATTTTTACAAGACAAAGATCAAACCTGT
GCATTTTACTCCACGCCTTTAGGATCCGGGCCGTGACCATTGACCGGGTCATG
AGCTATTTAAACGCCAGC

(Human Tissue Factor 219)

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAG
20 CACCAACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTT
TACACCGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCT
ATACCACCGACACCGAGTGCGATCTACCGATGAGATCGTGAAAGATGTGAA
ACAGACCTACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGC
ACTGGTTCCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTA
25 CCTCGAGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGC
ACAAAGGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAAC
AACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCAAAGATTTAATCTACACACT
GTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGACAGCTAAAACCAACACA
AACGAGTTTTTAATCGACGTGGATAAAGGCGAAAACACTACTGTTTCAGCGTGC
30 AAGCTGTGATCCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGT
TGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(Human IL-15)

AACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATTTAATT
 CAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTC
 TTGTAAGGTGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTTATCTCTT
 5 TAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTT
 AGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAG
 GAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTG
 TGCACATTGTCCAGATGTTTCATCAATACCTCC

10 **Example 14: Secretion of IL-18/IL-15R α Su and IL-12/TF/IL-15 fusion proteins**

The IL-18/IL-15R α Su and IL-12/TF/IL-15 constructs were cloned into a pMSGV-1 modified retrovirus expression vector (Hughes, *Hum Gene Ther* 16:457–72, 2005 herein incorporated by reference), and the expression vector was transfected into CHO-K1 cells. Co-expression of the two constructs in CHO-K1 cells resulted in secretion of a
 15 soluble IL-12/TF/IL-15:IL-18/IL-15R α Su protein complex (referred to as 12t15/18s), which can be purified by anti-TF Ab affinity and other chromatography methods.

The amino acid sequence of the IL-18/IL-15R α Su fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 135):

(Signal peptide)

20 MKWVTFISLLFLFSSAYS

(Human IL-18)

YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISM
 YKDSQPRGMAVTISVKCEKISTLSCENKIISFKEMNPPDNIKDTKSDIIFQRSVPG
 HDNKMQFESSYEGYFLACEKERDLFKLILKKEDELGDRSIMFTVQNED

25 (Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLTECVLNKAT
 NVAHWTTPSLKCIR

The amino acid sequence of the IL-12/TF/IL-15 fusion protein (including leader sequence) is as follows (SEQ ID NO: 136):

30 (Signal peptide)

MKWVTFISLLFLFSSAYS

(Human IL-12 subunit beta (p40))

IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDSSEVLGS
GKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLHKKEDGIWSTDILKDQKEPKN
KTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERV
5 RGDNKEYEYSVEQCEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIKPD
PPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVF
TDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCS

(Linker)

GGGGSGGGGSGGGGS

10 *(Human IL-12 subunit alpha (p35))*

RNLPVATPDPMFPCLLHHSQNLRLAVSNMLQKARQTLEFYPTSEEIDHE
DITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYE
DLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSS
LEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS

15 *(Human Tissue Factor 219)*

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLYTLYYW
KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
20 KGEFRE

(Human IL-15)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVIS
LES GDASIHDTVENLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQ
MFINTS

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

Example 15: Creation of an IL-7/IL-15R α Su DNA construct

In a non-limiting example, an IL-7/IL-15R α Su DNA construct was created (see
30 Figure 21). The human IL-7 sequence, human IL-15R α Su sequence, human IL-15

sequence, and human tissue factor 219 sequence were obtained from the UniProt website and DNA for these sequences was synthesized by Genewiz. A DNA construct was made linking the IL-7 sequence to the IL-15R α Su sequence. The final IL-7/IL-15R α Su DNA construct sequence was synthesized by Genewiz.

5 The nucleic acid sequence encoding the second chimeric polypeptide of IL-7/IL-15R α Su construct (including signal peptide sequence) is as follows (SEQ ID NO: 97):

(Signal peptide)

ATGGGAGTGAAAGTTCTTTTTGCCCTTATTTGTATTGCTGTGGCCGAGG
CC

10 *(Human IL-7)*

GATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTCTA
ATGGTCAGCATCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATT
GCCTGAATAATGAATTTAACTTTTTTAAAAGACATATCTGTGATGCTAATAAG
GAAGGTATGTTTTTATTCCGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAAAT
15 GAATAGCACTGGTGATTTTGATCTCCACTTATTAAAAGTTTCAGAAGGCACAA
CAATACTGTTGAACTGCACTGGCCAGGTAAAGGAAGAAAACCAGCTGCCCT
GGGTGAAGCCCAACCAACAAAGAGTTTGAAGAAAATAAATCTTTAAAGGA
ACAGAAAAAACTGAATGACTTGTGTTTCCTAAAGAGACTATTACAAGAGATA
AAAAC TTGTTGGAATAAAATTTTGATGGGCACTAAAGAACAC

20 *(Human IL-15R α sushi domain)*

ATCACGTGCCCTCCCCCATGTCCGTGGAACACGCAGACATCTGGGTC
AAGAGCTACAGCTTGTACTCCAGGGAGCGGTACATTTGTA ACTCTGGTTTCAA
GCGTAAAGCCGGCACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACG
AATGTCGCCCCACTGGACAACCCCCAGTCTCAAATGCATTAGA

25

The second chimeric polypeptide of IL-7/IL-15R α Su construct (including signal peptide sequence) is as follows (SEQ ID NO: 100):

(Signal peptide)

MKWVTFISLLFLFSSAYS

30 *(Human IL-7)*

DCDIEGKDGKQYESVLMV SIDQLLD SMKEIGSNCLNNEFNFFKRHICDAN
KEGMFLFRAARKLRQFLKMNSTGDFDLHLLKVSEGT TILLNCTGQVKGRKPAAL
GEAQPTKSLEENKSLKEQKKLNDLCFLKRLLQEIKTCWNKILMGTKEH

(Human Tissue Factor 219)

5 SGTNTNTVAAYNL TWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
FYTTDTECDLTDEIVKDV KQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLYTLYYW
KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
KGEFRE

10 (Human IL-15)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCCKVTAMKCFLELQVIS
LES GDASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQ
MFINTS

15 **Example 16: Creation of an IL-21/TF/IL-15 DNA construct**

In a non-limiting example, an IL-21/TF/IL-15 construct was made (Figure 22) by linking the IL-21 sequence to the N-terminus coding region of tissue factor 219, and further linking the IL-21/TF construct with the N-terminus coding region of IL-15.

The nucleic acid sequence encoding the first chimeric polypeptide of IL-
20 21/TF/IL-15 construct (including leader sequence), synthesized by Genewiz, is as follows (SEQ ID NO: 93):

(Signal peptide)

ATGGGAGTGAAAGTTCTTTTTGCCCTTATTTGTATTGCTGTGGCCGAGG
CC

25 *(Human IL-21 fragment)*

CAAGGTCAAGATCGCCACATGATTAGAATGCGTCAACTTATAGATATT
GTTGATCAGCTGAAAAATTATGTGAATGACTTGGTCCCTGAATTTCTGCCAGC
TCCAGAAGATGTAGAGACAACTGTGAGTGGTCAGCTTTTTCCTGTTTTTCAGA
AGGCCCAACTAAAGTCAGCAAATACAGGAAACAATGAAAGGATAATCAATG
30 TATCAATTAAAAAGCTGAAGAGGAAACACCTTCCACAAATGCAGGGAGAA
GACAGAAACACAGACTAACATGCCCTTCATGTGATTCTTATGAGAAAAAACC

ACCCAAAGAATTCCTAGAAAGATTCAAATCACTTCTCCAAAAGATGATTCAT
CAGCATCTGTCCTCTAGAACACACGGAAGTGAAGATTCC

(Human Tissue Factor 219)

TCAGGCACTACAAATACTGTGGCAGCATATAATTAACTTGGAAATCA
5 ACTAATTTCAAGACAATTTTGGAGTGGGAACCCAAACCCGTCAATCAAGTCT
ACACTGTTCAAATAAGCACTAAGTCAGGAGATTGGAAAAGCAAATGCTTTTA
CACAACAGACACAGAGTGTGACCTCACCGACGAGATTGTGAAGGATGTGAA
GCAGACGTA CT TGGCACGGGTCTTCTCCTACCCGGCAGGGAATGTGGAGAGC
ACCGGTTCTGCTGGGGAGCCTCTGTATGAGAACTCCCCAGAGTTCACACCTTA
10 CCTGGAGACAAACCTCGGACAGCCAACAATTCAGAGTTTTGAACAGGTGGGA
ACAAAAGTGAATGTGACCGTAGAAGATGAACGGACTTTAGTCAGAAGGAAC
AACACTTTCCTAAGCCTCCGGGATGTTTTTGGCAAGGACTTAATTTATACACT
TTATTATTGGAAATCTTCAAGTTCAGGAAAGAAAACAGCCAAAACAAACACT
AATGAGTTTTTTGATTGATGTGGATAAAGGAGAAAAC TACTGTTTCAGTGTTCA
15 AGCAGTGATTCCCTCCCGAACAGTTAACCGGAAGAGTACAGACAGCCCGGTA
GAGTGTATGGGCCAGGAGAAAGGGGAATTCAGAGAA

(Human IL-15)

AACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATTTAATT
CAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTC
20 TTGTAAGGTGACCGCCATGAAATGTTTTTTTACTGGAGCTGCAAGTTATCTCTT
TAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTTT
AGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAG
GAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTG
TGCACATTGTCCAGATGTTTCATCAATACCTCC

25

The first chimeric polypeptide of IL-21/TF/IL-15 construct including leader
sequence is SEQ ID NO: 92:

(Signal peptide)

MGVKVLFALICIAVAEA (SEQ ID NO: 140)

30

(Human IL-21)

QGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWSAFSCF
 QKAQLKSANTGNNERIINVSIIKKLRKPPSTNAGRQKHRLTCPSCDSYEKKPPK
 EFLERFKSLLQKMIHQHLSSRTHGSEDS

(*Human Tissue Factor 219*)

5 SGTNTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTD
 TECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLG
 QPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSG
 KKTAKTNTNEFLIDVDKGENYCFVQAVIPSRTVNRKSTDSPVECMGQEKGEFR
 E

10 (*Human IL-15*)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCCKVTAMKCFLLELQVISLESG
 DASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQMFIN
 TS

15 **Example 17: Secretion of IL-7/IL-15R α Su and IL-21/TF/IL-15 fusion proteins**

The IL-7/IL-15R α Su and IL-21/TF/IL-15 DNA constructs were cloned into a
 pMSGV-1 modified retrovirus expression vector (as described by Hughes, *Hum Gene*
Ther 16:457–72, 2005, hereby incorporated by reference), and the expression vector was
 transfected into CHO-K1 cells. Co-expression of the two constructs in CHO-K1 cells
 20 allowed for formation and secretion of a soluble IL-21/TF/IL-15:IL-7/IL-15R α Su protein
 complex (referred to as 21t15-7s; Figures 23 and Figure 24). The 21t15-7s protein was
 purified from CHO-K1 cell culture supernatant using anti-TF antibody affinity
 chromatography and size exclusion chromatography resulting in soluble (non-aggregated)
 protein complexes consisting of IL-7/IL-15R α Su and IL-21/TF/IL-15 fusion proteins.

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

Example 18: Purification of 21t15-7s by immunoaffinity chromatography

An anti-TF antibody affinity column was connected to a GE Healthcare™ AKTA
 30 Avant protein purification system. The flow rate was 4 mL/min for all steps except the
 elution step, which was 2 mL/min.

Cell culture harvest of 21t15-7s was adjusted to pH 7.4 with 1M Tris base and loaded onto the anti-TF antibody affinity column equilibrated with 5 column volumes of PBS. After loading the sample, the column was washed with 5 column volumes PBS, followed by elution with 6 column volumes 0.1M acetic acid, pH 2.9. Absorbance at 280 nm was collected and then the sample was neutralized 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. The buffer-exchanged protein sample was stored at 2-8°C for further biochemical analysis and biological activity testing.

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% sodium azide and stored at 2-8 °C.

Example 19: Size exclusion chromatography

A GE Healthcare Superdex® 200 Increase 10/300 GL gel filtration column was connected to a GE Healthcare AKTA™ Avant protein purification system. The column was equilibrated with 2 column volumes of PBS. The flow rate was 0.7 mL/min. A capillary loop was used to inject 200µL of 1 mg/mL of 7t15-21scomplex onto the column. The injection was chased with 1.25 column volumes of PBS.

Example 20: Cytotoxicity of NK cells against human tumor cells

Fresh human blood buffy coat was obtained from a blood bank. NK cells were isolated via negative selection using the RosetteSep/human NK cell reagent (StemCell Technologies). The NK cells were cultured in complete RPMI-1640 medium with 21t15-7s 100 nM and 50 nM of anti-TF IgG1 antibody for up to 11 days at 37°C and 5% CO₂. The activated NK cells were mixed with Celltrace violet-labeled K562 cells at E:T ratio equal to 2:1 and incubated at 37°C for 4 hours. The mixture was harvested and the percentage of dead K562 cells were determined by propidium iodide staining and flow cytometry. Figure 25 shows increased specific lysis of K562 cells when incubated with expanded NK cells.

Example 21: Creation of an IL-21/IL-15R α Su DNA construct

In a non-limiting example, an IL-21/IL-15R α Su DNA construct was created. The human IL-21 sequence and human IL-15R α Su sequence were obtained from the UniProt website and DNA for these sequences was synthesized by Genewiz. A DNA construct
 5 was made linking the IL-21 sequence to the IL-15R α Su sequence. The final IL-21/IL-15R α Su DNA construct sequence was synthesized by Genewiz. See Figure 26.

Example 22: Creation of an IL-7/TF/IL-15 DNA construct

In a non-limiting example, an IL-7/TF/IL-15 construct was made by linking the
 10 IL-7 sequence to the N-terminus coding region of tissue factor 219, and further linking the IL-7/TF construct with the N-terminus coding region of IL-15. See Figure 27.

Example 23: Creation of an IL-21/IL-15R α Sushi DNA construct

In a non-limiting example, a second chimeric polypeptide of IL-21/IL-15R α Su
 15 was generated. The human IL-21 and human IL-15R α sushi sequences were obtained from the UniProt website and DNA for these sequences was synthesized by Genewiz. A DNA construct was made linking the IL-21 sequence to the IL-15R α sushi sequence. The final IL-21/IL-15R α Su DNA construct sequence was synthesized by Genewiz.

The nucleic acid sequence encoding the second chimeric polypeptide of IL-21/IL-
 20 15R α Su domain (including leader sequence), synthesized by Genewiz, is as follows (SEQ ID NO: 105):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCCTGTTCTCCAGCGCCTACTC
 C

(Human IL-21)

CAGGGCCAGGACAGGCACATGATCCGGATGAGGCAGCTCATCGACATCGTCG
 ACCAGCTGAAGAACTACGTGAACGACCTGGTGCCCGAGTTTCTGCCTGCCCC
 CGAGGACGTGGAGACCAACTGCGAGTGGTCCGCCTTCTCCTGCTTTTCAAG
 GCCAGCTGAAGTCCGCCAACACCGGCAACAACGAGCGGATCATCAACGTG
 30 AGCATCAAGAAGCTGAAGCGGAAGCCTCCCTCCACAAACGCCGGCAGGAGG
 CAGAAGCACAGGCTGACCTGCCCCAGCTGTGACTCCTACGAGAAGAAGCCCC

CCAAGGAGTTCCTGGAGAGGTTCAAGTCCCTGCTGCAGAAGATGATCCATCA
GCACCTGTCCTCCAGGACCCACGGCTCCGAGGACTCC

(Human IL-15R α sushi domain)

ATTACATGCCCCCTCCCATGAGCGTGGAGCACGCCGACATCTGGGTGAAGA
5 GCTATAGCCTCTACAGCCGGGAGAGGTATATCTGTAAACAGCGGCTTCAAGAG
GAAGGCCGGCACCAGCAGCCTCACCGAGTGCGTGCTGAATAAGGCTACCAAC
GTGGCTCACTGGACAACACCCTCTTTAAAGTGCATCCGG

The second chimeric polypeptide of IL-21/IL-15R α sushi domain (including
10 leader sequence) is as follows (SEQ ID NO: 104):

(Signal Sequence)

MKWVTFISLLFLFSSAYS

(Human IL-21)

QGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWSAFSCFQKAQ
15 LKSANTGNNERIINVSIKKLKRKPPSTNAGRRQKHRLTCPSCDSYEKKPPKEFLER
FKSLLQKMIHQHLSSRTHGSEDS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAH
WTPPSLKCIR

20

Example 24: Creation of an IL-7/TF/IL-15 DNA construct

In a non-limiting example, an exemplary first chimeric polypeptide of IL-
7/TF/IL-15 was made by linking the IL-7 sequence to the N-terminus coding region of
tissue factor 219, and further linking the IL-7/TF construct with the N-terminus coding
25 region of IL-15. The nucleic acid sequence encoding the first chimeric polypeptide of
IL-7/TF/IL-15 (including leader sequence), synthesized by Genewiz, is as follows (SEQ
ID NO: 137):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCCTGTTCTCCAGCGCCTACTC

30 C

(Human IL-7 fragment)

GATTGCGACATCGAGGGCAAGGACGGCAAGCAGTACGAGAGCGTGCTGATG
GTGTCCATCGACCAGCTGCTGGACAGCATGAAGGAGATCGGCTCCAAGTCC
TCAACAACGAGTTCAACTTCTTCAAGCGGCACATCTGCGACGCCAACAAGGA
GGGCATGTTCTGTTCAGGGCCGCCAGGAACTGCGGCAGTTCCTGAAGATG
5 AACTCCACCGGCGACTTCGACCTGCACCTGCTGAAGGTGTCCGAGGGCACCA
CCATCCTGCTGAACTGCACCGGACAGGTGAAGGGCCGAAACCTGCTGCTCT
GGGAGAGGCCCAACCCACCAAGAGCCTGGAGGAGAACAAGTCCCTGAAGGA
GCAGAAGAAGCTGAACGACCTGTGCTTCCTGAAGAGGCTGCTGCAGGAGATC
AAGACCTGCTGGAACAAGATCCTGATGGGCACCAAGGAGCAT

10 *(Human Tissue Factor 219)*

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAGCACCA
ACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTTTACAC
CGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCTATACC
ACCGACACCGAGTGCGATCTCACCGATGAGATCGTGAAAGATGTGAAACAGA
15 CCTACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGCACTGG
TTCCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTACCTCG
AGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGCACAAA
GGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAACAACAC
CTTTCTCAGCCTCCGGGATGTGTTTCGGCAAAGATTTAATCTACACACTGTATT
20 ACTGGAAGTCCTCTTCCTCCGGCAAGAAGACAGCTAAAACCAACACAAACGA
GTTTTTAATCGACGTGGATAAAGGCGAAAACCTACTGTTTCAGCGTGCAAGCT
GTGATCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGTTGAGT
GCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(Human IL-15)

25 AACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATTTAATTCAGT
CCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTCTTGT
AAGGTGACCGCCATGAAATGTTTTTTTACTGGAGCTGCAAGTTATCTCTTTAGA
GAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTTAGCC
AATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAGGAGT
30 GCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTGTGCA
CATTGTCCAGATGTTTCATCAATACCTCC

The first chimeric polypeptide of IL-7/TF/IL-15 (including leader sequence), is as follows (SEQ ID NO: 100):

(Signal peptide)

MKWVTFISLLFLFSSAYS

5 *(Human IL-7)*

DCDIEGKDGKQYESVLMVSIQQLDSMKEIGSNCLNNEFNFFKRHICDANKEGM
FLFRAARKLRQFLKMNSTGDFDLHLLKVSEGTTILLNCTGQVKGRKPAALGEAQ
PTKSLEENKSLKEQKKLNDLCFLKRLLQEIKTCWNKILMGTKEH

(Human Tissue Factor 219)

10 SGTNTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKCFYTTD
TECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYLETNLG
QPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSG
KKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFR
E

15 *(Human IL-15)*

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLESG
DASIHDTVENLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFIN
TS

20 **Example 25: Secretion of IL-21/IL-15R α Su and IL-7/TF/IL-15 fusion proteins**

The IL-21/IL-15R α Su and IL-7/TF/IL-15 DNA constructs were cloned into a pMSGV-1 modified retrovirus expression vector (as described by Hughes, *Hum Gene Ther* 16:457–72, 2005, hereby incorporated by reference), and the expression vector was transfected into CHO-K1 cells. Co-expression of the two constructs in CHO-K1 cells
25 allowed for formation and secretion of a soluble IL-7/TF/IL-15:IL-21/IL-15R α Su protein complex (referred to as 7t15-21s). The 7t15-21s protein was purified from CHO-K1 cell culture supernatant using anti-TF antibody (IgG1) affinity chromatography and size exclusion chromatography resulting in soluble (non-aggregated) protein complexes consisting of IL-21/IL-15R α Su and IL-7/TF/IL-15 fusion proteins. See Figure 28 and
30 Figure 29.

Example 26: Analytical size exclusion chromatography (SEC) analysis of IL-21/IL-15R α Su and IL-7/TF/IL-15 fusion proteins

To determine if anti-tissue factor monoclonal antibody and 7t15-21s can form an antibody-fusion-molecule complex, analytical size exclusion chromatography (SEC) was performed. 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 0.7 mL/min. Samples of the anti-TF mAb (1 mg/mL), 7t15-21s (1mg/mL), and a mixture of combined at a 1:1 ratio, so the final concentration of each protein is 0.5mg/mL) were in PBS. Each sample was injected into the Superdex 200 column using a capillary loop, and analyzed by SEC. The SEC chromatograph of each sample was shown in Figure 30. The SEC results indicated that there are two protein peaks for 7t15-21s, likely representing a dimer (with an apparent molecular weight of 199.2 kDa) and a higher oligomer of 7t15-21s, and there is one peak (with an apparent molecular weight of 206.8 kDa) for the anti-TF mAb. However, as expected, a new protein peak with a higher molecular weight (with an apparent molecular weight of 576.9 kDa) was formed in the mixture sample containing the anti-TF mAb and 7t15-21s, indicating that the anti-TF mAb and 7t15-21s form an antibody-antigen complex through the binding of anti-TF mAb to TF in the fusion protein complex.

Example 27: Expansion capacity of primary natural killer (NK) cells by 7t15-21s complex + anti-TF IgG1 antibody

To assess the 7t15-21s complex's ability to expand primary natural killer (NK) cells, 7t15-21s complex and 7t15-21s complex + anti-TF IgG1 antibody are added to NK cells obtained from samples of fresh human leukocytes. Cells are stimulated with 50nM of 7t15-21s complex with or without 25 nM of anti-TF IgG1 or anti-TF IgG4 antibody at 37°C and 5% CO₂. Cells are maintained at concentration at 0.5 x 10⁶/mL not exceeding 2.0 x 10⁶/mL by counting every 48-72 hours and media is replenished with fresh stimulator. Cells stimulated with 7t15-21s complex or anti-TF IgG1 antibody or anti-TF IgG4 antibody or anti-TF IgG4 + 7t15-21s complex are maintained up to day 5.

Expansion of primary NK cells upon incubation with 21t15-7s complex + anti-TF IgG1 antibody is observed.

Example 28: Activation of expanded NK cells by the 7t15-21s complex + anti-TF IgG1 antibody

Primary NK cells are induced *ex vivo* following overnight stimulation of purified NK cells with 7t15-21s complex + anti-TF IgG1 antibody. Fresh human leukocytes are obtained from a blood bank and CD56⁺ NK cells are isolated with the RosetteSep/human NK cell reagent (StemCell Technologies). The purity of NK cells is >80% and is confirmed by staining with CD56-BV421 and CD16-BV510 specific antibodies (BioLegend). Cells are counted and resuspended in 1×10^6 /mL in a 24 well flat bottom plate in 1 mL of complete media (RPMI 1640 (Gibco), supplemented with 4 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), non-essential amino acid (Thermo Life Technologies), sodium pyruvate (Thermo Life Technologies), and 10% FBS (Hyclone)). Cells are stimulated with 50 nM of 7t15-21s with or without 25 nM of anti-TF IgG1 antibody at 37°C and 5% CO₂. Cells are counted every 48-72 hours and maintained at a concentration of 0.5×10^6 /mL to 2.0×10^6 /mL until day 14. Media is periodically replenished with fresh stimulator. Cells are harvested and surface stained at day 3 with CD56-BV421, CD16-BV510, CD25-PE, CD69-APCFire750 specific antibodies (Biolegend) and analyzed by flow cytometry-(Celeste-BD Bioscience). The activation marker CD25 MFI are observed to increase with 7t15-21s complex + anti-TF IgG1 antibody stimulation, but not 7t15-21s complex stimulation. The activation marker CD69 MFI is observed to increase with both 7t15-21s complex + anti-TF IgG1 antibody and with 7t15-21s complex, alone.

Example 29: Increase in Glucose Metabolism in NK Cells Using 18t15-12s

A set of experiments was performed to determine the effect of the construct of 18t15-12s (Figure 6) on oxygen consumption rate and extracellular acidification rate (ECAR) on NK cells purified from human blood.

In these experiments, fresh human leukocytes were obtained from the blood bank from two different human donors and NK cells were isolated via negative selection using the RosetteSep/human NK cell reagent (StemCell Technologies). The purity of NK cells was >80% and confirmed by staining with CD56-BV421 and CD16-BV510 specific Abs (BioLegend). The cells were counted and resuspended in 2×10^6 /mL in 24-well, flat-bottom plates in 1 mL of complete media (RPMI 1640 (Gibco) supplemented with 4 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), non-essential amino acid (Thermo Life Technologies), sodium pyruvate (Thermo Life Technologies) and 10% FBS (Hyclone)).

The cells were stimulated with either (1) media alone, (2) 100 nM 18t15-12s, or (3) mixture of single cytokines recombinant human IL-12 (0.25 μ g), recombinant human IL-15 (1.25 μ g), and recombinant human IL-18 (1.25 μ g) overnight at 37 °C and 5% CO₂. On the next day, the cells were harvested and extracellular flux assays on expanded NK cells were performed using a XFp Analyzer (Seahorse Bioscience). The harvested cells washed and plated 2.0×10^5 cells/well in at least duplicate for extracellular flux analysis of OCR (Oxygen Consumption Rate) and ECAR (Extracellular Acidification Rate). The glycolysis stress tests were performed in Seahorse Media contain 2 mM of glutamine. The following were used during the assay: 10 mM glucose; 100 nM oligomycin; and 100 mM 2-deoxy-D-glucose (2DG).

The data show that the 18t15-12s results in significantly increased oxygen consumption rate (Figure 31) and extracellular acidification rate (ECAR) as compared to the same cells activated with a combination of recombinant human IL-12, recombinant human IL-15, and recombinant human IL-18 (Figure 32).

Example 30: TGFRt15-TGFRs fusion protein generation and characterization

A fusion protein complex was generated comprising of TGF β receptor II/IL-15R α Su (TGFRs) and TGF β receptor II/TF/IL-15 (TGFRt15) fusion proteins (Figure 33 and Figure 34). The human TGF β receptor II (Ile24-Asp159), tissue factor 219, and IL-15 sequences were obtained from the UniProt website and DNA for these sequences was synthesized by Genewiz. Specifically, a construct was made linking two TGF β receptor II sequences with a G4S(3) linker to generate a single chain version of TGF β receptor II

and then directly linking to the N-terminus coding region of tissue factor 219 followed by the N-terminus coding region of IL-15.

The nucleic acid and protein sequences of a construct comprising two TGF β receptor II linked to the N-terminus of tissue factor 219 following with the N-terminus of IL-15 are shown below.

The nucleic acid sequence of the two TGF β receptor II/TF/IL-15 construct (including signal peptide sequence) is as follows:

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCCTGTTCTCCAGCGCCT
ACTCC

(Two Human TGF β Receptor II fragments)

ATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACC
GACAACAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCA
GGTTCAGCACCTGCGATAATCAGAAGTCCTGCATGTCCAAGTGCAGCATCAC
CTCCATCTGCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAAT
GACGAGAACATCACCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATC
ACGACTTCATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAA
GAAGAAGCCCGGAGAGACCTTCTTTATGTGTTCCCTGTAGCAGCGACGAGTGT
AACGACAACATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGATGGAG
GTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGTATTCCTCCCCA
CGTGCAGAAGAGCGTGAATAATGACATGATCGTGACCGATAACAATGGCGCC
GTGAAATTTCCCCAGCTGTGCAAATTCTGCGATGTGAGGTTTTCCACCTGCGA
CAACCAGAAGTCCTGTATGAGCAACTGCTCCATCACCTCCATCTGTGAGAAG
CCTCAGGAGGTGTGCGTGGCTGTCTGGCGGAAGAATGACGAGAATATCACCC
TGGAACCGTCTGCCACGATCCCAAGCTGCCCTACCACGATTTTCATCCTGGA
AGACGCCGCCAGCCCTAAGTGCATCATGAAAGAGAAAAAGAAGCCTGGCGA
GACCTTTTTTCATGTGCTCCTGCAGCAGCGACGAATGCAACGACAATATCATCT
TTAGCGAGGAATACAATACCAGCAACCCCGAC

(Human Tissue Factor 219)

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAG
CACCAACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTT

TACACCGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCT
 ATACCACCGACACCGAGTGCGATCTCACCATGAGATCGTGAAAGATGTGAA
 ACAGACCTACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGC
 ACTGGTTCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTA
 5 CCTCGAGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGC
 ACAAAGGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAAC
 AACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCAAAGATTTAATCTACACACT
 GTATTACTGGAAGTCCTCTTCCTCCGGCAAGAAGACAGCTAAAACCAACACA
 AACGAGTTTTTAATCGACGTGGATAAAGGCGAAAACACTACTGTTTCAGCGTGC
 10 AAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGT
 TGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(Human IL-15)

AACTGGGTGAACGTCATCAGCGATTTAAAGAAGATCGAAGATTTAATT
 CAGTCCATGCATATCGACGCCACTTTATACACAGAATCCGACGTGCACCCCTC
 15 TTGTAAGGTGACCGCCATGAAATGTTTTTTACTGGAGCTGCAAGTTATCTCTT
 TAGAGAGCGGAGACGCTAGCATCCACGACACCGTGGAGAATTTAATCATTTT
 AGCCAATAACTCTTTATCCAGCAACGGCAACGTGACAGAGTCCGGCTGCAAG
 GAGTGCGAAGAGCTGGAGGAGAAGAACATCAAGGAGTTTCTGCAATCCTTTG
 TGCACATTGTCCAGATGTTTCATCAATACCTCC (SEQ ID NO: 114)

20

The amino acid sequence of TGF β receptor II/TF/IL-15 fusion protein (including the leader sequence) is as follows:

(Signal peptide)

MKWVTFISLLFLFSSAYS

25

(Human TGF β Receptor II)

IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSI
 TSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKK
 PGETTFMCSCSSDECNDNIIFSEEYNTSNPDGGGGSGGGGSGGGGSIPPHVQKSVN
 NDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCAV
 30 WRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGGETTFMCSCSSDE
 CNDNIIFSEEYNTSNPD

(Human Tissue Factor 219)

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLYTLYYW
5 KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
KGEFRE

(Human IL-15)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVIS
LESGDASIHDTVENLIILANNSLSSNGNVTESGCKECELEEKNIKEFLQSFVHIVQ
10 MFINTS (SEQ ID NO: 113)

Constructs were also made by attaching two TGF β receptor II directly to the IL-15R α Su chain which was synthesized by Genewiz. The nucleic acid and protein sequences of a construct comprising the TGF β receptor II linked to the N-terminus of IL-15R α Su are shown below.

The nucleic acid sequence of the TGF β receptor II/IL-15 R α Su construct (including signal peptide sequence) is as follows:

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCCTGTTCTCCAGCGCCT
20 ACTCC

(Two human TGF β Receptor II fragments)

ATCCCCCCCCATGTGCAAAAGAGCGTGAACAACGATATGATCGTGACC
GACAACAACGGCGCCGTGAAGTTTCCCCAGCTCTGCAAGTTCTGCGATGTCA
GGTTCAGCACCTGCGATAATCAGAAGTCCTGCATGTCCAAGTGCAGCATCAC
25 CTCCATCTGCGAGAAGCCCCAAGAAGTGTGCGTGGCCGTGTGGCGGAAAAAT
GACGAGAACATCACCCCTGGAGACCGTGTGTACGACCCCAAGCTCCCTTATC
ACGACTTCATTCTGGAGGACGCTGCCTCCCCCAAATGCATCATGAAGGAGAA
GAAGAAGCCCGGAGAGACCTTCTTTATGTGTTCTGTAGCAGCGACGAGTGT
AACGACAACATCATCTTCAGCGAAGAGTACAACACCAGCAACCCTGATGGAG
30 GTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGTATTCCTCCCCA
CGTGCAGAAGAGCGTGAATAATGACATGATCGTGACCGATAACAATGGCGCC

GTGAAATTTCCCCAGCTGTGCAAATTCTGCGATGTGAGGTTTTCCACCTGCGA
 CAACCAGAAGTCCTGTATGAGCAACTGCTCCATCACCTCCATCTGTGAGAAG
 CCTCAGGAGGTGTGCGTGGCTGTCTGGCGGAAGAATGACGAGAATATCACCC
 TGGAAACCGTCTGCCACGATCCCAAGCTGCCCTACCACGATTTTCATCCTGGA
 5 AGACGCCGCCAGCCCTAAGTGCATCATGAAAGAGAAAAAGAAGCCTGGCGA
 GACCTTTTTTCATGTGCTCCTGCAGCAGCGACGAATGCAACGACAATATCATCT
 TTAGCGAGGAATACAATACCAGCAACCCCGAC

(Human IL-15R α sushi domain)

ATTACATGCCCCCTCCCATGAGCGTGGAGCACGCCGACATCTGGGTG
 10 AAGAGCTATAGCCTCTACAGCCGGGAGAGGTATATCTGTAACAGCGGCTTCA
 AGAGGAAGGCCGGCACCAGCAGCCTCACCGAGTGCGTGCTGAATAAGGCTA
 CCAACGTGGCTCACTGGACAACACCCTCTTTAAAGTGCATCCGG (SEQ ID NO:
 118)

15 The amino acid sequence of the two TGF β receptor II/IL-15R α Su construct
 (including signal peptide sequence) is as follows:

(Signal peptide)

MKWVTFISLLFLFSSAYS

(Two human TGF β Receptor II extra-cellular domains)

20 IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSI
 TSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKK
 PGETFFMCSCSSDECNDNIIFSEYNTSNPDGGGGSGGGGSGGGGSIPPHVQKSVN
 NDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCAV
 WRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCSCSSDE
 25 CNDNIIFSEYNTSNPD

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLSYRERYICNSGFKRKAGTSSLTECVLNKAT
 NVAHWTTPSLKCIR (SEQ ID NO: 117)

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

The TGF β R/IL-15R α Su and TGF β R/TF/IL-15 constructs were cloned into a modified retrovirus expression vectors as described previously (Hughes MS, Yu YY, Dudley ME, Zheng Z, Robbins PF, Li Y, et al. Transfer of a TCR gene derived from a patient with a marked antitumor response conveys highly active T-cell effector functions. *Hum Gene Ther* 2005;16:457–72), and the expression vectors were transfected into CHO-K1 cells. Co-expression of the two constructs in CHO-K1 cells allowed for formation and secretion of the soluble TGF β R/TF/IL-15:TGF β R/IL-15R α Su protein complex (referred to as TGF β Rt15-TGFRs), which can be purified by anti-TF IgG1 affinity and other chromatography methods.

Effect of TGF β Rt15-TGFRs on TGF β 1 activity in HEK-Blue TGF β cells

To evaluate the activity of TGF β RII in TGF β Rt15-TGFRs, the effect of TGF β Rt15-TGFRs on the activity of TGF β 1 in HEK-Blue TGF β cells was analyzed. HEK-Blue TGF β cells (Invivogen) were washed twice with pre-warmed PBS and resuspended in the testing medium (DMEM, 10% heat-inactivated FCS, 1x glutamine, 1x anti-anti, and 2x glutamine) at 5×10^5 cells/mL. In a flat-bottom 96-well plate, 50 μ L cells were added to each well (2.5×10^4 cells/well) and followed with 50 μ L 0.1nM TGF β 1 (R&D systems). TGF β Rt15-TGFRs or TGF β R-Fc (R&D Systems) prepared at a 1:3 serial dilution was then added to the plate to reach a total volume of 200 μ L. After 24 hrs of incubation at 37°C, 40 μ L of induced HEK-Blue TGF β cell supernatant was added to 160 μ L pre-warmed QUANTI-Blue (Invivogen) in a flat-bottom 96-well plate, and incubated at 37°C for 1-3 hrs. The OD values were then determined using a plate reader (Multiscan Sky) at 620-655 nM. The IC₅₀ of each protein sample was calculated with GraphPad Prism 7.04. The IC₅₀ of TGF β Rt15-TGFRs and TGF β R-Fc were 216.9 pM and 460.6 pM respectively. These results showed that the TGF β RII domain in TGF β Rt15-TGFRs was able to block the activity of TGF β 1 in HEK-Blue TGF β cells.

The IL-15 in TGF β Rt15-TGFRs promotes IL-2R β and common γ chain containing 32D β cell proliferation

To evaluate the activity of IL-15 in TGF β Rt15-TGFRs, the IL-15 activity of TGF β Rt15-TGFRs was compared to recombinant IL-15 using 32D β cells that express

IL2R β and common γ chain, and evaluating their effects on promoting cell proliferation. IL-15 dependent 32D β cells were washed 5 times with IMDM-10% FBS and seeded in the wells at 2×10^4 cells/well. Serially-diluted TGF α 15-TGFRs or IL-15 were added to the cells (Figure 35). Cells were incubated in a CO₂ incubator at 37°C for 3 days. Cell proliferation was detected by adding 10 μ L of WST1 to each well on day 3 and incubating for an additional 3 hours in a CO₂ incubator at 37°C. The absorbance at 450 nm was measured by analyzing the amount of formazan dye produced. As shown in Figure 35, TGF α 15-TGFRs and IL-15 promoted 32D β cell proliferation, with the EC₅₀ of TGF α 15-TGFRs and IL-15 being 1901 pM and 10.63 pM, respectively.

Detection of IL-15 and TGF β RII domains in TGF α 15-TGFRs with corresponding antibodies using ELISA

A 96-well plate was coated with 100 μ L (8 μ g/mL) of anti-TF IgG1 in R5 (coating buffer) and incubated at room temperature (RT) for 2 hrs. The plates were washed 3 times and blocked with 100 μ L of 1% BSA in PBS. TGF α 15-TGFRs was added at a 1:3 serial dilution, and incubated at RT for 60 min. After 3 washes, 50 ng/mL of biotinylated-anti-IL-15 antibody (BAM247, R&D Systems), or 200 ng/mL of biotinylated-anti-TGF β RII antibody (BAF241, R&D Systems) was added to the wells and incubated at RT for 60 min. Next the plates were washed 3 times, and 0.25 μ g/mL of HRP-SA (Jackson ImmunoResearch) at 100 μ L per well was added and incubated for 30 min at RT, followed by 4 washes and incubation with 100 μ L of ABTS for 2 mins at RT. Absorbance at 405 nm was read. As shown in Figure 36A and 36B, the IL-15 and TGF β RII domains in TGF α 15-TGFRs were detected by the individual antibodies.

Purification elution chromatograph of TGF α 15-TGFRs from anti-TF antibody affinity column

TGF α 15-TGFRs harvested from cell culture was loaded onto the anti-TF antibody affinity column equilibrated with 5 column volumes of PBS. After sample loading, the column was washed with 5 column volumes of PBS, followed by elution with 6 column volumes of 0.1M acetic acid (pH 2.9). A280 elution peak was collected and then neutralized to pH 7.5-8.0 with 1M Tris base. The neutralized sample was then

buffer exchanged into PBS using Amicon centrifugal filters with a 30 KDa molecular weight cutoff. As shown in Figure 37, the anti-TF antibody affinity column bound to TGFRT15-TGFRs which contains TF as a fusion partner. The buffer-exchanged protein sample was stored at 2-8 °C for further biochemical analyses and biological activity tests.

5 After each elution, the anti-TF antibody affinity column was stripped using 6 column volumes of 0.1M glycine (pH 2.5). The column was then neutralized using 5 column volumes of PBS, and 7 column volumes of 20% ethanol for storage. The anti-TF antibody affinity column was connected to a GE Healthcare AKTA Avant system. The flow rate was 4 mL/min for all steps except for the elution step, which was 2 mL/min.

Analytical size exclusion chromatography (SEC) analysis of TGFRT15-TGFRs

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 0.7 mL/min. A sample
15 containing TGFRT15-TGFRs in PBS was injected into the Superdex 200 column using a capillary loop, and analyzed by SEC. The SEC chromatograph of the sample is shown in Figure 38. The SEC results showed four protein peaks for TGFRT15-TGFRs.

Reduced SDS-PAGE analysis of TGFRT15-TGFRs

20 To determine the purity and molecular weight of the TGFRT15-TGFRs protein, protein sample purified with anti-TF antibody affinity column was analyzed by sodium dodecyl sulfate polyacrylamide gel (4-12% NuPage Bis-Tris gel) electrophoresis (SDS-PAGE) method under reduced condition. After electrophoresis, the gel was stained with InstantBlue for about 30 min, followed by destaining overnight in purified water.

25 To verify that the TGFRT15-TGFRs protein undergoes glycosylation after translation in CHO cells, a deglycosylation experiment was conducted using the Protein Deglycosylation Mix II kit from New England Biolabs and the manufacturer's instructions. Figure 39 shows the reduced SDS-PAGE analysis of the sample in non-deglycosylated (lane 1 in red outline) and deglycosylated (lane 2 in yellow outline) state.
30 The results showed that the TGFRT15-TGFRs protein is glycosylated when expressed in CHO cells. After deglycosylation, the purified sample showed expected molecular

weights (69 kDa and 39 kDa) in the reduced SDS gel. Lane M was loaded with 10 ul of SeeBlue Plus2 Prestained Standard.

Immunostimulatory activity of TGF α t15-TGFRs in C57BL/6 mice

5 TGF α t15-TGFRs is a multi-chain polypeptide (a type A multi-chain polypeptide described herein) that includes a first polypeptide that is a soluble fusion of two TGF β RII domains, human tissue factor 219 fragment and human IL-15, and the second polypeptide that is a soluble fusion of two TGF β RII domains and sushi domain of human IL-15 receptor alpha chain.

10 Wild type C57BL/6 mice were treated subcutaneously with either control solution or with TGF α t15-TGFRs at a dosage of 0.3 mg/kg, 1 mg/kg, 3 mg/kg, or 10 mg/kg. Four days after treatment, spleen weight and the percentages of various immune cell types present in the spleen were evaluated. As shown in Figure 40A, the spleen weight in mice treated with TGF α t15-TGFRs increased with increasing dosage of TGF α t15-TGFRs.

15 Moreover, the spleen weight in mice treated with 1 mg/kg, 3 mg/kg, and 10 mg/kg of TGF α t15-TGFRs were higher as compared to mice treated with the control solution, respectively. In addition, the percentages of CD4⁺ T cells, CD8⁺ T cells, NK cells, and CD19⁺ B cells present in the spleen of control-treated and TGF α t15-TGFRs-treated mice were evaluated. As shown in Figure 40B, in the spleens of mice treated with TGF α t15-
20 TGFRs, the percentages of CD8⁺ T cells and NK cells both increased with increasing dosage of TGF α t15-TGFRs. Specifically, the percentages of CD8⁺ T cells were higher in mice treated with 0.3 mg/kg, 3 mg/kg, and 10 mg/kg of TGF α t15-TGFRs compared to control-treated mice, and the percentages of NK cells were higher in mice treated with 0.3 mg/kg, 1 mg/kg, 3 mg/kg, and 10 mg/kg of TGF α t15-TGFRs compared to control-
25 treated mice. These results demonstrate that TGF α t15-TGFRs is able to stimulate immune cells in the spleen, in particular CD8⁺ T cells and NK cells.

The pharmacokinetics of TGF α t15-TGFRs molecules were evaluated in wild type C57BL/6 mice. The mice were treated subcutaneously with TGF α t15-TGFRs at a dosage of 3 mg/kg. The mouse blood was drained from tail vein at various time points
30 and the serum was prepared. The TGF α t15-TGFRs concentrations in mouse serum was determined with ELISA (capture: anti-human tissue factor antibody; detection:

biotinylated anti-human TGF β receptor antibody and followed by peroxidase conjugated streptavidin and ABTS substrate). The results showed that the half-life of TGF β Rt15-TGFRs was 12.66 hours in C57BL/6 mice.

The mouse splenocytes were prepared in order to evaluate the immunostimulatory activity of TGF β Rt15-TGFRs over time in mice. As shown in Figure 41A, the spleen weight in mice treated with TGF β Rt15-TGFRs increased 48 hours posttreatment and continued to increase over time. In addition, the percentages of CD4 $^{+}$ T cells, CD8 $^{+}$ T cells, NK cells, and CD19 $^{+}$ B cells present in the spleen of control-treated and TGF β Rt15-TGFRs-treated mice were evaluated. As shown in Figure 41B, in the spleens of mice treated with TGF β Rt15-TGFRs, the percentages of CD8 $^{+}$ T cells and NK cells both increased at 48 hours after treatment and were higher and higher overtime after the single dose treatment. These results further demonstrate that TGF β Rt15-TGFRs is able to stimulate immune cells in the spleen, in particular CD8 $^{+}$ T cells and NK cells.

Furthermore, the dynamic proliferation of immune cells based on Ki67 expression of splenocytes and cytotoxicity potential based on granzyme B expression were evaluated in splenocytes isolated from mice following a single dose (3 mg/kg) of TGF β Rt15-TGFRs. As shown in Figure 42A and 42B, in the spleens of mice treated with TGF β Rt15-TGFRs, the expression of Ki67 and granzyme B by NK cells increased at 24 hours after treatment and its expression of CD8 $^{+}$ T cells and NK cells both increased at 48 hours and later time points after the single dose treatment. These results demonstrate that TGF β Rt15-TGFRs not only increases the numbers of CD8 $^{+}$ T cells and NK cells but also enhance the cytotoxicity of these cells. The single dose treatment of TGF β Rt15-TGFRs led CD8 $^{+}$ T cells and NK cells to proliferate for at least 4 days.

The cytotoxicity of the splenocytes from TGF β Rt15-TGFRs-treated mice against tumor cells was also evaluated. Mouse Moloney leukemia cells (Yac-1) were labeled with CellTrace Violet and were used as tumor target cells. Splenocytes were prepared from TGF β Rt15-TGFRs (3 mg/kg)-treated mouse spleens at various time points post treatment and were used as effector cells. The target cells were mixed with effector cells at an E:T ratio = 10:1 and incubated at 37°C for 20 hours. Target cell viability was assessed by analysis of propidium iodide positive, violet-labeled Yac-1 cells using flow cytometry. Percentage of Yac-1 tumor inhibition was calculated using the formula, (1-

[viable Yac-1 cell number in experimental sample]/[viable Yac-1 cell number in the sample without splenocytes]) x 100. As shown in Figure 43, splenocytes from TGFRt15-TGFRs-treated mice had stronger cytotoxicity against Yac-1 cells than the control mouse splenocytes.

5

Tumor size analysis in response to chemotherapy and/or TGFRt15-TGFRs

Pancreatic cancer cells (SW1990, ATCC® CRL-2172) were subcutaneously (s.c.) injected into C57BL/6 scid mice (The Jackson Laboratory, 001913, 2×10^6 cells/mouse, in 100 μ L HBSS) to establish the pancreatic cancer mouse model. Two weeks after tumor cell injection, chemotherapy was initiated in these mice intraperitoneally with a combination of Abraxane (Celgene, 68817-134, 5 mg/kg, i.p.) and Gemcitabine (Sigma Aldrich, G6423, 40 mg/kg, i.p.), followed by immunotherapy with TGFRt15-TGFRs (3 mg/kg, s.c.) in 2 days. The procedure above was considered one treatment cycle and was repeated for another 3 cycles (1 cycle/week). Control groups were set up as the SW1990-injected mice that received PBS, chemotherapy (Gemcitabine and Abraxane), or TGFRt15-TGFRs alone. Along with the treatment cycles, tumor size of each animal was measured and recorded every other day, until the termination of the experiment 2 months after the SW1990 cells were injected. Measurement of the tumor volumes were analyzed by group and the results indicated that the animals receiving a combination of chemotherapy and TGFRt15-TGFRs had significantly smaller tumors comparing to the PBS group, whereas neither chemotherapy nor TGFRt15-TGFRs therapy alone work as sufficiently as the combination (Figure 44).

In vitro senescent B16F10 melanoma model

Next, in vitro killing of senescent B16F10 melanoma cells by activated mouse NK cells was evaluated. B16F10 senescence cells (B16F10-SNC) cells were labelled with CellTrace violet and incubated for 16 hrs with different E:T ratio of in vitro 2t2-activated mouse NK cells (isolated from spleen of C57BL/6 mice injected with TGFRt15-TGFRs 10 mg/kg for 4 days). The cells were trypsinized, washed and resuspended in complete media containing propidium iodide (PI) solution. The cytotoxicity was assessed by flow cytometry (Figure 45).

Example 31: Stimulation of NK cells *in vitro* by multi-chain chimeric polypeptide constructs

A set of experiments was performed to assess changes in the surface phenotype of lymphocyte populations after stimulation with 18t15-12s, 18t15-12s16, and 7t15-21s. In these experiments, fresh human leukocytes were obtained from the blood bank.

Peripheral blood lymphocytes were isolated with the Ficoll-PAQUE Plus (GE Healthcare) density gradient media. The cells were counted and resuspended at $0.2 \times 10^6/\text{mL}$ in a 96-well flat-bottom plate in 0.2 mL of complete media (RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone)). The cells were stimulated with: 18t15-12s (100 nM); 18t15-12s16 (100 nM), a mixture of single cytokines rhIL15 (50 ng/mL) (Miltenyi), rhIL18 (50 ng/mL) (Invivogen), and rhIL-12 (10 ng/mL) (Peprotech); 7t15-21s (100 nM) + anti-TF antibody (50 nM); 7t15-21s (100 nM); or anti-TF antibody (50 nM) at 37 °C and 5% CO₂ for 16 hours. The next day, the cells were harvested and surface stained for 30 minutes with antibodies specific for CD4 or CD8, CD62L, and CD69. After surface staining, 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). Figure 46 shows that overnight incubation of purified lymphocyte populations (CD4 and CD8 T cells) with 18t15-12s, 18t15-12s16, or 7t15-21s + anti-TF antibody resulted in an increase in the percentage of CD8 and CD4 T cells expressing CD69. Additionally, incubation with 7t15-21s + anti-TF antibody resulted in an increase in the percentage of CD8 and CD4 T cells expressing CD62L (Figure 46).

A set of experiments was performed to determine the increase in phospho-STAT4 and phospho-STAT5 levels in NK cells after stimulation with 18t15-12s. In these experiments, fresh human leukocytes were obtained from the blood bank and CD56⁺ NK cells were isolated with the RosetteSep/human NK cell reagent (StemCell Technologies). The purity of NK cells was >70% and confirmed by staining with CD56-BV421, CD16-BV510, CD25-PE, and CD69-APCFire750 specific antibodies (BioLegend). The cells were counted and resuspended in $0.05 \times 10^6/\text{mL}$ in a 96-well flat-bottom plate in 0.1 mL

of complete media (RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone)). The cells were stimulated with hIL-12 (10 ng/mL) (Biolegend) or hIL-15 (50 ng/mL) (NCI) (Single cytokines), or 18t15-12s (100 nM) at 37 °C and 5% CO₂ for 90 minutes. Unstimulated NK cells (US) were used as a control. The cells were harvested and fixed in paraformaldehyde (Sigma) to a final concentration of 1.6%. Plates were incubated in the dark at room temperature for 10 minutes. FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)) (100 µL) was added and cells were transferred to 96-well “V” bottom plate. The cells were washed for 1500 RPM for 5 minutes at room temperature. The cell pellet was mixed with 100 µL chilled methanol by gently pipetting up and down, and cells were incubated for 30 minutes at 4 °C. The cells were mixed with 100 mL of FACS buffer and washed for 1500 RPM for 5 minutes at room temperature. The cell pellets were mixed with 50 mL of FACS buffer containing 4 mL of pSTAT4 (BD Bioscience) and pSTAT5 antibodies (BD Bioscience) followed by incubation for 30 minutes at room temperature in the dark. The cells were mixed with 100 mL of FACS buffer and washed for 1500 RPM for 5 minutes at room temperature. The cell pellets were mixed with 50 mL of FACS buffer and cells were analyzed by flow cytometry (Celesta-BD Bioscience). Figure 47 shows that incubation of NK cells with 18t15-12s induced an increase in pSTAT4 and pSTAT5 (plotted data, normalized fold-change).

Example 32: Stimulation of NK cells *in vivo* by TGFRt15-TGFRs

A set of experiments was performed to determine the effect of the TGFRt15-TGFRs construct on immune stimulation in ApoE^{-/-} mice fed with a Western diet. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories). After 8-weeks of the Western diet, the mice were injected subcutaneously with TGFRt15-TGFRs at 3 mg/kg. Three days post treatment, mice were fasted for 16 hours and then blood samples were collected through retro-orbital venous plexus puncture. The blood was mixed with 10 µL 0.5 M EDTA, and 20 µL blood was taken for lymphocyte subsets analysis. The red

blood cells were lysed with ACK (0.15 M NH_4Cl , 1.0 mM KHCO_3 , 0.1 mM Na_2EDTA , pH 7.4) and the lymphocytes were stained with anti-mouse CD8a and anti-mouse NK1.1 antibodies for 30 minutes at 4 °C in FACS staining buffer (1% BSA in PBS). The cells were washed once and analyzed with a BD FACS Celesta. For Treg staining, ACK
5 treated blood lymphocytes were stained with anti-mouse CD4 and anti-mouse CD25 antibodies for 30 minutes at 4 °C in FACS staining buffer. The cells were washed once and resuspended in fixation/permeabilization working solution and incubated at room temperature for 60 minutes. The cells were washed once and resuspended in permeabilization buffer. The samples were centrifuged at 300-400 x g for 5 minutes at
10 room temperature and the supernatant was then discarded. The cell pellet was resuspended in residual volume and the volume adjusted to about 100 μL with 1 x permeabilization buffer. Anti-Foxp3 antibody was added to the cells, and the cells were incubated for 30 minutes at room temperature. Permeabilization buffer (200 μL) was added to the cells, and the cells were centrifuged at 300-400 x g for 5 minutes at room
15 temperature. The cells were resuspended in flow cytometry staining buffer and analyzed on a flow cytometer. Figures 48A-48C show that treatment with TGFRt15-TGFRs increased the percentage of NK cells and CD8^+ T cells in $\text{ApoE}^{-/-}$ mice fed with Western diet.

20 **Example 33: Induction of proliferation of immune cells *in vivo***

A set of experiments was performed to determine the effect of the TGFRt15-TGFRs construct on immune stimulation in C57BL/6 mice. In these experiments, C57BL/6 mice were subcutaneously treated with control solution (PBS) or TGFRt15-TGFRs at 0.1, 0.3, 1, 3, and 10 mg/kg. The treated mice were euthanized 4 days post-
25 treatment. Spleen weight was measured and splenocyte suspensions were prepared. The splenocyte suspensions were stained with conjugated anti-CD4, anti-CD8, and anti-NK1.1 (NK) antibodies. The cells were additionally stained for proliferation marker Ki67. Figure 49A shows that spleen weight in mice treated with TGFRt15-TGFRs increased with increasing dosage of TGFRt15-TGFRs. Additionally, spleen weight in
30 mice treated with 1 mg/kg, 3 mg/kg, and 10 mg/kg of TGFRt15-TGFRs was higher as compared to mice treated with just the control solution. The percentages of CD8^+ T cells

and NK cells both increased with increasing dosage of TGF α t15-TGFRs (Figure 49B). Finally, TGF α t15-TGFRs significantly upregulated expression of cell proliferation marker Ki67 in both CD8⁺ T cells and NK cells at all doses of TGF α t15-TGFRs tested (Figure 49C). These results demonstrate that TGF α t15-TGFRs treatment induced proliferation of both CD8⁺ T cells and NK cells in C57BL/6 mice.

A set of experiments was performed to determine the effect of the TGF α t15-TGFRs construct on immune stimulation in ApoE^{-/-} mice fed with a Western diet. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories). After 8-week of the Western diet, the mice were injected subcutaneously with TGF α t15-TGFRs at 3 mg/kg. Three days post-treatment, the mice were fasted for 16 hours and then blood samples were collected through retro-orbital venous plexus puncture. The blood was mixed with 10 μ L 0.5 M EDTA and 20 μ L blood was taken for lymphocyte subsets analysis. The red blood cells were lysed with ACK (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4) and the lymphocytes were stained with anti-mouse CD8a and anti-mouse NK1.1 antibodies for 30 minutes at 4 °C in FACS staining buffer (1% BSA in PBS). The cells were washed once and resuspended in Fixation Buffer (BioLegend Cat# 420801) for 20 minutes at room temperature. The cells were centrifuged at 350 x g for 5 minutes, the fixed cells were resuspended in Intracellular Staining Permeabilization Wash Buffer (BioLegend Cat# 421002) and then centrifuged at 350 x g for 5 minutes. The cells were then stained with anti-Ki67 antibody for 20 minutes at RT. The cells were washed twice with Intracellular Staining Permeabilization Wash Buffer and centrifuged at 350 x g for 5 minutes. The cells were then resuspended in FACS staining buffer. Lymphocyte subsets were analyzed with a BD FACS Celesta. As described in Figure 50A and 50B, treatment of ApoE^{-/-} mice with TGF α t15-TGFRs induced proliferation (Ki67-positive staining) in NK and CD8⁺ T cells.

Example 34: NK-mediated cytotoxicity following treatment with multi-chain construct

A set of experiments was performed to determine if treatment of NK cells with TGF α 15-TGFRs enhanced cytotoxicity of NK cells. In these experiments, Human Daudi B lymphoma cells were labeled with CellTrace Violet (CTV) and used as tumor target cells. Mouse NK effector cells were isolated with NK1.1-positive selection using a magnetic cell sorting method (Miltenyi Biotec) of C57BL/6 female mouse spleens 4 days post TGF α 15-TGFRs subcutaneous treatment at 3 mg/kg. Human NK effector cells were isolated from peripheral blood mononuclear cells derived from human blood buffy coats with the RosetteSep/human NK cell reagent (Stemcell Technologies). The target cells (Human Daudi B lymphoma cells) were mixed with effector cells (either mouse NK effector cells or human NK effector cells) in the presence of 50 nM TGF α 15-TGFRs or in the absence of TGF α 15-TGFRs (control) and incubated at 37 °C for 44 hours for mouse NK cells and for 20 hours for human NK cells. Target cell (Daudi) viability was assessed by analysis of propidium iodide-positive, CTV-labeled cells using flow cytometry. The percentage of Daudi inhibition was calculated using the formula (1-viable tumor cell number in experimental sample/viable tumor cell number in the sample without NK cells) x 100. Figure 51 shows that mouse (Figure 51A) and human (Figure 51B) NK cells had significantly stronger cytotoxicity against Daudi B cells following NK cell activation with TGF α 15-TGFRs than in the absence of TGF α 15-TGFRs activation.

A set of experiments was performed to determine antibody-dependent cellular cytotoxicity (ADCC) of mouse and human NK cells following treatment with TGF α 15-TGFRs. In these experiments, human Daudi B lymphoma cells were labeled with CellTrace Violet (CTV) and used as tumor target cells. Mouse NK effector cells were isolated with NK1.1-positive selection using a magnetic cell sorting method (Miltenyi Biotec) of C57BL/6 female mouse spleens 4 days post-TGF α 15-TGFRs subcutaneous treatment at 3 mg/kg. Human NK effector cells were isolated from peripheral blood mononuclear cells derived from human blood buffy coats with the RosetteSep/human NK cell reagent (Stemcell Technologies). The target cells (Daudi B cells) were mixed with effector cells (either mouse NK effector cells or human NK effector cells) in the presence of anti-CD20 antibody (10 nM Rituximab, Genentech) and in the presence of 50 nM

TGFRt15-TGFRs, or in the absence of TGFRt15-TGFRs (control) and incubated at 37 °C for 44 hours for mouse NK cells and for 20 hours for human NK cells. The Daudi B cells express the CD20 targets for the anti-CD20 antibody. Target cell viability was assessed after incubation by analysis of propidium iodide-positive, CTV-labeled target cells using
 5 flow cytometry. The percentage of Daudi inhibition was calculated using the formula $(1 - \text{viable tumor cell number in experimental sample} / \text{viable tumor cell number in the sample without NK cells}) \times 100$. Figure 52 shows that mouse NK cells (Figure 52A) and human NK cells (Figure 52B) had stronger ADCC activity against Daudi B cells following NK cell activation with TGFRt15-TGFRs than in the absence of TGFRt15-TGFRs activation.

10

Example 35: Treatment of Cancer

A set of experiments was performed to assess antitumor activity of TGFRt15-TGFRs plus anti-TRP1 antibody (TA99) in combination with chemotherapy in a melanoma mouse model. In these experiments, C57BL/6 mice were subcutaneously
 15 injected with 0.5×10^6 B16F10 melanoma cells. The mice were treated with three doses of chemotherapy docetaxel (10 mg/kg) (DTX) on day 1, day 4, and day 7, followed by treatment with single dose of combination immunotherapy TGFRt15-TGFRs (3 mg/kg) + anti-TRP1 antibody TA99 (200 µg) on day 9. Figure 53A shows a schematic of the treatment regimen. Tumor growth was monitored by caliper measurement, and tumor
 20 volume was calculated using the formula $V = (L \times W^2)/2$, where L is the largest tumor diameter and W is the perpendicular tumor diameter. Figure 53B shows that treatment with DTX + TGFRt15-TGFRs + TA99 significantly reduced tumor growth compared to saline control and DTX treatment groups ($N=10$, **** $p < 0.001$, Multiple t test analyses).

To assess immune cell subsets in the B16F10 tumor model, peripheral blood
 25 analysis was performed. In these experiments, C57BL/6 mice were injected with B16F10 cells and treated with DTX, DTX + TGFRt15-TGFRs + TA99, or saline. Blood was drawn from the submandibular vein of B16F10 tumor-bearing mice on days 2, 5, and 8 post-immunotherapy for the DTX + TGFRt15-TGFRs + TA99 group and day 11 post-tumor injection for the DTX and saline groups. RBCs were lysed in ACK lysis buffer
 30 and the lymphocytes were washed and stained with anti-NK1.1, anti-CD8, and anti-CD4 antibodies. The cells were analyzed by flow cytometry (Celesta-BD Bioscience).

Figures 53C-53E show that DTX + TGFRT15-TGFRs + TA99 treatment induced an increase in the percentage of NK cells and CD8⁺ T cells in the tumors compared to the saline and DTX treatment groups.

On day 17, total RNA was extracted from tumors of mice treated with saline, DTX or DTX + TGFRT15-TGFRs + TA99 using Trizol. Total RNA (1 µg) was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was carried out with CFX96 Detection System (Bio-Rad) using FAM-labeled predesigned primers for senescence cell markers, (F) p21 (G) DPP4 and (H) IL6. The housekeeping gene 18S ribosomal RNA was used as an internal control to normalize the variability in expression levels. The expression of each target mRNA relative to 18S rRNA was calculated based on Ct as $2^{-\Delta(\Delta Ct)}$, in which $\Delta Ct = Ct_{\text{target}} - Ct_{18S}$. The data is presented as fold-change as compared to saline control. Figure 53F-53H show that DTX treatment induced an increase in senescent tumor cells that were subsequently reduced following treatment with TGFRT15-TGFRs + TA99 immunotherapy.

A set of experiments was performed to investigate amelioration of Western diet-induced hyperglycemia in ApoE^{-/-} mice by TGFRT15-TGFRs. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories). After 8-weeks of the Western diet, the mice were injected subcutaneously with TGFRT15-TGFRs at 3 mg/kg. Three days post-treatment, the mice were fasted for 16 hours and then blood samples were collected through retro-orbital venous plexus puncture. Blood glucose was detected with a glucose meter (OneTouch UltraMini) and GenUltimated test strips using a drop of fresh blood. As shown in Figure 54A, TGFRT15-TGFRs treatment reduced hyperglycemia induced by the Western diet. The plasma insulin and resistin levels were analyzed with Mouse Rat Metabolic Array by Eve Technologies. HOMA-IR was calculated using the following formula: homeostatic model assessment-insulin resistance = Glucose (mg/dL) * Insulin (mU/mL)/405. As shown in Figure 54B, TGFRT15-TGFRs treatment reduced insulin resistance compared to the untreated group. TGFRT15-TGFRs (p<0.05) reduced resistin levels significantly compared to the untreated group as shown in Figure 54C, which may relate to the reduced insulin resistance induced by TGFRT15-TGFRs (Figure 54B).

Example 36: Induction of differentiation of NK cells into cytokine-induced memory like NK cells

A set of experiments was performed to assess the differentiation of NK cells into cytokine-induced memory like NK cells (CIMK-NK cells) after stimulation with 18t15-12s. In these experiments, fresh human leukocytes were obtained from the blood bank and CD56⁺ NK cells were isolated with the RosetteSep/human NK cell reagent (StemCell Technologies). The purity of NK cells was >90% and confirmed by staining with CD56-BV421, CD16-BV510, CD25-PE, and CD69-APCFire750 antibodies (BioLegend). The cells were counted and resuspended in 2×10^6 /mL in a 24-well flat-bottom plate in 2 mL of complete media (RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone)). The cells were unstimulated (“No Spike”) or stimulated with 18t15-12s (100 nM) or a mixture of single cytokines including rhIL15 (50 ng/mL) (Miltenyi), rhIL18 (50 ng/mL) (Invivogen), and rhIL-12 (10 ng/mL) (Peprotech) (“single cytokines”) at 37 °C and 5% CO₂ for 16 hrs. The next day, the cells were harvested, and washed two times with warm complete media at 1000 RPM for 10 minutes at room temperature. The cells were resuspended at 2×10^6 /mL in a 24-well flat-bottom plate in 2 mL of complete media with rhIL15 (1 ng/mL). After every 2 days, half of the medium was replaced with fresh complete media containing rhIL15.

To assess the change in memory phenotype of NK cells at day 7, the cells were stained with antibodies to cell-surface CD56, CD16, CD27, CD62L, NKp30, and NKp44 (BioLegend). After surface staining, the cells were washed (1500 RPM for 5 minutes at room temperature) in FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)). After two washes, the cells were analyzed by flow cytometry (Celesta-BD Bioscience). Figure 55 shows that incubation of NK cells with 18t15-12s resulted in an increase in the percentage of CD16⁺CD56⁺ NK cells expressing CD27, CD62L, and NKp44, and an increase in the levels (MFI) of NKp30 in CD16⁺CD56⁺ NK cells.

Example 37: Upregulation of CD44 memory T cells

A set of experiments was performed to assess upregulation of CD44 memory T cells upon treatment with TGF α 15-TGFRs. In these experiments, C57BL/6 mice were subcutaneously treated with TGF α 15-TGFRs. The treated mice were euthanized and the single splenocyte suspensions were prepared 4 days (TGF α 15-TGFRs) following the treatment. The prepared splenocytes were stained with fluorochrome-conjugated anti-CD4, anti-CD8 and anti-CD44 antibodies and the percentages of CD44^{high} T cells in CD4⁺ T cells or CD8⁺ T cells were analyzed by flow cytometry. The results show that TGF α 15-TGFRs upregulated expression of the memory marker CD44 on CD4⁺ and CD8⁺ T cells (Figures 56). These findings indicate that TGF α 15-TGFRs was able to induce mouse T cells to differentiate into memory T cells.

Example 38. Production of an Exemplary Single-Chain Chimeric Polypeptides

An exemplary single-chain chimeric polypeptide including a first target-binding domain that is an anti-CD3 scFv, a soluble human tissue factor domain, and a second target-binding domain that is an anti-CD28 scFv was generated (α CD3scFv/TF/ α CD28scFv) (Figure 57). The nucleic acid and amino acid sequences of this single-chain chimeric polypeptide are shown below.

Nucleic Acid Encoding Exemplary Single-Chain Chimeric Polypeptide
(α CD3scFv/TF/ α CD28scFv) (SEQ ID NO: 126)

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCTTATTATTTTATTCAGCTCCGCCT
 ATTCC

(α CD3 light chain variable region)

CAGATCGTGCTGACCCAAAGCCCCGCCATCATGAGCGCTAGCCCCGGT
 GAGAAGGTGACCATGACATGCTCCGCTTCCAGCTCCGTGTCCTACATGAAC
 TGGTATCAGCAGAAAAGCGGAACCGCCCCAAAAGGTGGATCTACGACACCA
 GCAAGCTGGCCTCCGGAGTGCCCGCTCATTTCCGGGGCTCTGGATCCGGCAC
 CAGCTACTCTTTAACCATTTCCGGCATGGAAGCTGAAGACGCTGCCACCTACT

ATTGCCAGCAATGGAGCAGCAACCCCTTCACATTCGGATCTGGCACCAAGCT
CGAAATCAATCGT

(Linker)

GGAGGAGGTGGCAGCGGCGGCGGTGGATCCGGCGGAGGAGGAAGC

5 *(α CD3 heavy chain variable region)*

CAAGTTCAACTCCAGCAGAGCGGCGCTGAACTGGCCCCGGCCCCGGCGC
CTCCGTCAAGATGAGCTGCAAGGCTTCCGGCTATACATTTACTCGTTACACAA
TGCATTGGGTCAAGCAGAGGCCCGGTCAAGGTTTAGAGTGGATCGGATATAT
CAACCCTTCCCGGGGCTACACCAACTATAACCAAAAGTTCAAGGATAAAGCC
10 ACTTTAACCCTGACAAGAGCTCCTCCACCGCCTACATGCAGCTGTCCTCTTT
AACCAGCGAGGACTCCGCTGTTTACTACTGCGCTAGGTATTACGACGACCAC
TACTGTTTAGACTATTGGGGACAAGGTACCACTTTAACCGTCAGCAGC

(Human tissue factor 219 form)

TCCGGCACCAATACCGTGGCCGCTTATAACCTCACATGGAAGAGC
15 ACCAACTTCAAGACAATTCTGGAATGGGAACCCAAGCCCGTCAATCAAGTTT
ACACCGTGCAGATCTCCACCAAATCCGGAGACTGGAAGAGCAAGTGCTTCTA
CACAACAGACACCGAGTGTGATTTAACCGACGAAATCGTCAAGGACGTCAAG
CAAACCTATCTGGCTCGGGTCTTTTCTACCCCGCTGGCAATGTCGAGTCCAC
CGGCTCCGCTGGCGAGCCTCTCTACGAGAATTCGCCCGAATTCACCCCTTATT
20 TAGAGACCAATTTAGGCCAGCCTACCATCCAGAGCTTCGAGCAAGTTGGCAC
CAAGGTGAACGTCACCGTCGAGGATGAAAGGACTTTAGTGCGGCGGAATAAC
ACATTTTTATCCCTCCGGGATGTGTTTCGGCAAAGACCTCATCTACACACTGTA
CTATTGGAAGTCCAGCTCCTCCGGCAAAAAGACCGCTAAGACCAACACCAAC
GAGTTTTTAATTGACGTGGACAAAGGCGAGAACTACTGCTTCAGCGTGCAAG
25 CCGTGATCCCTTCTCGTACCGTCAACCGGAAGAGCACAGATTCCCCCGTTGA
GTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(α CD28 light chain variable region)

GTCCAGCTGCAGCAGAGCGGACCCGAACTCGTGAAACCCGGTGCTTCC
GTGAAAATGTCTTGTAAGGCCAGCGGATACACCTTCACCTCCTATGTGATCCA
30 GTGGGTCAAACAGAAGCCCGGACAAGGTCTCGAGTGGATCGGCAGCATCAA
CCCTTACAACGACTATACCAAATACAACGAGAAGTTTAAGGGAAAGGCTACT

TTAACCTCCGACAAAAGCTCCATCACAGCCTACATGGAGTTCAGCTCTTTAAC
 ATCCGAGGACAGCGCTCTGTACTATTGCGCCCGGTGGGGCGACGGCAATTAC
 TGGGGACGGGGCACAACTGACCGTGAGCAGC

(Linker)

5 GGAGGCGGAGGCTCCGGCGGAGGCGGATCTGGCGGTGGCGGCTCC

(α CD28 light chain variable region)

GACATCGAGATGACCCAGTCCCCCGCTATCATGTCCGCCTCTTTAGGCGAGC
 GGGTCACAATGACTTGTACAGCCTCCTCCAGCGTCTCCTCCTCCTACTTCCAT
 TGGTACCAACAGAAACCCGGAAGCTCCCCTAAACTGTGCATCTACAGCACCA
 10 GCAATCTCGCCAGCGGCGTGCCCCCTAGGTTTTCCGGAAGCGGAAGCACCAG
 CTACTCTTTAACCATCTCCTCCATGGAGGCTGAGGATGCCGCCACCTACTTTT
 GTCACCAGTACCACCGGTCCCCCACCTTCGGAGGCGGCACCAAACCTGGAGAC
 AAAGAGG

15 **Exemplary Single-Chain Chimeric Polypeptide (α CD3scFv/TF/ α CD28scFv) (SEQ
 ID NO: 125)**

(Signal peptide)

MKWVTFISLLFLFSSAYS

(α CD3 light chain variable region)

20 QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDT
 SKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEI
 NR

(Linker)

GGGGSGGGGSGGGGS

25 *(α CD3 heavy chain variable region)*

QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPQGQLEWI
 GYINPSRGYTNYNQKFQDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDD
 HYCLDYWGQGTTTLTVSS

(Human tissue factor 219)

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
 FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
 ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLYTLYYW
 KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
 5 KGEFRE

(α CD28 light chain variable region)

VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGS
 INPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNY
 WGRGTTTLTVSS

10 *(Linker)*

GGGGS GGGGS GGGGS

(α CD28 heavy chain variable region)

DIEMTQSPAIMASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYSTSNLA
 SGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR

15 A second exemplary single-chain chimeric polypeptide including a first target-binding domain that is an anti-CD28 scFv, a soluble human tissue factor domain, and a second target-binding domain that is an anti-CD3 scFv was generated (α CD28scFv/TF/ α CD3scFv) (Figure 58). The nucleic acid and amino acid sequences of this single-chain chimeric polypeptide are shown below.

20

Nucleic Acid Encoding Exemplary Single-Chain Chimeric Polypeptide

(α CD28scFv/TF/ α CD3scFv) (SEQ ID NO: 138)

(Signal peptide)

ATGAAATGGGTCACCTTCATCTCTTTACTGTTTTTATTTAGCAGCGCCT

25 ACAGC

(α CD28 light chain variable region)

GTGCAGCTGCAGCAGTCCGGACCCGAAGTGGTCAAGCCCGGTGCCTCC
 GTGAAAATGTCTTGTAAGGCTTCTGGCTACACCTTTACCTCCTACGTCATCCA
 ATGGGTGAAGCAGAAGCCCGGTCAAGGTCTCGAGTGGATCGGCAGCATCAAT
 30 CCCTACAACGATTACACCAAGTATAACGAAAAGTTTAAGGGCAAGGCCACTC
 TGACAAGCGACAAGAGCTCCATTACCGCCTACATGGAGTTTTCTCTTTAACT

TCTGAGGACTCCGCTTTATACTATTGCGCTCGTTGGGGCGATGGCAATTATTG
GGGCCGGGGAACTACTTTAACAGTGAGCTCC

(Linker)

GGCGGCGGCGGAAGCGGAGGTGGAGGATCTGGCGGTGGAGGCAGC

5 *(α CD28 heavy chain variable region)*

GACATCGAGATGACACAGTCCCCCGCTATCATGAGCGCCTCTTTAGGA
GAACGTGTGACCATGACTTGTACAGCTTCCTCCAGCGTGAGCAGCTCCTATTT
CCACTGGTACCAGCAGAAACCCGGCTCCTCCCCTAAACTGTGTATCTACTCCA
CAAGCAATTTAGCTAGCGGCGTGCCTCCTCGTTTTAGCGGCTCCGGCAGCACC
10 TCTTACTCTTTAACCATTAGCTCTATGGAGGCCGAAGATGCCGCCACATACTT
TTGCCATCAGTACCACCGGTCCCCTACCTTTGGCGGAGGCACAAAGCTGGAG
ACCAAGCGG

(Human tissue factor 219 form)

AGCGGCACCACCAACACAGTGGCCGCCTACAATCTGACTTGGAAATCC
15 ACCAACTTCAAGACCATCCTCGAGTGGGAGCCCAAGCCCGTTAATCAAGTTT
ATACCGTGCAGATTTCCACCAAGAGCGGCGACTGGAAATCCAAGTGCTTCTA
TACCACAGACACCGAGTGCGATCTCACCGACGAGATCGTCAAAGACGTGAAG
CAGACATATTTAGCTAGGGTGTTCTCCTACCCCGCTGGAAACGTGGAGAGCA
CCGGATCCGCTGGAGAGCCTTTATACGAGAACTCCCCCGAATTCACCCCCTAT
20 CTGGAAACCAATTTAGGCCAGCCCACCATCCAGAGCTTCGAACAAGTTGGCA
CAAAGGTGAACGTCACCGTCGAAGATGAGAGGACTTTAGTGCGGAGGAACA
ATACATTTTTATCCTTACGTGACGTCTTCGGCAAGGATTTAATCTACACACTG
TATTACTGGAAGTCTAGCTCCTCCGGCAAGAAGACCGCCAAGACCAATACCA
ACGAATTTTTAATTGACGTGGACAAGGGCGAGAACTACTGCTTCTCCGTGCA
25 AGCTGTGATCCCCTCCCGGACAGTGAACCGGAAGTCCACCGACTCCCCCGTG
GAGTGCATGGGCCAAGAGAAGGGAGAGTTTCGTGAG

(α CD3 light chain variable region)

CAGATCGTGCTGACCCAGTCCCCCGCTATTATGAGCGCTAGCCCCGGT
GAAAAGGTGACTATGACATGCAGCGCCAGCTCTTCCGTGAGCTACATGAACT
30 GGTATCAGCAGAAGTCCGGCACCAGCCCTAAAAGGTGGATCTACGACACCAG
CAAGCTGGCCAGCGGCGTCCCCGCTCACTTTCGGGGCTCCGGCTCCGGAACA

AGCTACTCTCTGACCATCAGCGGCATGGAAGCCGAGGATGCCGCTACCTATT
 ACTGTCAGCAGTGGAGCTCCAACCCCTTCACCTTTGGATCCGGCACCAAGCTC
 GAGATTAATCGT

(Linker)

5 GGAGGCGGAGGTAGCGGAGGAGGCGGATCCGGCGGTGGAGGTAGC

(α CD3 heavy chain variable region)

CAAGTTCAGCTCCAGCAAAGCGGCGCCGAACCTCGCTCGGCCCCGGCGCT
 TCCGTGAAGATGTCTTGTAAGGCCTCCGGCTATACCTTCACCCGGTACACAAT
 GCACTGGGTCAAGCAACGGCCCCGGTCAAGGTTTAGAGTGGATTGGCTATATC
 10 AACCCCTCCCGGGGCTATACCAACTACAACCAGAAGTTCAAGGACAAAGCCA
 CCCTCACCACCGACAAGTCCAGCAGCACCGCTTACATGCAGCTGAGCTCTTT
 AACATCCGAGGATTCCGCCGTGTACTACTGCGCTCGGTACTACGACGATCATT
 ACTGCCTCGATTACTGGGGCCAAGGTACCACCTTAACAGTCTCCTCC

15 **Exemplary Single-Chain Chimeric Polypeptide (α CD28scFv/TF/ α CD3scFv) (SEQ
 ID NO: 139)**

(Signal peptide)

MKWVTFISLLFLFSSAYS

(α CD28 light chain variable region)

20 VQLQQSGPELVKPGASVKMSCKASGYTFTSYVIQWVKQKPGQGLEWIGS
 INPYNDYTKYNEKFKGKATLTSDKSSITAYMEFSSLTSEDSALYYCARWGDGNY
 WGRGTTTLTVSS

(Linker)

GGGGSGGGGSGGGGS

25 *(α CD28 heavy chain variable region)*

DIEMTQSPAISASLGERVTMTCTASSSVSSSYFHWYQQKPGSSPKLCIYS
 TSNLASGVPPRFSGSGSTSYSLTISSMEAEDAATYFCHQYHRSPTFGGGTKLETKR

(Human tissue factor 219)

SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
 30 FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
 ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLYTLYYW

KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
KGEFRE

(α CD3 light chain variable region)

QIVLTQSPAIMASAPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDT

5 SKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEI
NR

(Linker)

GGGGSGGGGSGGGGS

(α CD3 heavy chain variable region)

10 QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPQGLEWI
GYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDD
HYCLDYWGQGTTTLTVSS

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

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

25 Cell culture harvest including α CD3scFv/TF/ α CD28scFv single-chain chimeric
polypeptide was adjusted to pH 7.4 with 1M Tris base and loaded onto the anti-TF
antibody affinity column (described above) which was equilibrated with 5 column
volumes of PBS. After sample loading, the column was washed with 5 column volumes
PBS, followed by elution with 6 column volumes 0.1 M acetic acid, pH 2.9. An A280
30 elution peak was collected and then neutralized to pH 7.5-8.0 by adding 1 M Tris base.
The neutralized sample was then buffer exchanged into PBS using Amicon centrifugal

filters with a 30 kDa molecular weight cutoff. The data in Figure 2 show that the anti-tissue factor antibody affinity column can bind the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide, which contains a human soluble tissue factor domain. The buffer-exchanged protein sample was stored at 2-8 °C for further biochemical analysis and biological activity testing.

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

Analytical size exclusion chromatography (SEC) was performed on the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide using a Superdex 200 Increase 10/300 GL gel filtration column (from GE Healthcare) connected to an AKTA Avant system (from GE Healthcare). The column was equilibrated with 2 column volumes of PBS. A flow rate of 0.8 mL/min was used. Two hundred μ L of α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide (1 mg/mL) was injected onto the column using a capillary loop. After injection of the single-chain chimeric polypeptide, 1.25 column volumes of PBS were flowed into the column. The SEC chromatograph is shown in Figure 59. The data show that there are 3 protein peaks, likely representing a monomer and dimer or other different forms of the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide.

To determine the purity and protein molecular weight of the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide, the purified α CD3scFv/TF/ α CD28scFv protein sample from anti-tissue factor antibody affinity column was analyzed by standard sodium dodecyl sulfate polyacrylamide gel (4-12% NuPage Bis-Tris gel) electrophoresis (SDS-PAGE) method under reduced conditions. The gel was stained with InstantBlue for about 30 minutes and destained overnight with purified water. Figure 60 shows the SDS gel of the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide purified using an anti-tissue factor antibody affinity column. The results show that the purified α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide has the expected molecular weight (72 kDa) in reduced SDS gel.

Example 39. Functional Characterization of α CD3scFv/TF/ α CD28scFv Single-Chain Chimeric Polypeptide

ELISA-based methods confirmed the formation of the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide. The α CD3scFv/TF/ α CD28scFv single-chain
5 chimeric polypeptide was detected using ELISA with one anti-TF monoclonal antibody for capture and a different anti-TF monoclonal antibody for detection (Figure 61). A purified tissue factor protein with a similar concentration was used as a control.

A further in vitro experiment was performed to determine whether the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide is capable of activating
10 human peripheral blood mononuclear cells (PBMCs). Fresh human leukocytes were obtained from the blood bank and peripheral blood mononuclear cells (PBMC) were isolated using density gradient Histopaque (Sigma). The cells were counted and resuspended in 0.2×10^6 /mL in a 96-well flat bottom plate in 0.2 mL of complete media (RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Thermo Life Technologies),
15 penicillin (Thermo Life Technologies), streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone)). The cells were stimulated with α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide from 0.01 nM to 1000 nM for 3 days at 37 °C, 5% CO₂. After 72 hours, the cells were harvested and surface stained for CD4-488, CD8-PerCP Cy5.5, CD25-BV421, CD69-APCFire750, CD62L-PE Cy7, and CD44-PE (Biolegend) for 30
20 minutes. After surface staining, the cells were washed (1500 RPM for 5 minutes at room temperature) in FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)). After two washes, the cells were resuspended in 300 μ L of FACS buffer and analyzed by Flow Cytometry (Celesta-BD Bioscience). The data in Figures 62 and 63 show that the α CD3scFv/TF/ α CD28scFv single-chain chimeric
25 polypeptide is able to stimulate both CD8⁺ and CD4⁺ T-cells.

A further experiment was performed, in which PBMCs isolated from blood using Histopaque (Sigma) were counted and resuspended in 0.2×10^6 /mL in a 96-well flat bottom plate in 0.2 mL of complete media (RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Thermo Life Technologies), penicillin (Thermo Life Technologies),
30 streptomycin (Thermo Life Technologies), and 10% FBS (Hyclone)). The cells were then stimulated with the α CD3scFv/TF/ α CD28scFv single-chain chimeric polypeptide

from 0.01 nM to 1000 nM for 3 days at 37 °C, 5% CO₂. After 72 hours, the cells were harvested and surface stained for CD4-488, CD8-PerCP Cy5.5, CD25-BV421, CD69-APCFire750, CD62L-PE Cy7, and CD44-PE (Biolegend) for 30 minutes. After surface staining, the cells were washed (1500 RPM for 5 minutes at room temperature) in FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)). After two washes, the cells were resuspended in 300 µL of FACS buffer and analyzed by Flow Cytometry (Celesta-BD Bioscience). The data again show that the αCD3scFv/TF/αCD28scFv single-chain chimeric polypeptide was able to stimulate activation of CD4⁺ T cells (Figure 64).

Example 40. Production and characterization of the Exemplary Single-Chain Chimeric Polypeptide IL-2/TF/IL-2

An exemplary single-chain chimeric polypeptide including a first target-binding domain that binds to an IL-2 receptor, a soluble human tissue factor domain, and a second target-binding domain that binds to an IL-2 receptor was generated (IL-2/TF/IL-2) (Figure 65). The nucleic acid and amino acid sequences of this single-chain chimeric polypeptide are shown below.

Nucleic Acid Encoding Exemplary Single-Chain Chimeric Polypeptide (IL-2/TF/IL-2) (SEQ ID NO: 132)

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTCCTGTTCTCCAGCGCCT
ACTCC

(First IL-2 fragment)

GCCCCACCTCCTCCTCCACCAAGAAGACCCAGCTGCAGCTGGAGCAT
TTACTGCTGGATTTACAGATGATTTTAAACGGCATCAACAACCTACAAGAACC
CCAAGCTGACTCGTATGCTGACCTTCAAGTTCTACATGCCCAAGAAGGCCAC
CGAGCTGAAGCATTTACAGTGTTTAGAGGAGGAGCTGAAGCCCCCTCGAGGAG
GTGCTGAATTTAGCCCAGTCCAAGAATTTCCATTAAAGGCCCCGGGATTTAAT
CAGCAACATCAACGTGATCGTTTTAGAGCTGAAGGGCTCCGAGACCACCTTC

ATGTGCGAGTACGCCGACGAGACCGCCACCATCGTGGAGTTTTTAAATCGTT
GGATCACCTTCTGCCAGTCCATCATCTCCACTTTAACC

(Human tissue factor 219 form)

AGCGGCACAACCAACACAGTCGCTGCCTATAACCTCACTTGGAAGAG
5 CACCAACTTCAAAACCATCCTCGAATGGGAACCCAAACCCGTTAACCAAGTT
TACACCGTGCAGATCAGCACCAAGTCCGGCGACTGGAAGTCCAAATGTTTCT
ATACCACCGACACCGAGTGCGATCTCACCGATGAGATCGTGAAAGATGTGAA
ACAGACCTACCTCGCCCGGGTGTTTAGCTACCCCGCCGGCAATGTGGAGAGC
ACTGGTTCCGCTGGCGAGCCTTTATACGAGAACAGCCCCGAATTTACCCCTTA
10 CCTCGAGACCAATTTAGGACAGCCCACCATCCAAAGCTTTGAGCAAGTTGGC
ACAAAGGTGAATGTGACAGTGGAGGACGAGCGGACTTTAGTGCGGCGGAAC
AACACCTTTCTCAGCCTCCGGGATGTGTTTCGGCAAAGATTTAATCTACACACT
GTATTACTGGAAGTCCTCTTCCCTCCGGCAAGAAGACAGCTAAAACCAACACA
AACGAGTTTTTAATCGACGTGGATAAAGGCGAAAACACTGTTTCAGCGTGC
15 AAGCTGTGATCCCCTCCCGGACCGTGAATAGGAAAAGCACCGATAGCCCCGT
TGAGTGCATGGGCCAAGAAAAGGGCGAGTTCCGGGAG

(Second IL-2 fragment)

GCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGGAGCAT
TTACTGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCC
20 CAAACTCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACA
GAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAG
TGCTAAATTTAGCTCAAAGCAAAAACCTTCACTTAAGACCCAGGGACTTAAT
CAGCAATATCAACGTAATAGTTCTGGAATAAAGGGATCTGAAACAACATTC
ATGTGTGAATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGAT
25 GGATTACCTTTTGTCAAAGCATCATCTCAACACTAACT

Exemplary Single-Chain Chimeric Polypeptide (IL-2/TF/IL-2) (SEQ ID NO: 131)

(Signal peptide)

MKWVTFISLLFLFSSAYS

30 *(Human IL-2)*

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA
 TELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCE
 YADETATIVEFLNRWITFCQSIISTLT

(Human Tissue Factor 219)

5 SGTNTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDWKSKC
 FYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPLYENSPEFTPYL
 ETNLGQPTIQSFEQVGTKVNVTVEDERTLVRRNNTFLSLRDVFGKDLYTLYYW
 KSSSSGKKTAKTNTNEFLIDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQE
 KGEFRE

10 *(Human IL-2)*

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA
 TELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCE
 YADETATIVEFLNRWITFCQSIISTLT

15 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
 20 purified by anti-TF antibody affinity and other chromatography methods.

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

To evaluate the IL-2 activity of IL-2/TF/IL-2, IL-2/TF/IL-2 was compared with
 25 recombinant IL-2 for promoting proliferation of 32D β cells that express IL-2R β and
 common γ chain. IL-2 dependent 32D β cells were washed 5 times with IMDM-10% FBS
 and seeded to the wells at 2×10^4 cells/well. Serial dilutions of IL-2/TF/IL-2 or IL-2
 were added to the cells (Figure 66). Cells were incubated in a CO₂ incubator at 37°C for 3
 days. Cell proliferation was detected by adding 10 μ l of WST1 to each well on day 3 and
 30 incubating for an additional 3 hours in a CO₂ incubator at 37°C. The amount of formazan
 dye produced was analyzed by measuring the absorbance at 450 nm. As shown in Figure

66, IL-2/TF/IL-2 and IL-2 activated 32D β cells in a similar manner. The EC₅₀ of IL-2/TF/IL-2 and IL-2 was 158.1 pM and 140 pM, respectively.

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

To evaluate the IL-2 activity of IL-2/TF/IL-2, IL-2/TF/IL-2 was compared with recombinant IL-2 for promoting proliferation of CTLL-2 cells that express IL-2R α , IL-2R β and common γ chain. IL-2 dependent CTLL-2 cells were washed 5 times with IMDM-10% FBS and seeded to the wells at 2×10^4 cells/well. Serial dilutions of IL-2/TF/IL-2 or IL-2 were added to the cells (Figure 67). Cells were incubated in a CO₂ incubator at 37°C for 3 days. Cell proliferation was detected by adding 10 μ l of WST1 to each well in the day 3 and incubating for an additional 3 hours in a CO₂ incubator at 37°C. The amount of formazan dye produced was analyzed by measuring the absorbance at 450 nm. As shown in Figure 59, IL-2/TF/IL-2 promoted CTLL-2 cell proliferation 4-5-fold stronger than IL-2. The EC₅₀ of IL-2/TF/IL-2 was 123.2 pM and IL-2 was 548.2 pM.

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

Six-week-old female ApoE^{-/-} mice (Jackson Lab) were fed with standard chow diet or high diet fat containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Harlan Laboratories) and maintained in the standard conditions. At week 7, mice fed with high fat diet were randomly assigned into the control group and treatment group. Mice then received either IL-2/TF/IL-2 (treatment group) or PBS (chow diet group and control group) per subcutaneous injection at a dosage of 3 mg/kg. Three days post dosing, the mice were fasted overnight, and blood samples were collected through retro-orbital venous plexus puncture. Overnight fasting glucose levels were measured using a OneTouch Glucometer. As shown in Figure 68, the results showed that IL-2/TF/IL-2 injection effectively suppresses the increase of glucose levels in ApoE^{-/-} mice.

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

Six-week-old female ApoE^{-/-} mice (Jackson Lab) were fed with standard chow diet or high diet fat containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Harlan Laboratories) and maintained in the standard conditions. At week 7, mice fed with the high fat diet were randomly assigned into control group and treatment group. Mice then received either IL-2/TF/IL-2 (treatment group) or PBS (chow diet group and control group) per subcutaneous injection at a dosage of 3mg/kg. Three days after the dosing, overnight fasting blood samples were collected through retro-orbital venous plexus puncture and incubated with ACK lysing buffer (Thermo Fisher Scientific) at 37°C for 5 minutes. Samples were then resuspended in FACS buffer (1 X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)) and surface stained with FITC-anti-CD4 and APC-anti-CD25 antibodies (BioLegend) for 30 minutes. Surface-stained samples were further fixed and premetallized with Fix/Perm buffer (BioLegend) and intracellular stained with PE-anti-Foxp3 antibody (BioLegend). After staining, cells were washed twice with FACS buffer followed by centrifugation at 1500 RPM for 5 minutes at room temperature. The cells were analyzed by flow cytometry (Celesta-BD Bioscience). As shown in Figure 69, IL-2/TF/IL-2 treatment significantly increased Treg populations in blood lymphocytes (3.5%±0.32) compared to the untreated groups (0.4%±0.16 for chow diet group and 0.46%±0.09 for high fat diet group).

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

IL-2/TF/IL-2 harvested from cell culture was loaded onto the anti-TF antibody affinity column equilibrated with 5 column volumes of PBS. After sample loading, the column was washed with 5 column volumes of PBS, followed by elution with 6 column volumes of 0.1M acetic acid, pH 2.9. A280 elution peak was collected and then neutralized to pH 7.5-8.0 with 1M Tris base. The neutralized sample was then buffer exchanged into PBS using Amicon centrifugal filters with a 30 kDa molecular weight cutoff. As shown in Figure 70, the anti-TF antibody affinity column bound to IL-2/TF/IL-

2 which contains TF as a fusion domain. The buffer-exchanged protein sample was stored at 2-8 °C for further biochemical analyses and biological activity tests. After each elution, the anti-TF antibody affinity column was stripped using 6 column volumes of 0.1M glycine, pH 2.5. The column was then neutralized using 5 column volumes of PBS, and 7 column volumes of 20% ethanol for storage. The anti-TF antibody affinity column was connected to a GE Healthcare AKTA Avant system. The flow rate was 4 mL/min for all steps except for the elution step, which was 2 mL/min.

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

To analyze IL-2/TF/IL-2 using analytical size exclusion chromatography (SEC), a Superdex 200 Increase 10/300 GL gel filtration column (from GE Healthcare) was connected to an AKTA Avant system (from GE Healthcare). The column was equilibrated with 2 column volumes of PBS. The flow rate was 0.7 mL/min. A sample containing IL-2/TF/IL-2 in PBS was injected into the Superdex 200 column using a capillary loop, and analyzed by SEC. The SEC chromatograph of the sample is shown in Figure 71. The SEC results indicated two protein peaks for IL-2/TF/IL-2.

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

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

To verify that the IL-2/TF/IL-2 protein undergoes glycosylation after translation in CHO cells, a deglycosylation experiment was conducted using the Protein Deglycosylation Mix II kit from New England Biolabs according to the manufacturer's instructions. Figures 72A and 72B show the reduced SDS-PAGE analysis of the sample in non-deglycosylated (lane 1 in red outline) and deglycosylated (lane 2 in yellow outline) state. The results show that the IL-2/TF/IL-2 protein is glycosylated when expressed in CHO cells. After deglycosylation, the purified sample ran with expected

molecular weights (56 kDa) in reduced SDS gel. Lane M was loaded with 10 μ L of SeeBlue Plus2 Prestained Standard.

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

5 IL-2/TF/IL-2 was subcutaneously injected into C57BL/6 mice at various doses to determine the immunostimulatory activity of IL-2/TF/IL-2 in vivo. Mice were subcutaneously treated with control solution (PBS) or IL-2/TF/IL-2 at 0.1, 0.4, 2 and 10 mg/kg. The treated mice were euthanized day 3 post treatment. The mouse spleens were collected and weighed day 3 post treatment. Single splenocyte suspensions were
10 prepared, and the prepared splenocytes were stained for CD4⁺ T cells, CD8⁺ T cells and NK cells (with fluorochrome-conjugated anti-CD4, -CD8, and -NK1.1 antibodies), and analyzed by flow cytometry. The results showed that IL-2/TF/IL-2 was effective at expanding splenocytes based on spleen weight (Figure 73A) especially at 0.1-10 mg/kg. The percentage of CD8⁺ T cells were higher compared to control-treated mice (Figure
15 73B) at 2 and 10 mg/kg. The percentage of NK cells were higher compared to control-treated mice (Figure 73B) at all doses tested.

It has been known that IL-2 upregulates CD25 expression by immunocytes. We therefore accessed CD25 expression of CD4⁺ T cells, CD8⁺ T cells and NK cells in the IL-2/TF/IL-2 treated mice. C57BL/6 mice were subcutaneously treated with IL-2/TF/IL-
20 2 as described in the paragraph above. The splenocytes were stained with fluorochrome-conjugated anti-CD4, -CD8, CD25 and NK1.1 monoclonal antibodies. The CD25 expression (MFI) of splenocyte subsets was analyzed by flow cytometry. As shown in Figure 74, at the doses and time point (day 3) tested, IL-2/TF/IL-2 significantly upregulated CD25 expression by CD4⁺ T cells but not CD8⁺ T cells or NK cells.

25 The pharmacokinetics of IL-2/TF/IL-2 in C57BL/6 mice was also investigated. IL-2/TF/IL-2 was subcutaneously injected into C57BL/6 mice at 1 mg/kg. The mouse blood was drawn from tail vein at various time points as shown in Figure 75 and the serum was prepared. IL-2/TF/IL-2 concentrations were determined with ELISA (Capture: anti-tissue factor antibody; Detection: biotinylated anti-human IL-2 antibody
30 followed by SA-HRP and ABTS substrate). The half-life of IL-2/TF/IL-2 was 1.83 hours calculated with PK Solutions 2.0 (Summit Research Services).

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

Six-week-old female ApoE^{-/-} mice (The Jackson Laboratory) were fed with standard chow diet or high diet fat (21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch) (TD88137, Harlan Laboratories) and maintained in the standard conditions. At week 7, mice fed with high fat diet (HFD) were randomly assigned into control group and treatment group. Mice were then administrated either IL-2/TF/IL-2 (treatment group) or PBS (chow diet group and control group) subcutaneously at a dosage of 3mg/kg weekly for 4 weeks. At week 12, all mice were euthanized by isoflurane. Aortas were collected, opened longitudinally, and stained with Sudan IV solution (0.5%) using *en face* method. The percentage of plaque area (red color as shown in Figure 76A) relative to total aorta area was then quantified with Image J software. Figure 76A shows a representative view of atherosclerotic plaques from each group. Figure 76B shows the results of quantitative analysis of atherosclerotic plaques of each group. The percentage of plaque areas in control group (HF Diet) was much higher than the treatment group (HFD+IL-2/TF/IL-2), being 10.28% vs 4.68 %.

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

Male BKS.Cg-Dock7^m +/+ Lepr^{db}/J (db/db (Jackson Lab)) mice were fed with standard chow diet and received drinking water ad libitum. At the age of six weeks, mice were randomly assigned into control group and treatment group. The treatment group received IL-2/TF/IL-2 by subcutaneous injection at 3 mg/kg bi-weekly, while control group received vehicle (PBS) only. Overnight fasting glucose levels were measure weekly using a OneTouch Glucometer. The results showed that IL-2/TF/IL-2 effectively suppressed the increase of glucose levels in BKS.Cg-Dock7^m +/+ Lepr^{db}/J mice (Figure 77).

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

Male BKS.Cg-Dock7^m +/+ Lepr^{db}/J (db/db) (The Jackson Laboratory) mice were fed with standard chow diet and received drinking water ad libitum. At the age of six

weeks, mice were randomly assigned into control group and treatment group. The treatment group received IL-2/TF/IL-2 by subcutaneous injection at 3 mg/kg bi-weekly, while the control group received vehicle (PBS) only. Four days after the first drug injection, overnight fasting blood samples were collected and incubated with ACK lysing
 5 buffer (Thermo Fisher Scientific) at 37°C for 5 minutes. Samples were then resuspended in FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% sodium azide (Sigma)) and surface stained with FITC-anti-CD4 and APC-anti-CD25 antibodies (BioLegend) for 30 minutes. Surface-stained samples were further fixed and premetallized with Fix/Perm buffer (BioLegend) and intracellular stained with PE-anti-
 10 Foxp3 antibody (BioLegend). After staining, cells were washed twice with FACS buffer and were analyzed by flow cytometry (Celesta-BD Bioscience). The percentage of CD4⁺CD25⁺FoxP3⁺ Tregs in blood lymphocytes were measured. As shown in Figure 78, the results showed that IL-2/TF/IL-2 significantly upregulated the ratio of Tregs in blood lymphocytes. * p<0.05

15

Example 41: Stimulation of NK cells *in vivo* by IL-2/TF/IL-2 (2t2)

A set of experiments was performed to determine the effect of the 2t2 construct on immune stimulation in C57BL/6 mice. In these experiments, C57BL/6 mice were subcutaneously treated with control solution (PBS) or 2t2 at 0.1, 0.4, 2, and 10 mg/kg.
 20 Treated mice were euthanized 3 days post-treatment. Spleen weight was measured and single splenocyte suspensions were prepared. Splenocytes suspensions were stained with conjugated anti-CD4, anti-CD8, and anti-NK1.1 (NK) antibodies. The percentage of CD4⁺ T cells, CD8⁺ T cells, and NK cells, and CD25 expression on lymphocyte subsets were analyzed by flow cytometry. Figure 79A shows that 2t2 was effective at expanding
 25 splenocytes based on spleen weight especially at a dose level of 0.1-10 mg/kg. Following treatment, the percentage of CD8⁺ T cells were higher in 2t2-treated mice compared to control-treated mice at 2 and 10 mg/kg (Figure 79B). The percentage of NK cells were also higher in 2t2-treated mice compared to control-treated mice at all doses of 2t2 tested (Figure 79B). Additionally, 2t2 significantly upregulated CD25 expression by CD4⁺ T
 30 cells, but not CD8⁺ T cells and NK cells following treatment at 0.4 to 10 mg/kg (Figure 79C).

A set of experiments was performed to determine the effect of the 2t2 construct on immune stimulation in ApoE^{-/-} mice fed with a Western diet. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories). After 8-weeks of the Western diet, the mice were injected subcutaneously with 2t2 at 3 mg/kg. Three days post treatment, mice were fasted for 16 hours and then blood samples were collected through retro-orbital venous plexus puncture. The blood was mixed with 10 μ L 0.5 M EDTA, and 20 μ L blood was taken for lymphocyte subsets analysis. The red blood cells were lysed with ACK (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4) and the lymphocytes were stained with anti-mouse CD8a and anti-mouse NK1.1 antibodies for 30 minutes at 4 °C in FACS staining buffer (1% BSA in PBS). The cells were washed once and analyzed with a BD FACS Celesta. For Treg staining, ACK treated blood lymphocytes were stained with anti-mouse CD4 and anti-mouse CD25 antibodies for 30 minutes at 4 °C in FACS staining buffer. The cells were washed once and resuspended in fixation/permeabilization working solution and incubated at room temperature for 60 minutes. The cells were washed once and resuspended in permeabilization buffer. The samples were centrifuged at 300-400 x g for 5 minutes at room temperature and the supernatant was then discarded. The cell pellet was resuspended in residual volume and the volume adjusted to about 100 μ L with 1 x permeabilization buffer. Anti-Foxp3 antibody was added to the cells, and the cells were incubated for 30 minutes at room temperature. Permeabilization buffer (200 μ L) was added to the cells, and the cells were centrifuged at 300-400 x g for 5 minutes at room temperature. The cells were resuspended in flow cytometry staining buffer and analyzed on a flow cytometer. Figures 80B-80C show that treatment with 2t2 increased the percentage of NK cells and CD8⁺ T cells in ApoE^{-/-} mice fed with Western diet. Figure 80A shows that treatment with 2t2 also increased the percentage of Treg cells.

Example 42: Induction of proliferation of immune cells *in vivo*

A set of experiments was performed to determine the effect of the 2t2 construct on immune cell stimulation in C57BL/6 mice. In these experiments, C57BL/6 mice were

subcutaneously treated with control solution (PBS) or 2t2 at 0.1, 0.4, 2, and 10 mg/kg. Treated mice were euthanized 3 days post-treatment. Spleen weight was measured and single splenocyte suspensions were prepared. The splenocyte suspensions were stained with conjugated anti-CD4, anti-CD8, and anti-NK1.1 (NK) antibodies. The percentage of CD4⁺ T cells, CD8⁺ T cells, and NK cells were analyzed by flow cytometry. Figure 81A shows that 2t2 treatment was effective at expanding splenocytes based on spleen weight especially at 0.1-10 mg/kg. The percentage of CD8⁺ T cells was higher compared to control-treated mice at 2 and 10 mg/kg (Figure 81B). The percentage of NK cells was higher compared to control-treated mice at all doses of 2t2 tested (Figure 81B). These results demonstrate that 2t2 treatment was able to induce proliferation of CD8⁺ T cells and NK cells in C57BL/6 mice.

A set of experiments was performed to determine the effect of the 2t2 construct on immune stimulation in ApoE^{-/-} mice fed with a Western diet. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories). After 8-week of the Western diet, the mice were injected subcutaneously with 2t2 at 3 mg/kg. Three days post-treatment, the mice were fasted for 16 hours and then blood samples were collected through retro-orbital venous plexus puncture. The blood was mixed with 10 μ L 0.5 M EDTA and 20 μ L blood was taken for lymphocyte subsets analysis. The red blood cells were lysed with ACK (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4) and the lymphocytes were stained with anti-mouse CD8a and anti-mouse NK1.1 antibodies for 30 minutes at 4 °C in FACS staining buffer (1% BSA in PBS). The cells were washed once and resuspended in Fixation Buffer (BioLegend Cat# 420801) for 20 minutes at room temperature. The cells were centrifuged at 350 x g for 5 minutes, the fixed cells were resuspended in Intracellular Staining Permeabilization Wash Buffer (BioLegend Cat# 421002) and then centrifuged at 350 x g for 5 minutes. The cells were then stained with anti-Ki67 antibody for 20 minutes at RT. The cells were washed twice with Intracellular Staining Permeabilization Wash Buffer and centrifuged at 350 x g for 5 minutes. The cells were then resuspended in FACS staining buffer. Lymphocyte subsets were analyzed with a

BD FACS Celesta. Figure 82A and 82B shows treatment of ApoE^{-/-} mice with 2t2 also induced proliferation (Ki67-positive staining) in NK and CD8⁺ T cells.

Example 43: Treatment of Diabetes

5 A set of experiments was performed to investigate amelioration of Western diet-induced hyperglycemia in ApoE^{-/-} mice by 2t2. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories). After 8-weeks of the Western diet, the mice were
10 injected subcutaneously with 2t2 at 3 mg/kg. Three days post-treatment, the mice were fasted for 16 hours and then blood samples were collected through retro-orbital venous plexus puncture. Blood glucose was detected with a glucose meter (OneTouch UltraMini) and GenUltimated test strips using a drop of fresh blood. As shown in Figure 83A, 2t2 treatment significantly reduced hyperglycemia induced by the Western diet
15 (p<0.04). The plasma insulin and resistin levels were analyzed with Mouse Rat Metabolic Array by Eve Technologies. HOMA-IR was calculated using the following formula: homeostatic model assessment-insulin resistance = Glucose (mg/dL) * Insulin (mU/mL)/405. As shown in Figure 83B, 2t2 treatment reduced insulin resistance compared to the untreated group. 2t2 (p<0.02) reduced resistin levels significantly
20 compared to the untreated group as shown in Figure 31C, which may relate to the reduced insulin resistance induced by 2t2 (Figure 83B).

Example 44. Upregulation of CD44 memory T cells

C57BL/6 mice were subcutaneously treated with 2t2. The treated mice were
25 euthanized and the single splenocyte suspensions were prepared 4 days (TGFRt15-TGFRs) or 3 days (2t2) following the treatment. The prepared splenocytes were stained with fluorochrome-conjugated anti-CD4, anti-CD8 and anti-CD44 antibodies and the percentages of CD44^{high} T cells in CD4⁺ T cells or CD8⁺ T cells were analyzed by flow cytometry. The results show that 2t2 upregulated expression of the memory marker
30 CD44 on CD4⁺ and CD8⁺ T cells (Figure 84). These findings indicate that 2t2 was able to induce mouse T cells to differentiate into memory T cells.

Example 45: Induction of Treg cells by 2t2

The peripheral blood mononuclear cells (PBMC) of a healthy donor (Donor 163) were isolated from 5 mL of whole blood buffy coats by Ficoll Paque Plus (GE17144003).

- 5 The PBMC were then lysed with ACK to remove red blood cells. Cells were washed with IMDM-10% FBS and counted. 1.8×10^6 cells (100 μ L/tube) were seeded to the flow tubes and incubated with 50 μ L of descending 2t2 or IL2 (15000, 1500, 150, 15, 1.5, 0.15, or 0 pM) and 50 μ L of pre-staining antibodies (anti-CD8-BV605 and anti-CD127-AF647). Cells were incubated for 30 min at 37 °C in water bath. 200 μ L of pre-warmed
- 10 BD Phosflow Fix Buffer I (Cat# 557870, Becton Dickinson Biosciences) was added for 10 min at 37° C in water bath to stop the stimulation. Cells (4.5×10^5 cells/100 μ L) were transferred to a V-shape 96-well plate and were spun down followed by permeabilization with 100 μ L of -20 °C pre-cooled BD Phosflow Perm Buffer III (Cat# BD Biosciences) for 30 min on ice. The cells were then extensively washed x2 with 200 μ L of FACS
- 15 buffer and stained with a panel of fluorescent antibodies (anti-CD25-PE, CD4-PerCP-Cy5.5, CD56-BV421, CD45RA-PE-Cy7 and pSTAT5a-AF488) to distinguish between different lymphocyte subpopulations and evaluate the pSTAT5a status. Cells were spun down and resuspended in 200 μ L of FACS buffer for FACSCelesta analysis. As shown in Figure 85A, 6 pM of 2t2 was sufficient to induce the phosphorylation of Stat5a in
- 20 $CD4^+CD25^{hi}$ T_{reg} cells while 43.11 pM of IL-2 was required to induce phosphorylation of Stat5a in the same population of lymphocytes. In contrast, 2t2 was less active (Figure 85B) or equally active (Figure 85C) as compared to IL2 in inducing phosphorylation of Stat5a in $CD4^+CD25^+T_{con}$ and $CD8^+T_{con}$ cells. These results suggest that 2t2 is superior as compared to IL2 in activating T_{reg} in human PBMC, and that 2t2 demonstrates
- 25 increased T_{reg} selectivity compared to IL-2 in human blood lymphocyte pStat5a responses.

Example 46. Effects of TGF β Rt15-TGFRs and 2t2 treatment on mouse hemoglobin A1C in plasma of aged mice

- 30 C57BL/6, 72-week-old mice were purchased from the Jackson Laboratory. Mice were housed in a controlled temperature and controlled light environment. Mice were

divided into five groups receiving the following treatment: Saline control ($n=8$), one dose of TGF α 15-TGFRs on day 0 ($n=8$), one dose of TGF α 15-TGFRs on day 0 followed by one dose of 2t2 on day 60 ($n=7$), one dose of 2t2 on day 0 ($n=3$) and one dose of 2t2 on day 0 followed by one dose of TGF α 15-TGFRs on day 60 ($n=7$). Mice were treated subcutaneously with PBS, TGF α 15-TGFRs (3 mg/kg), 2t2 (3 mg/kg) or TGF α 15-TGFRs (3 mg/kg) plus 2t2 (3 mg/kg).

Mouse blood was collected from submandibular vein on day 120 in tubes containing EDTA. Levels of hemoglobin A1C in the blood was assessed using a Mouse Hemoglobin A1C Assay kit (Crystal Chem). The whole blood was mixed with lysis buffer without creating foam and incubated for 10 minutes at room temperature to lyse the red blood cells. In a microplate, CC1a and CC1b reagents were added and mixed with lysate from previous steps and further incubated at 37°C incubator for 5 minutes. After incubation, absorbance was measured in microplate reader at A₇₀₀ nM wavelength. After absorbance measurement, CC2 reagent was added and further incubated at 37°C incubator for 3 minutes. Final absorbance was measured in microplate reader at A₇₀₀ nM wavelength. Hemoglobin A1C was calculated based on the change in absorbance per the manufacturer's instructions (Crystal Chem).

The results indicate that treatment of aged mice with 2t2 followed by TGF α 15-TGFRs reduced plasma levels of hemoglobin A1C, compared to control treated mice (Figure 86).

Example 47. Reduction in senescent markers in an aged mouse model

C57BL/6, 72-week-old mice were purchased from the Jackson Laboratory. Mice were housed in a controlled temperature and controlled light environment. Mice were divided into five groups receiving the following treatment: Saline control ($n=8$), one dose of TGF α 15-TGFRs on day 0 ($n=8$), one dose of TGF α 15-TGFRs on day 0 followed by one dose of 2t2 on day 60 ($n=7$), one dose of 2t2 on day 0 ($n=3$) and one dose of 2t2 on day 0 followed by one dose of TGF α 15-TGFRs on day 60 ($n=7$). Mice were treated subcutaneously with PBS, TGF α 15-TGFRs (3 mg/kg), 2t2 (3 mg/kg) or TGF α 15-TGFRs (3 mg/kg) plus 2t2 (3 mg/kg). At day 120 post treatment, mice were euthanized, and livers were harvested in order to evaluate the expression levels of

senescence markers IL-1 α , IL6 and PAI-1 by quantitative-PCR. Harvested kidneys were stored in liquid nitrogen in 1.7 mL Eppendorf tubes. Samples were homogenized by using homogenizer in 1 mL of Trizol (Thermo Fischer). Homogenized tissues were transferred in fresh Eppendorf tubes. Total RNA was extracted using RNeasy Mini Kit (Qiagen #74106) according to the manufacturer's instructions. One μ g of total RNA was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was carried out with CFX96 Detection System (Bio-Rad) using FAM labeled predesigned primers purchased from Thermo Scientific. Reactions were run in triplicate for all the genes examined. The housekeeping gene 18S ribosomal RNA was used as an internal control to normalize the variability in expression levels. The expression of each target mRNA relative to 18S rRNA was calculated based on Ct as $2^{-\Delta(\Delta C_t)}$, in which $\Delta C_t = C_{t\text{target}} - C_{t18S}$. Untreated 6-week-old mice (Young) were used as a control to compare the gene expression level to aged mice.

As showed in Figures 87A-87C, gene expression of IL-1 α , IL6 and PAI-1 by in liver increased with the age of the mice as expected with the age-dependent increase in cellular senescence. Treatment of 72-month old mice with a single dose of TGFRt15-TGFRs resulted in a significant and long-lasting effect in reducing gene expression of senescence markers in livers, suggesting a treatment associated decrease in naturally-occurring senescent cells in the liver of aged mice. However, in other treatment though gene expression of IL-1 α , IL6 and PAI-1 was reduced but not statically significant.

Example 48. Reduction of the Western diet-induced non-alcoholic steatohepatitis (NASH) in ApoE^{-/-} mice by a combination of 2t2 and TGFRt15-TGFRs

The 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories) (Table 1). After 6 weeks on the Western diet, the mice were injected subcutaneously with a 1st dose of TGFRt15-TGFRs at 3 mg/kg for Group 2 or 2t2 at 3 mg/kg for Groups 3 and 4. After 12 weeks on the Western diet, the mice were injected subcutaneously with 2nd dose of TGFRt15-TGFRs at 3 mg/kg for Group 2, 2t2 at 3 mg/kg for Group 3, or TGFRt15-TGFRs at 3 mg/kg for Group 4 (Figure 88A). Mice of Group 1 served as controls. After 14 weeks on the

Western diet, the mice were euthanized, and livers were collected to analyze the hepatic hydroxyproline content as a marker of fibrosis. Hepatic hydroxyproline was photometrically measured in liver hydrolysates. Similar segments (200 mg) of snap-frozen livers were first hydrolyzed in HCl (6N) at 110 °C for 16 hours and then filtered and aliquoted. Aliquots (50 µL) were incubated with chloramine T (2.5 mM) for 5 minutes and subsequently with Ehrlich's reagent (410 mM) for 30 minutes at 60 °C. Adsorption was determined three times at 558 nm and compared to a standard curve for hydroxyproline. The results are expressed as µg/g of wet liver tissue. As shown in Figure 88B, a combination with 2t2 and TGFRt15-TGFRs treatment (Group 4) significantly reduced hepatic hydroxyproline content induced by the Western diet ($p < 0.00312$ based on ordinary one-way ANOVA and Tukey's multiple comparisons test) while TGFRt15-TGFRs or 2t2 treatment alone did not result in significant changes of hepatic hydroxyproline content compared to the control group. The data suggests that a combination of 2t2 and TGFRt15-TGFRs may have potential for treatment of NASH.

Table 1

Group	Animal	Treatment	Mouse/group
1	ApoE ^{-/-}	Control	7
2	ApoE ^{-/-}	TGFRt15-TGFRs	11
3	ApoE ^{-/-}	2t2	10
4	ApoE ^{-/-}	2t2+TGFRt15-TGFRs	10

Example 49. Cytokine, Triglyceride, and LDL Levels in ApoE^{-/-} Atherosclerosis**Mouse Model**

A set of experiments was performed to determine cytokine, triglyceride, and LDL levels in ApoE^{-/-} mice treated with 2t2. In these experiments, 6-week old female B6.129P2-ApoE^{tm1Unc}/J mice (Jackson Laboratory) were fed with a Western diet containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Envigo Laboratories). After 6 weeks, 9 weeks, and 12 weeks of the Western diet, the mice were injected subcutaneously with 2t2 at 3 mg/kg.

Plasma samples obtained from the 2t2-treated mice and the untreated mice (mice on the same diet but not treated with 2t2) were obtained from the mice at 3 days after the

second injection at 9 weeks. As shown in Figure 89A, 3 days after the second dosing, over-night fasting blood samples were collected through submandibular vein puncture and the plasma was isolated. IL-1 β , MCP-1 and TNF- α were analyzed with Mouse Cytokine Array Proinflammatory Focused 10-plex (MDF10) by Eve Technologies (Calgary, AB Canada T2N 0M4). As shown in Figure 89B, IL-1 β levels were significantly reduced in 2t2 treatment group compared with the control group ($p = 0.0312$), MCP-1 levels were significantly reduced in 2t2 treatment group compared with the control group ($p = 0.0235$), and TNF- α levels were significantly reduced in 2t2 treatment group compared with the control group ($p = 0.0172$).

The concentration of triglyceride and LDL in plasma samples obtained from the 2t2-treated mice and the untreated mice (mice on the same diet but not treated with 2t2) at 3 days after the second injection at 9 weeks, were determined. Over-night fasting blood samples were collected through submandibular vein puncture and the plasma was isolated. Plasma concentration of triglyceride was determined using Abcam's triglyceride quantification assay kit (Cat# ab65336, Abcam) according to manufacturer's protocol, where the plasma was prepared in a standard 96-well plate and mixed with triglyceride assay buffer. Lipase was added to the wells and further incubated for 20 minutes at room temperature. After incubation, triglyceride reaction mix was added to each well and further incubated for 60 minutes at room temperature out of the light and absorbance was measured at 570 nm wavelength. Concentration of triglyceride in nmol/L (mM) in the test samples were calculated per the manufacturer's instructions.

Plasma LDL was analyzed with Mouse LDL-Cholesterol Assay Kit (Cat# 79980, Crystal Chem) according to manufacturer's protocol. The plasma sample was mixed with CC1 reagent and incubated for 5 minutes at 37 °C. After incubation, absorbance was measured in microplate reader at 600 nm wavelength. After absorbance measurement, CC2 reagent was added and further incubated at 37 °C for 5 minutes. Final absorbance was measured in microplate reader at 600 nm. The mouse LDL-cholesterol concentration was calculated based on the change in absorbance per the manufacturer's instructions (Crystal Chem).

The data show that 2t2 treatment significantly reduced the plasma triglyceride levels and the plasma LDL levels (Figures 90 and 91, respectively). Administration of

the 2t2 did not significantly affect the weight of the mice (as compared to untreated controls) (Figure 92).

Example 50: Effect of TGFRt15-TGFRs administration on high fat diet-based Type-2 diabetes mouse model

Materials and Methods

TGFRt15-TGFRs is a multi-chain chimeric polypeptide (a multi-chain chimeric polypeptide described herein) that includes two TGF β -binding domains which a soluble human TGF β RII dimer (aa24-159). 21t15-TGFRs is a multi-chain chimeric polypeptide that includes IL-21 and a TGF β -binding domain. The 2t2 single-chain chimeric polypeptide is the same single-chain chimeric polypeptide described in the above Examples.

Results

To evaluate the effect of TGFRt15-TGFRs, 2t2, and 21t15-TGFRs in a high fat diet-based Type-2 diabetes mouse model (B6.129P2-ApoE^{tm1Unc}/J from The Jackson Laboratory) was used. Mice were fed either a control diet or a high fat diet for 11 weeks. A subset of mice fed with the high fat diet were also treated with TGFRt15-TGFRs, 21t15-TGFR, or 2t2. Mice fed the control diet, high fat diet, and mice fed with the high fat diet and treated with TGFRt15-TGFRs, 21t15-TGFRs, or 2t2 were evaluated 4 days post-treatment.

To examine the effect of TGFRt15-TGFRs, 21t15-TGFRs, and 2t2 on the appearance and texture of skin and hair in animals, mice were fed either a control or a high fat diet for 7 weeks, and a subset of the mice fed a high fat diet were also treated with TGFRt15-TGFRs, 21t15-TGFRs, or 2t2. One week post-treatment, the appearance of the mice was evaluated. Mice fed a high fat diet and untreated, or a high diet and treated with 21t15-TGFRs appeared ungroomed and ruffled, and had increased gray hair/hair loss as compared to mice fed a control diet (Figures 93A, 93B and 93E).

Surprisingly, mice fed a high fat diet that received TGFRt15-TGFRs or 2t2 treatment appeared groomed and healthier (less gray hair/hair loss) (Figures 93C and 93D) as

compared to mice fed a high fat diet that did not receive TGFRt15-TGFRs or 2t2 treatment (Figure 93B). Specifically, TGFRt15-TGFRs or 2t2-treated mice showed superior skin and hair appearance and texture as compared to control mice. These results demonstrate that treatment with TGFRt15-TGFRs or 2t2 improves the appearance and texture of skin and hair in mammals.

Example 51. Improvement in Hair Growth using a Single-Chain Chimeric Polypeptide

The dorsal hair of 7-week-old C57BL6/J mice was shaved and depilated using commercial depilatory cream. The mice were injected on the same day subcutaneously with a single dose of 2t2 or low dose commercially available recombinant IL-2, followed by daily dosing for four additional days. Untreated mice served as controls. On day 10, the mice were sacrificed and skin sections of the shaved areas were prepared. Representative H&E staining of skin sections from C57BL6J mice on day 10 following depilation are shown in Figures 94A-94E. Figure 94A shows control mice - only depilation done after hair was shaved, Figure 94B shows mice where depilation was followed by low dose IL-2 (1 mg/kg) administration, and Figures 94C-94E shows mice where depilation was followed by 2t2 administered at 0.3 mg/kg (Figure 94C), 1 mg/kg (Figure 94D), and 3 mg/kg (Figure 94E). Black arrows indicate anagen-phase hair follicles that will later extend into dermis and facilitate hair growth. Figure 95 shows the total number of anagen phase hair follicles counted per 10 fields for each treatment group. In summary, the data show that the 2t2 molecule resulted in increased numbers of anagen-phase hair follicles compared to depilation alone. This effect was also dose-dependent.

Example 52: Treatment of Cancer

A set of experiments was performed to assess anti-tumor activity of TGFRt15-TGFRs plus anti-TRP1 antibody (TA99) in combination with chemotherapy in a melanoma mouse model. In these experiments, C57BL/6 mice were subcutaneously injected with 0.5×10^6 B16F10 melanoma cells. The mice were treated with three doses of chemotherapy docetaxel (10 mg/kg) (DTX) on day 1, day 4, and day 7, followed by

treatment with single dose of combination immunotherapy TGF α t15-TGFRs (3 mg/kg) + anti-TRP1 antibody TA99 (200 μ g) on day 8. Figure 96 shows a schematic of the treatment regimen.

To assess immune cell subsets in the B16F10 tumor model, peripheral blood analysis was performed. In these experiments, C57BL/6 mice were injected with B16F10 cells and treated with DTX, DTX + TGF α t15-TGFRs + TA99, or saline. Blood was drawn from the submandibular vein of B16F10 tumor-bearing mice on days 3, 5, and 10 post-immunotherapy for the DTX + TGF α t15-TGFRs + TA99 group. RBCs were lysed in ACK lysis buffer and the lymphocytes were washed and stained with anti-NK1.1, anti-CD8, anti-Ki67, anti-CD25, anti-granzyme B, and anti-CD4 antibodies. The cells were analyzed by flow cytometry (Celesta-BD Bioscience). Figures 97A-97H show that DTX + TGF α t15-TGFRs + TA99 treatment induced an increase in the percentage of NK cells and CD8⁺ T cells in blood as compared to the saline and DTX treatment groups.

Plasma levels of TGF- β 1, TGF- β 2, and TGF- β 3 were also determined in samples obtained at 16 hours, 3 days, 5 days, and 10 days post-immunotherapy for the DTX-TGF α t15-TGFRs + TA99 group. The data show that treatment with TGF α t15-TGFRs and TA99 reduced the plasma levels of TGF- β 1 and TGF- β 2 in DTX-treated mice as compared to the levels in DTX-only treated mice (Figures 98A-98C).

Plasma levels of IL-2, IL-1 β , IL-6, MCP-1, and GM-CSF were also determined in samples obtained at 16 hours, 3 days, 5 days, and 10 days post-immunotherapy for the DTX-TGF α t15-TGFRs + TA99 group. The data show that treatment with TGF α t15-TGFRs and TA99 reduced the plasma levels of IL-2, IL-1 β , IL-6, and GM-CSF in DTX-treated mice as compared to the levels in DTX-only treated mice (Figures 99A-99E).

On day 18 after transplantation of B16F10 cells in the mice, the mice were sacrificed and the relative levels of NK cells and CD8⁺ T-cells in the spleens of mice were determined. The data show that treatment with TGF α t15-TGFRs and TA99 increased the NK cell and CD8⁺ T-cell levels in the spleens of DTX-treated mice, as compared to the levels in the spleens of mice treated with DTX alone (Figures 100A-100B).

To assess glycolytic activity, glycolytic stress tests were performed in samples obtained 3 days, 5 days, and 10 days post-immunotherapy from the mice. Glycolytic activity of splenocytes from B16F10 tumor-bearing mice was determined by measuring

glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic acidification. The data show that treatment with TGFRT15-TGFRs and TA99 increased the glycolytic activity of splenocytes in DTX-treated mice as compared to the levels in DTX-only treated mice (Figures 101A-101C and Figures 102A-102L).

5 Mito stress tests were performed to further assess metabolism on splenocytes from the B16F10 tumor-bearing mice on samples obtained 3 days, 5 days, and 10 days post-immunotherapy from the mice. Mitochondrial respiration of splenocytes from the B16F10 tumor-bearing mice was also determined by measuring basal respiration, maximal respiration, spare respiratory capacity, and ATP production. The data show that
10 treatment with TGFRT15-TGFRs and TA99 increased the mitochondrial respiration of splenocytes in DTX-treated mice as compared to the levels in DTX-only treated mice (Figures 103A-103C and Figures 104A-104L).

NK and T-cell tumor infiltration was also assessed in B16F10 tumors in mice treated with DTX, DTX + TGFRT15-TGFRs + TA99, or saline. Figures 105A-105H
15 show that DTX + TGFRT15-TGFRs + TA99 treatment resulted in an increased level of infiltration of NK cells and CD8⁺ T cells in B16F10 tumors as compared to the saline and DTX treatment groups.

Senescence-associated gene expression in B16F10 tumors was determined in a melanoma mouse model treated with three doses of chemotherapy docetaxel (10 mg/kg) (DTX) on day 1, day 4, and day 7. Figure 106A shows a schematic of the treatment
20 regimen. The expression levels of DPP4, IL6, p16, and p21 in the B16F10 tumor were assessed. Figures 106B-106E show that DTX treatment induced an increase in senescence-associated gene expression in B16F10 tumor cells in the mice.

To assess the level of chemotherapy-induced senescence in B16F10 tumor cells
25 after TGFRT15-TGFRs treatment, the mice were treated with three doses of chemotherapy docetaxel (10 mg/kg) (DTX) on day 1, day 4, and day 7 followed by a single dose of combination immunotherapy TGFRT15-TGFRs (3 mg/kg) + anti-TRP1 antibody TA99 (200 µg) on day 8. On day 17, total RNA was extracted from B16H10 tumors of mice treated with saline, DTX, or DTX + TGFRT15-TGFRs + TA99 using
30 Trizol. Figure 107A shows a schematic of the treatment regimen. Total RNA (1 µg) was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen).

Real-time PCR was carried out with CFX96 Detection System (Bio-Rad) using FAM-labeled predesigned primers for senescence cell markers, p21 and IL-6, the data shows that TGF β 15-TGFRs and anti-TRP1 treatment reduces p21 gene expression in B16F10 tumors in mice treated with dexamethasone (Figures 107B-107C).

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Example 52: IL-2 Activity of 2t2

IL2 activity of 2t2 was compared with recombinant IL2 (Proleukin) in cell lines and PBMC cells. IL2 dependent 32D β or CTLL-2 cells were washed x5 with IMDM-10% FBS and seeded to the wells at 2×10^4 cells/well. Series diluted 2t2 or IL2 as shown in Figure 108A were added to the cells. Cells were incubated in CO₂ incubator at 37 °C for 3 days. Cell proliferation was detected by adding 10 μ L of WST1 to each well in the day 3 and incubated for an additional 3 hours in CO₂ incubator at 37 °C. Analyze the amount of formazan dye produced by measuring the absorbance at 450 nm. As shown in Figure 108A, 2t2 and IL2 activated 32D β (contained IL-2R $\beta\gamma$) cells in a similar manner. The EC₅₀ of 2t2 and IL2 is 70.59 pM and 65.51 pM, respectively. However, 2t2 promoted CTLL-2 cell (contained IL-2R $\alpha\beta\gamma$) proliferation over 2 folds stronger than IL-2. The EC₅₀ of 2t2 was 90.72 pM and IL2 was 252.8 pM.

The activity of a descending 2t2 were determined and compared with IL2 (starting at 15000 pM) by PBMC pSTAT5 assays. PBMC were isolated from 5ml of whole blood buffy coat by Ficoll Paque Plus (Cat# GE17144003, GE Healthcare Life Sciences) and were lysed with ACK. Cells were washed with I10 and counted. 1.8×10^6 cells (100 μ L/tube) were seeded to the flow tubes and incubated with 50 μ L of descending 2t2 or IL2 and 50 μ L of pre-staining antibodies (BV605-anti-CD8, BioLegend). Cells were incubated for 30 min at 37 °C in water bath. Added 200 μ L of pre-warmed BD Phosflow Fix Buffer I (Cat# 557870, BD Biosciences) for 10 min at 37° C in water bath. Cells (4.5×10^5 cells/100 μ L) were transferred to V-shape 96-well plate and were spun down followed by permeabilization with 100 μ L of -20 °C pre-cooled BD Phosflow Perm Buffer III (Cat# 558050, BD Biosciences) for 30 min in ice. The cells were then extensively washed x2 with 200 μ L of FACS washing buffer and stained with a panel of fluorescent antibodies (PE-anti-CD25, PerCP-Cy5.5-anti-CD4, AF488-anti-pSTAT5a,

BD Biosciences and BV421-anti-CD56, BioLegend) to distinguish different lymphocyte subpopulations and pSTAT5a status. Cells were spun down and resuspended in 200 μ L of FACS buffer for FACSCelesta analysis. As shown in Figure 108B, 2t2 activated CD4⁺CD25⁺ T_{reg} cells better than IL2 over 7 folds. The EC₅₀ of 2t2 was 6.118 pM and IL2 was 43.11 pM. 2t2 and IL2 activated CD8⁺ T_{con} cells in a similar manner. The EC₅₀ of 2t2 and IL2 is 853.6 pM and 932.3 pM respectively. As shown in Figure 108C, 2t2 activated CD4⁺CD25⁻ T_{con} cells better than IL2 over 2 folds. The EC₅₀ of 2t2 was 100.9 pM and IL2 was 223 pM. 2t2 and IL2 activated CD56^{bright} NK cells in a similar manner. The EC₅₀ of 2t2 and IL2 is 26.62 pM and 24.16 pM respectively. As shown in Figure 108D, 2t2 activated CD56^{dim} NK cells better than IL2 over 4 folds. The EC₅₀ of 2t2 was 165.4 pM and IL2 was 660.3 pM.

Example 53: 2t2-activated Treg cells inactivate inflamm-aging

Study design as shown in Figure 109A, six-week-old female ApoE^{-/-} mice (Jackson Lab) were fed with a Western diet fat containing 21% fat, 0.15% cholesterol, 34.1% sucrose, 19.5% casein, and 15% starch (TD88137, Harlan Laboratories) and maintained in the standard conditions. At week 6, mice fed with Western diet were randomly assigned into control group and treatment group. Mice were then received either 2t2 (treatment group) or PBS (control group) per subcutaneous injection at a dosage of 3mg/kg. The mice received 2 consecutive doses in three weeks interval subcutaneously after which they received the 1st doses for the duration of the study while continuing the Western diet. Mice were euthanized at 20 weeks of age (14 weeks after the Western diet).

As shown in Figure 109B, three days after the 1st dosing, over-night fasting blood samples were collected through submandibular vein puncture and incubated with ACK lysing buffer (Thermo Fisher Scientific) at 37 °C for 5 minutes. Samples were then resuspended in FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% Sodium Azide (Sigma) and surface stained with BV605-anti-CD45, PE-Cy7-anti-CD3, BV510-anti-CD4, APC-Cy7-anti-CD25, APC-anti-CD39, and BV421-anti-CTLA4 (Biolegend) for 30 minutes. Surface-stained samples were further fixed and premetallized with Fix/Perm buffer (Biolegend) and intracellular stained with PE-anti-

Foxp3 (Biolegend). After staining, cells were washed twice with FACs buffer at 1500 RPM for 5 minutes at room temperature and were analyzed by Flow Cytometry (Celesta-BD Bioscience). The flow data were collected and analyzed by FlowJo. Lymphocyte populations were delineated on the flow cytometer using a heterogeneous lymphocyte gating strategy consisting of high CD45 fluorescent staining and low side scatter (SSC) gating ($CD45^{high}SSC^{low}$). At least 50,000 gated lymphocytes were acquired from each tube for each analysis. Lymphocyte subsets were delineated from the total $CD45^{high}SSC^{low}$ lymphocyte population as regulatory T-cells ($CD3^{+}CD4^{+}CD8^{-}CD25^{+}Foxp3^{+}$), regulatory T-cells ($CD3^{+}CD4^{+}CD8^{-}CD39^{+}Foxp3^{+}$), and regulatory T-cells ($CD3^{+}CD4^{+}CD8^{-}CTLA4^{+}Foxp3^{+}$).

As shown in Figure 109B, 2t2 treatment significantly increased $CD3^{+}CD4^{+}CD8^{-}CD25^{+}Foxp3^{+}$ Treg population in blood lymphocytes compared to the control groups ($p=0.0041$), significantly increased $CD3^{+}CD4^{+}CD8^{-}CTLA4^{+}Foxp3^{+}$ Treg population in blood lymphocytes compared to the control groups ($p=0.022$), significantly increased $CD3^{+}CD4^{+}CD8^{-}CD39^{+}Foxp3^{+}$ Treg population in blood lymphocytes compared to the control groups ($p<0.0001$).

As shown in Figure 109C, three days after the 1st dosing, over-night fasting blood samples were collected through submandibular vein puncture and incubated with ACK lysing buffer (Thermo Fisher Scientific) at 37 °C for 5 minutes. Samples were then resuspended in FACS buffer (1X PBS (Hyclone) with 0.5% BSA (EMD Millipore) and 0.001% Sodium Azide (Sigma) and surface stained with BV605-anti-CD45, PE-Cy7-anti-CD3, BV510-anti-CD4, PerCP5.5-anti-CD8, and APC-anti-NK1.1 (Biolegend) for 30 minutes. After staining, cells were washed twice with FACS buffer at 1500 RPM for 5 minutes at room temperature and were analyzed by Flow Cytometry (Celesta-BD Bioscience). The flow data were collected and analyzed by FlowJo. Lymphocyte populations were delineated on the flow cytometer using a heterogeneous lymphocyte gating strategy consisting of high CD45 fluorescent staining and low side scatter (SSC) gating ($CD45^{high}SSC^{low}$). At least 50,000 gated lymphocytes were acquired from each tube for each analysis. Lymphocyte subsets were delineated from the total $CD45^{high}SSC^{low}$ lymphocyte population as helper T-cells ($CD3^{+}CD4^{+}CD8^{-}$). Lymphocyte subsets were delineated from the total $CD45^{high}SSC^{low}$ lymphocyte

population as cytotoxic T-cells ($CD3^+CD4^-CD8^+$). Lymphocyte subsets were delineated from the total $CD45^{high}SSC^{low}$ lymphocyte population as Natural killer (NK) cells ($CD3^-NK1.1^+$). As shown in Figure 109C, 2t2 treatment significantly reduced helper T-cells ($CD3^+CD4^+CD8^-$) population in blood lymphocytes compared to the control groups ($p=0.0279$), had no effect on cytotoxic T-cells ($CD3^+CD4^-CD8^+$) population in blood lymphocytes compared to the control groups (NS), significantly increased Natural killer (NK) cells ($CD3^-NK1.1^+$) population in blood lymphocytes compared to the control groups ($p<0.0001$).

As shown in Figure 109D, 3 days after the 2nd dosing, over-night fasting blood samples were collected through submandibular vein puncture and the plasma was isolated. IL-1 β , MCP-1 and TNF- α were analyzed with Mouse Cytokine Array Proinflammatory Focused 10-plex (MDF10) by Eve Technologies (Calgary, AB Canada T2N 0M4). As shown in Figure 109D, IL-1 β levels were significantly reduced in 2t2 treatment group compared with the control group ($p=0.0312$), MCP-1 levels were significantly reduced in 2t2 treatment group compared with the control group ($p=0.0235$), and TNF- α levels were significantly reduced in 2t2 treatment group compared with the control group ($p=0.0172$).

As shown in Figure 109E, 2 weeks after the 3rd dosing, over-night fasting blood samples were collected through submandibular vein puncture and fasting glucose levels were measured immediately using a OneTouch Glucometer. The plasma was isolated, and LDL and insulin were analyzed with Mouse LDL-Cholesterol Assay Kit (Cat# 79980, Crystal Chem) and Ultra Sensitive Mouse Insulin ELISA Kit (Cat# 90080, Crystal Chem) according to manufactures' protocol. As shown in Figure 109E, LDL levels were significantly reduced in 2t2 treatment group compared with the control group ($p=0.0025$). Fasting glucose levels were significantly reduced in 2t2 treatment group compared with the control group ($p=0.0089$). HOMA-IR index based on glucose and insulin levels and reflected insulin resistance were significantly reduced in 2t2 treatment group compared with the control group ($p=0.0102$).

Example 54: Effects of TGF β 15-TGFRs and 2t2 Treatment on Mouse Plasma Markers in Aged Mice

C57BL/6, 72-week-old mice were purchased from the Jackson Laboratory. Mice were housed in a controlled temperature and controlled light environment. Mice were divided into five groups receiving the following treatment: saline control ($n=8$), one dose of TGF β 15-TGFRs on day 0 ($n=8$), one dose of TGF β 15-TGFRs on day 0 followed by one dose of 2t2 on day 60 ($n=7$), one dose of 2t2 on day 0 ($n=3$) and one dose of 2t2 on day 0 followed by one dose of TGF β 15-TGFRs on day 60 ($n=7$). Mice were treated subcutaneously with PBS, TGF β 15-TGFRs (3 mg/kg), 2t2 (3 mg/kg) or TGF β 15-TGFRs (3 mg/kg) plus 2t2 (3 mg/kg). Mouse blood was collected from submandibular vein on day 120 in tubes containing EDTA. The whole blood was centrifuged at 3000 RPM for 10 minutes to separate plasma from blood. Plasma markers PAI-1, IL-1 α and CXCL1 were analyzed by multiplex cytokine array (Eve Technologies). The results indicate that treatment of aged mice with 2t2 followed by TGF β 15-TGFRs reduced plasma levels of PAI-1, IL-1 α and CXCL1 compare to control treated mice (Figures 110A-D). The plasma levels of IL-2 were also measured. Plasma IL-2 levels were reduced by treatment with 2t2 followed by TGF β 15-TGFRs but due to variability between animals these changes were not significant. Treatment of aged mice with TGF β 15-TGFRs alone also resulted in significant reduction in PAI-1 and CXCL1 protein levels in plasma compare to the control group (Fig. 110A-D).

OTHER EMBODIMENTS

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

WHAT IS CLAIMED IS:

1. A method of treating an aging-related disease or inflammatory disease in a subject, the method comprising administering to the subject:

(i) a therapeutically effective amount of an NK cell activating agent and/or an NK cell and/or a monoclonal antibody; and

(ii) a therapeutically effective amount of a Treg cell activating agent and/or a Treg cell and/or a monoclonal antibody and/or an advanced glycation end product (AGE) inhibitor.

2. The method of claim 1, wherein the aging-related disease is inflamm-aging related.

3. The method of claim 1 or 2, wherein (i) is administered to the subject at substantially the same time as (ii).

4. The method of claim 1 or 2, wherein (i) is administered to the subject prior to administration of (ii) to the subject.

5. The method of claim 1 or 2, wherein (ii) is administered to the subject prior to administration of (i) to the subject.

6. The method of any one of claims 1-5, wherein the method comprises administering a therapeutically effective amount of an NK cell to the subject.

7. The method of claim 6, wherein the NK cell is an autologous, haploidentical or allogeneic NK cell isolated from peripheral blood, umbilical cord blood, or isolated and differentiated from iPSC.

8. The method of claim 7, wherein the method further comprises:
isolating the NK cell from the subject;

culturing the isolated NK cell in a liquid culture medium under conditions sufficient to induce or increase proliferation of the NK cell,
wherein following the isolating and culturing steps, the NK cell is administered to the subject.

9. The method of claim 8, wherein the liquid culture medium comprises a multi-chain chimeric polypeptide.

10. The method of any one of claims 6-9, wherein the NK cell comprises a chimeric antigen receptor.

11. The method of claim 10, wherein the chimeric antigen receptor comprises an extracellular domain that binds specifically to tissue factor or CD26.

12. The method of any one of claims 1-5, wherein the method comprises administering a therapeutically effective amount of an NK cell activating agent and/or monoclonal antibody to the subject.

13. The method of claim 12, wherein the NK cell activating agent is one or more multi-chain chimeric polypeptide(s).

14. The method of claim 12, wherein the monoclonal antibody is one or more of an anti-tissue factor antibody and/or an anti-CD26 antibody.

15. The method of claim 12, wherein the NK cell activating agent comprises one or more multi-chain chimeric polypeptide(s) and the monoclonal antibody comprises one or more of an anti-tissue factor antibody and/or an anti-CD26 antibody.

16. The method of any one of claims 1-15, wherein the method comprises administering a therapeutically effective amount of a Treg cell to the subject.

17. The method of claim 16, wherein the Treg cell is an autologous Treg cell, a haploidentical Treg cell, or an allogeneic Treg cell isolated from peripheral blood or umbilical cord blood.

18. The method of claim 17, wherein the method further comprises:
isolating the Treg cell from the subject;
culturing the isolated Treg cell in a liquid culture medium under conditions sufficient to induce or increase proliferation of the Treg cell,
wherein following the isolating and culturing steps, the Treg cell is administered to the subject.

19. The method of claim 18, wherein the step of isolating the Treg cell from the subject comprises obtaining a sample comprising Treg cells from the subject, and isolating the Treg cell from the sample using an antibody or ligand capable of binding CD39.

20. The method of claim 19, wherein the step of isolating the Treg cell from the sample comprises:
mixing the sample with the 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 components in the sample, thereby isolating the Treg cell.

21. The method of claim 19 or 20, wherein:
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.

22. The method of claim 20, wherein the separating comprises the use of flow cytometry, fluorescence-activated cell sorting (FACS), centrifugation, or column, plate, particle, or bead-based methods.

23. The method of any one of claims 18-22, wherein the T_{reg} cell is an autologous T_{reg} cell, a haploidentical T_{reg} cell, or an allogeneic T_{reg} cell isolated from a sample comprising fresh or frozen peripheral blood, umbilical cord blood, peripheral blood mononuclear cells, lymphocytes, CD4⁺ T cells, or T_{reg} cells.

24. The method of any one of claims 18-23, wherein the T_{reg} cell is a CD4⁺CD25⁺Foxp3⁺ cell.

25. The method of any one of claims 18-23, wherein the T_{reg} cell is a CD4⁺CD25⁺CD127^{dim-} cell.

26. The method of any one of claims 18-25, wherein the T_{reg} cell is immunosuppressive *in vitro* and *in vivo*.

27. The method of any one of claims 18-26, wherein the liquid culture medium comprises one or more single-chain chimeric polypeptide(s).

28. The method of any one of claims 16-27, wherein the Treg cell comprises a chimeric antigen receptor.

29. The method of claim 28, wherein the chimeric antigen receptor comprises an extracellular domain that binds specifically to tissue factor or CD36.

30. The method of any one of claims 1-15, wherein the method comprises administering a therapeutically effective amount of a Treg cell activating agent and/or monoclonal antibody and/or AGE inhibitor to the subject.

31. The method of claim 30, wherein the Treg cell activating agent is one or more single-chain chimeric polypeptide(s).

32. The method of claim 30, wherein the monoclonal antibody is one or both of an anti-tissue factor antibody and/or an anti-CD36 antibody.

33. The method of claim 30, wherein the AGE inhibitor is a soluble RAGE trap.

34. The method of claim 30, wherein the Treg cell activating agent comprises one or more single-chain chimeric polypeptide(s), the monoclonal antibody comprises one or more of an anti-tissue factor antibody and/or an anti-CD36 antibody, and the AGE inhibitor comprises one or more soluble RAGE trap.

35. The method of claim 9, 13, or 15, wherein the multi-chain chimeric polypeptide comprises:

(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.

36. The method of claim 27, 31, or 34, wherein the single-chain chimeric polypeptide comprises:

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

37. The method of any one of claims 1-37, wherein the aging-related disorder 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, cancer, dementia, vascular disease, infection susceptibility, chronic inflammation, and renal dysfunction.

38. The method of any one of claims 1-37, wherein 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, and non-alcoholic steatohepatitis.

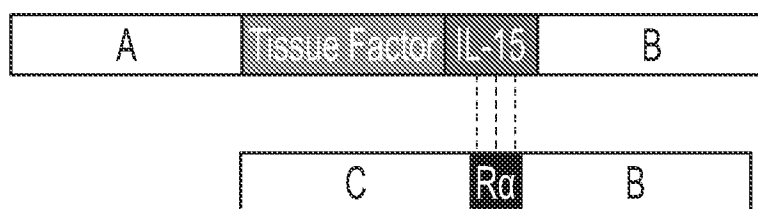
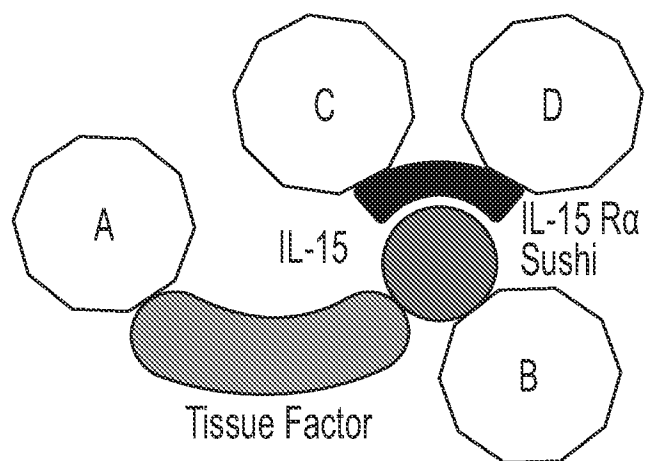


Figure 1

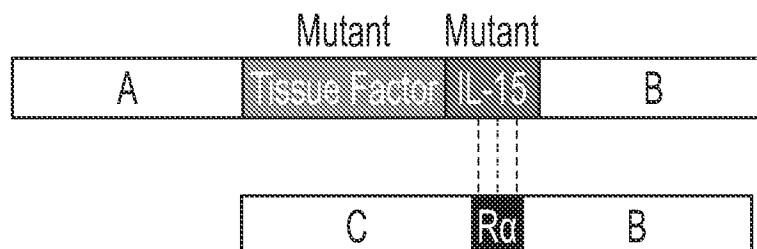
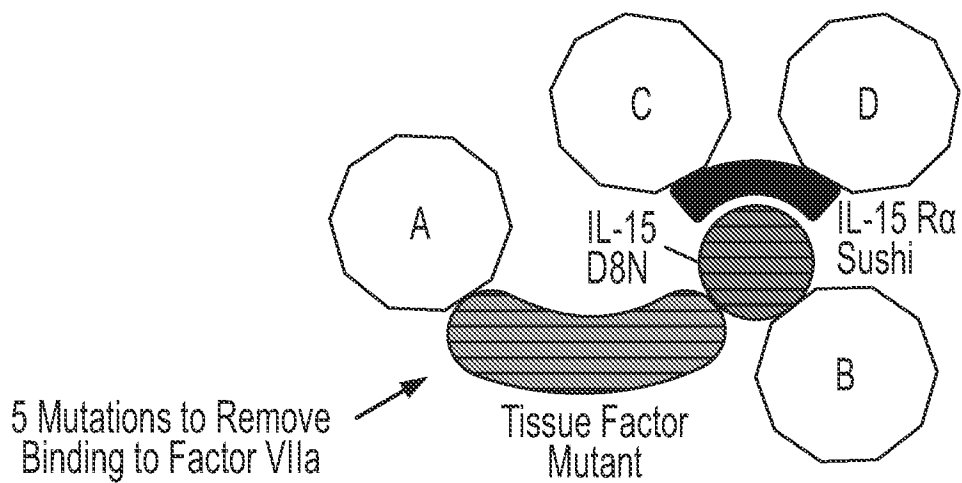


Figure 2

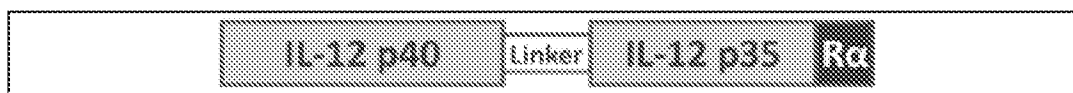


Figure 3



Figure 4

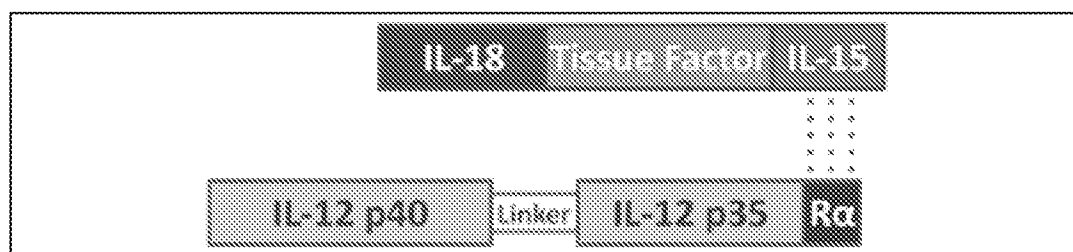


Figure 5

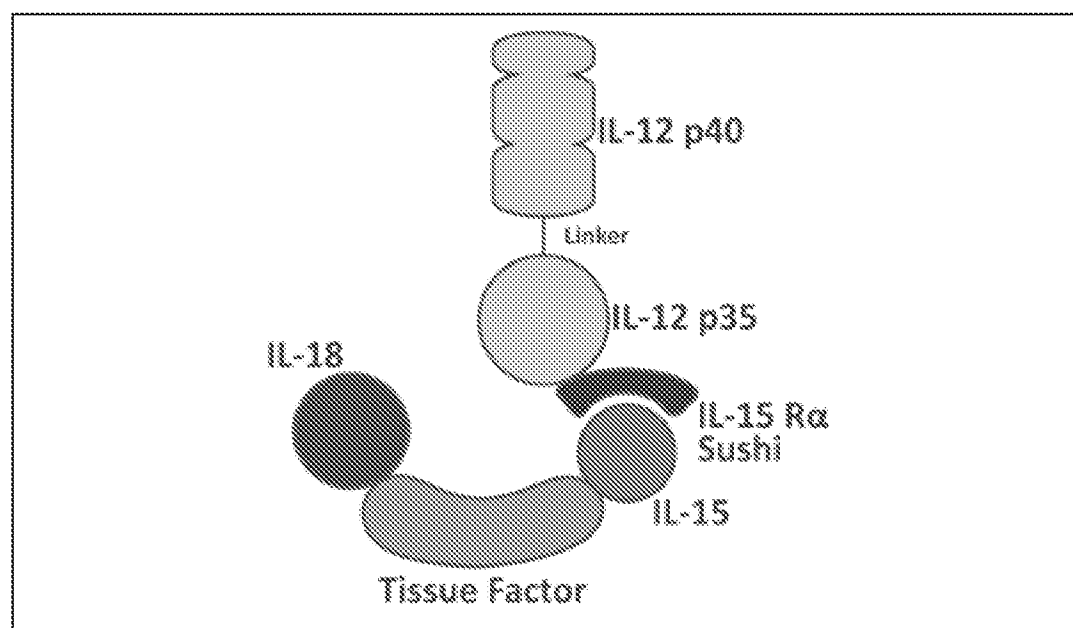


Figure 6

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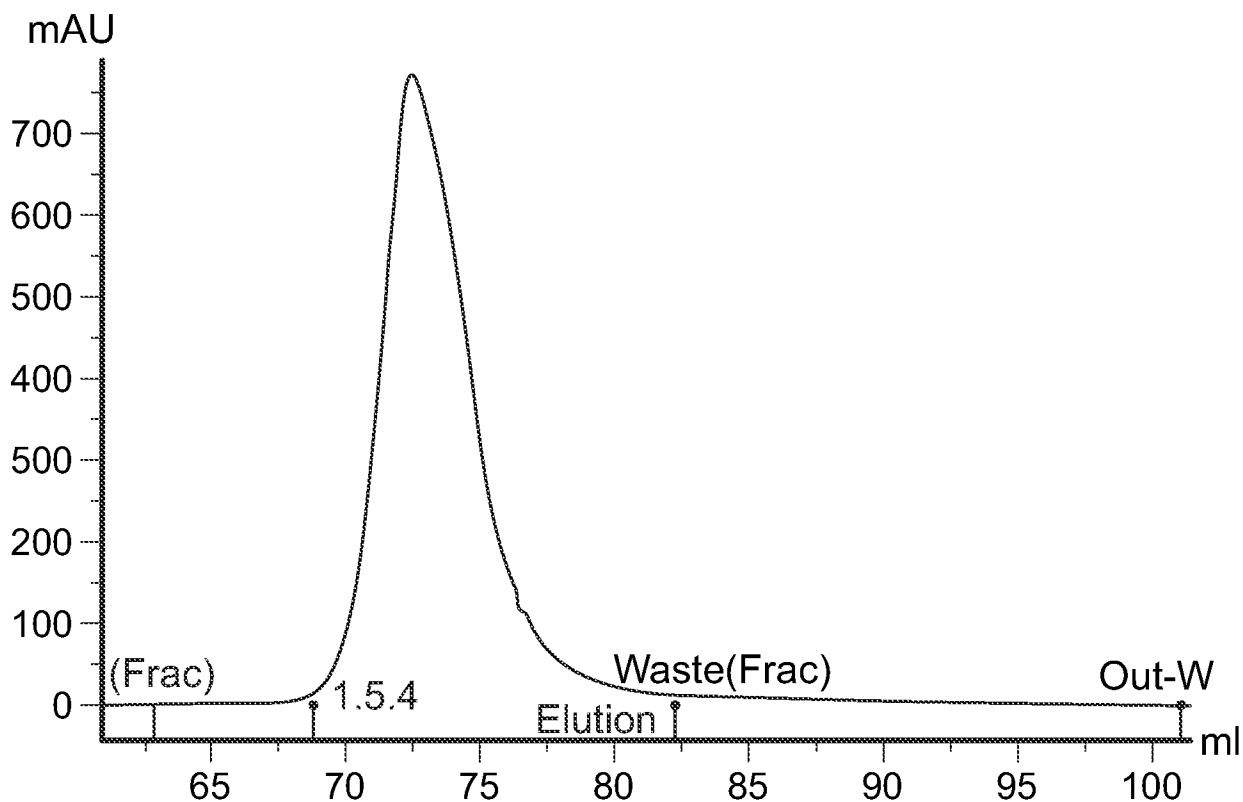


Figure 7

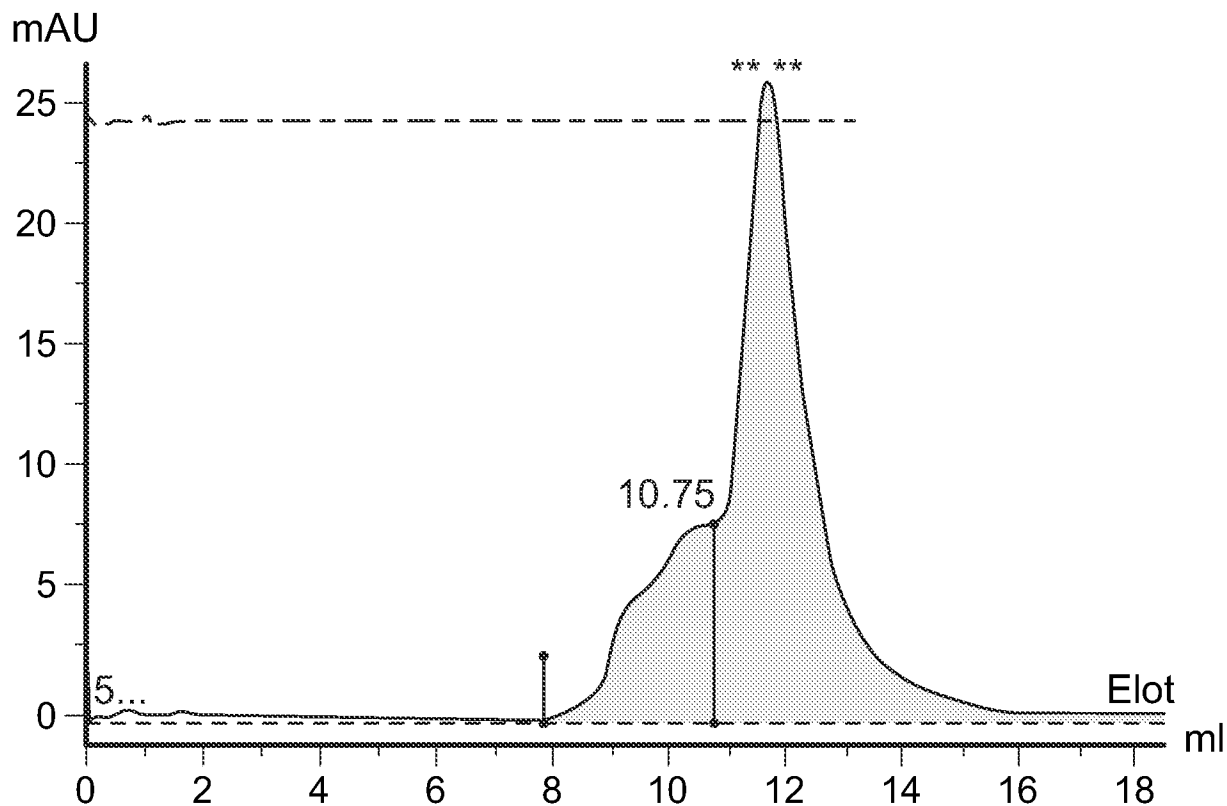


Figure 8

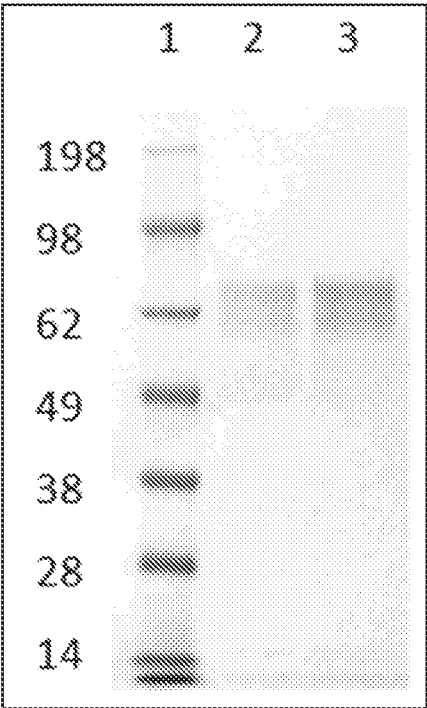


Figure 9

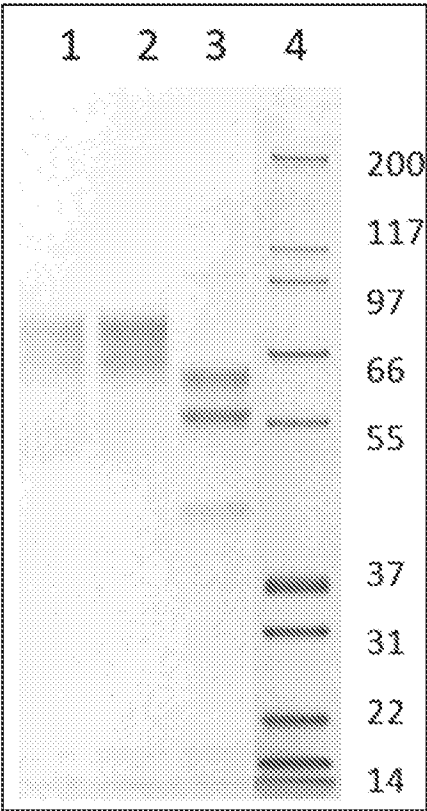


Figure 10

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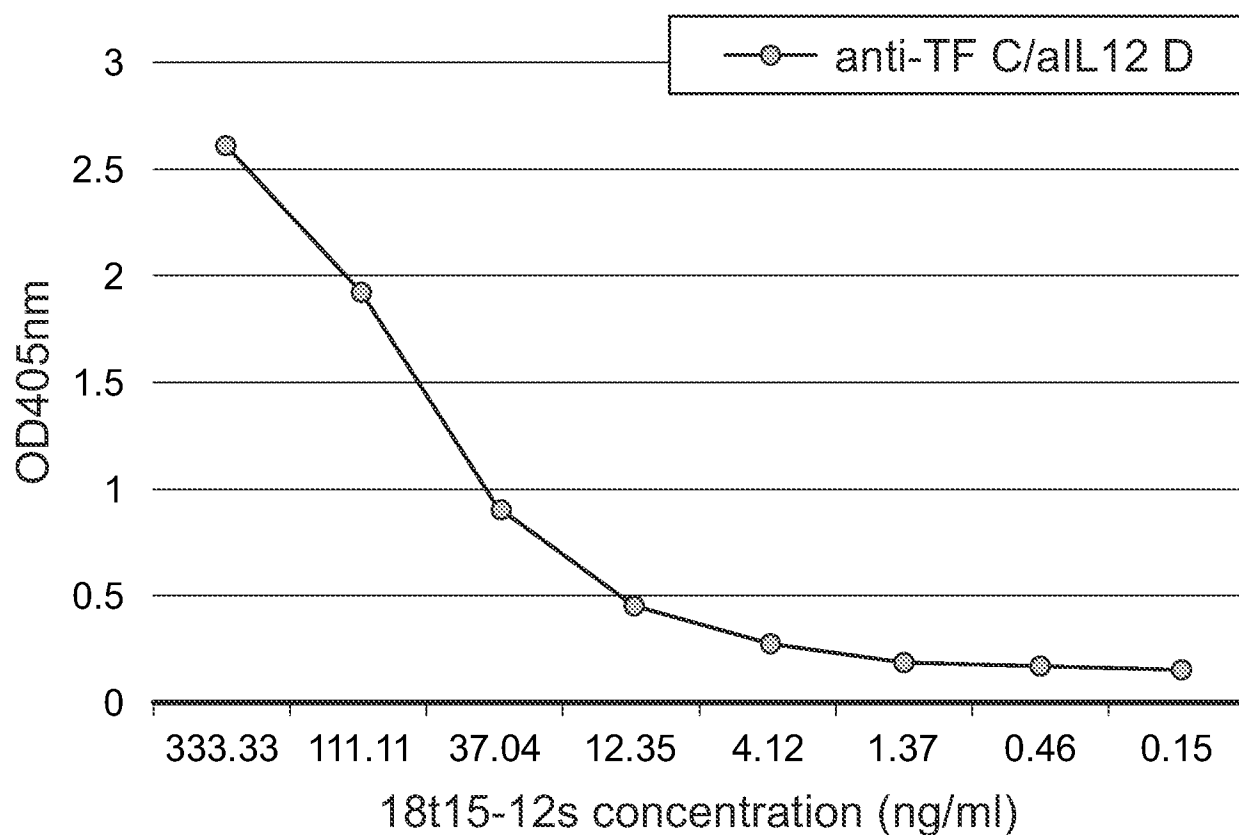


Figure 11

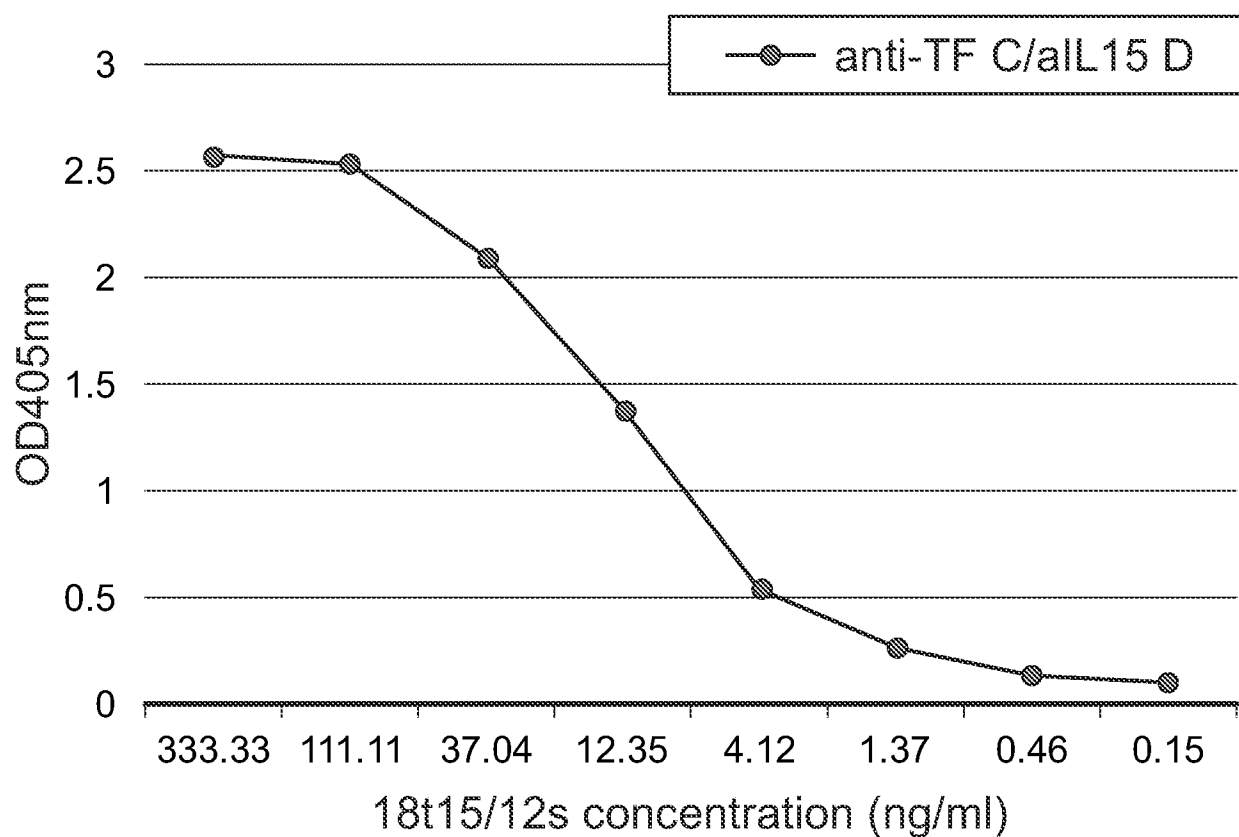


Figure 12

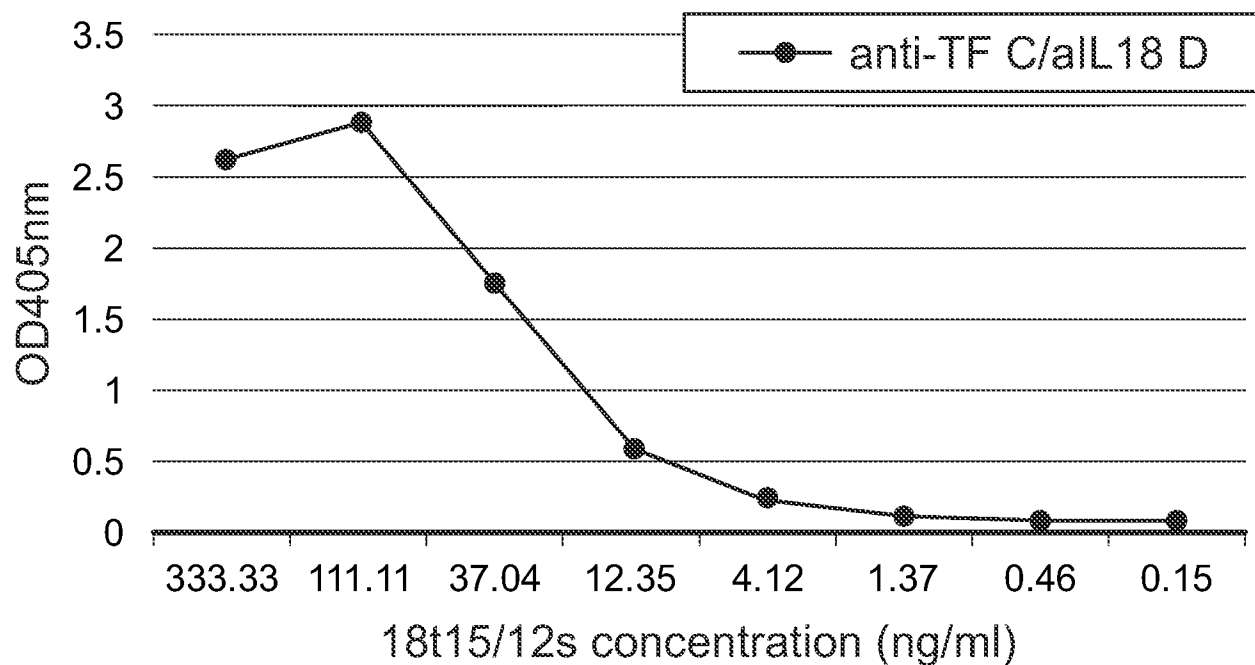


Figure 13

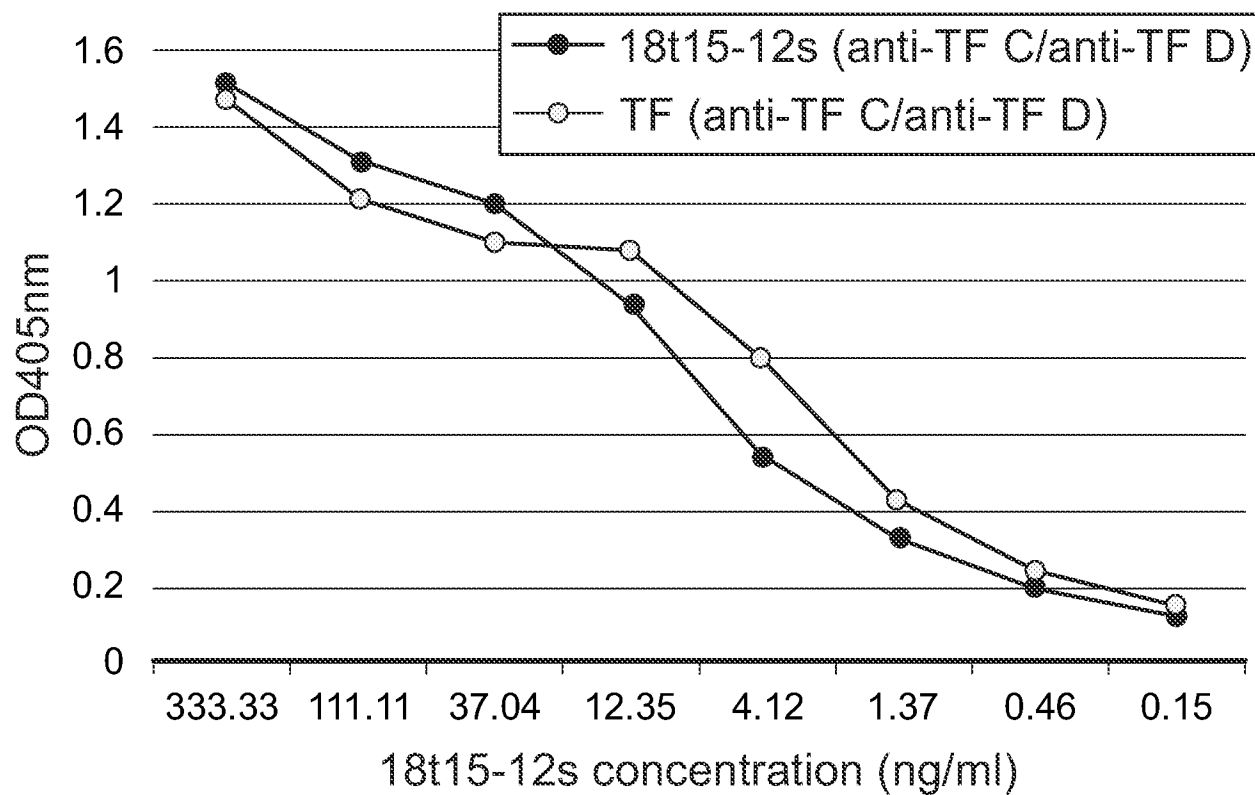


Figure 14

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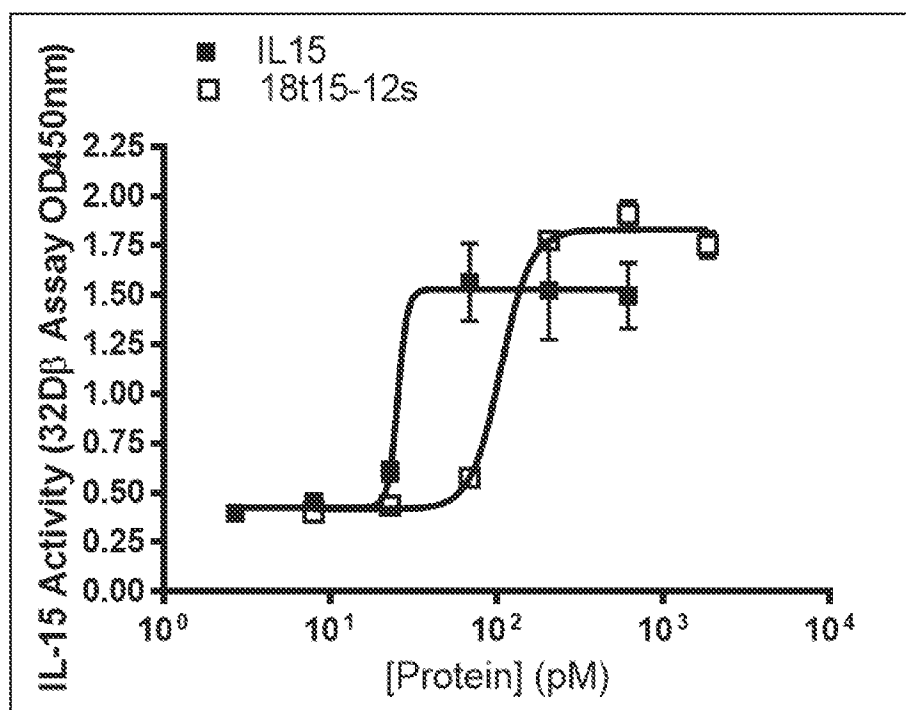


Figure 15

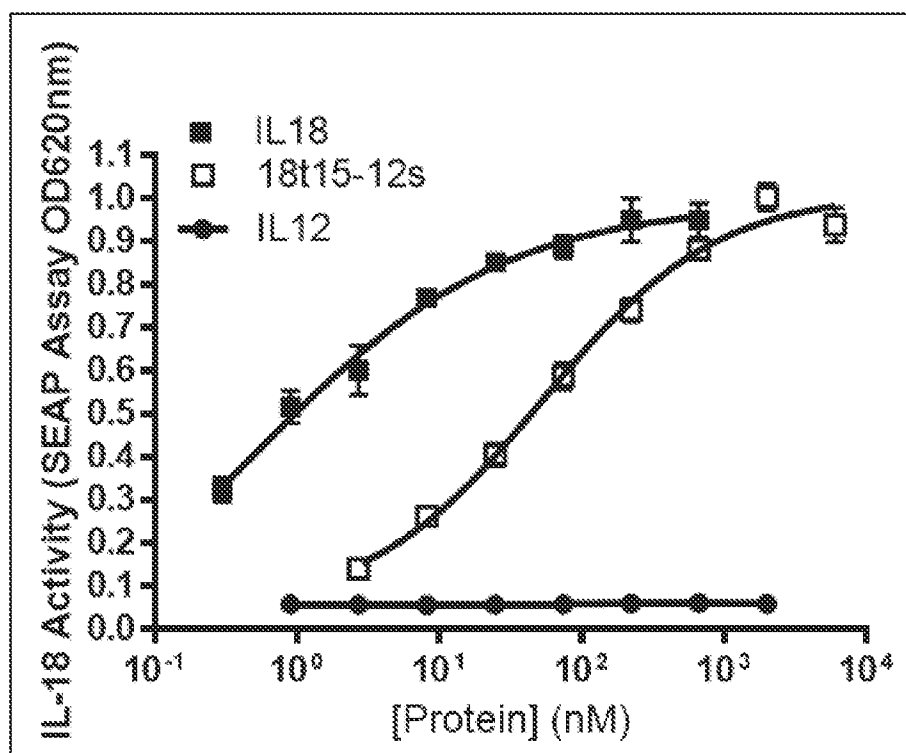


Figure 16

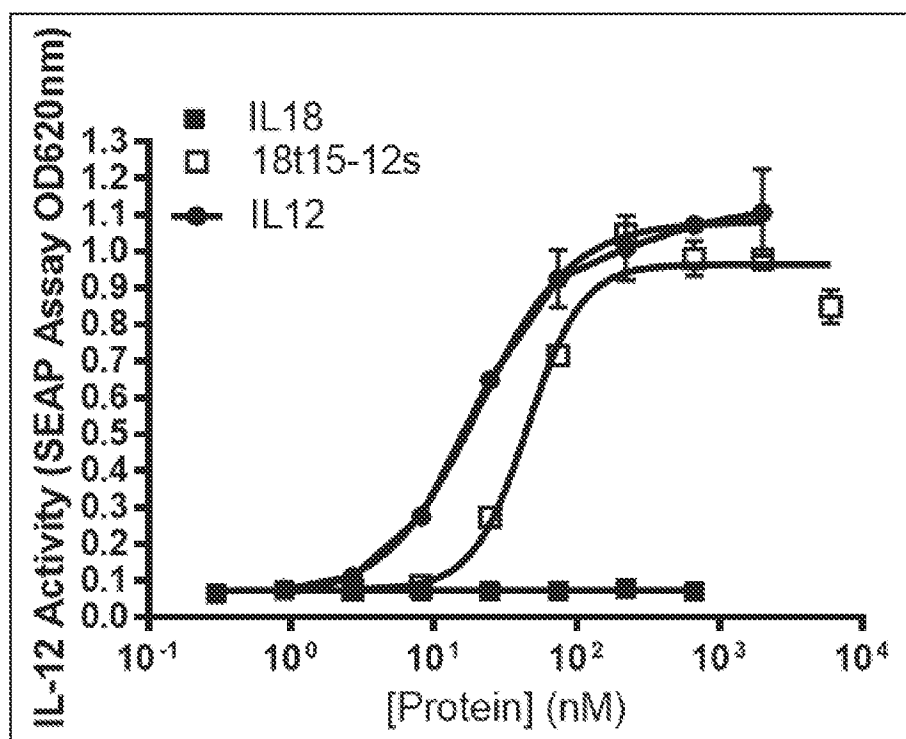


Figure 17

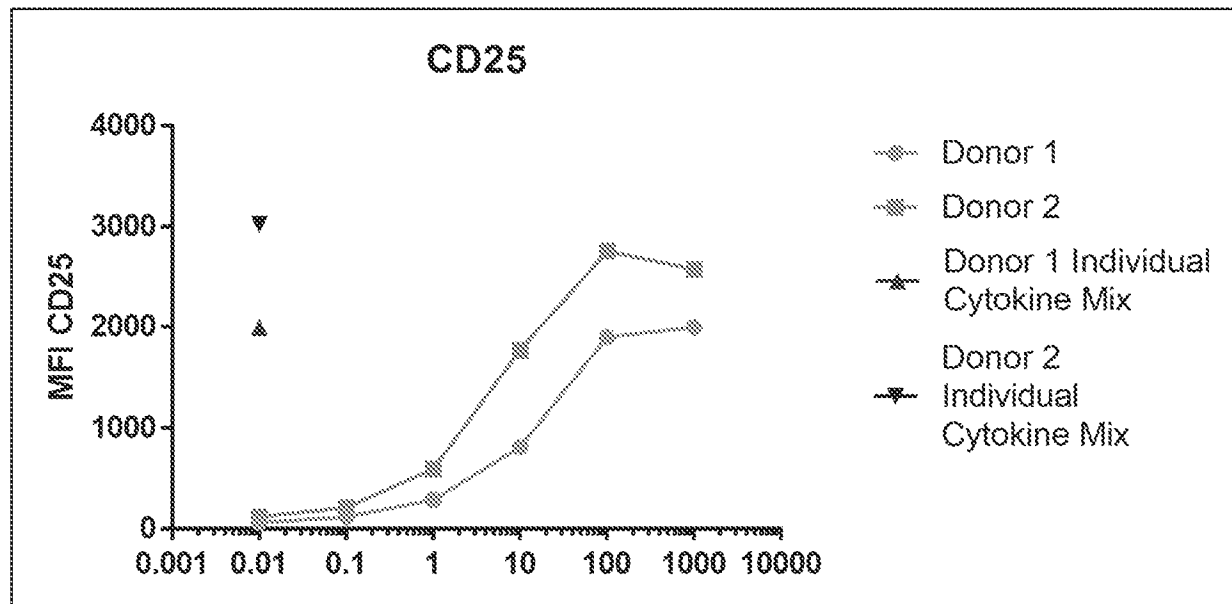


Figure 18A

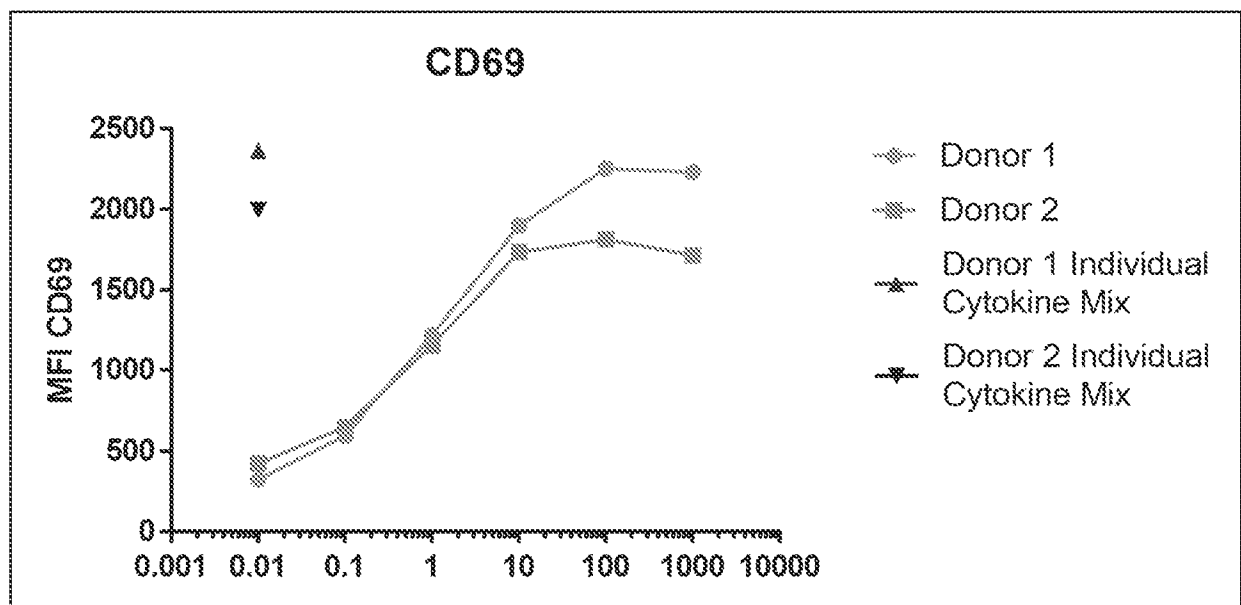


Figure 18B

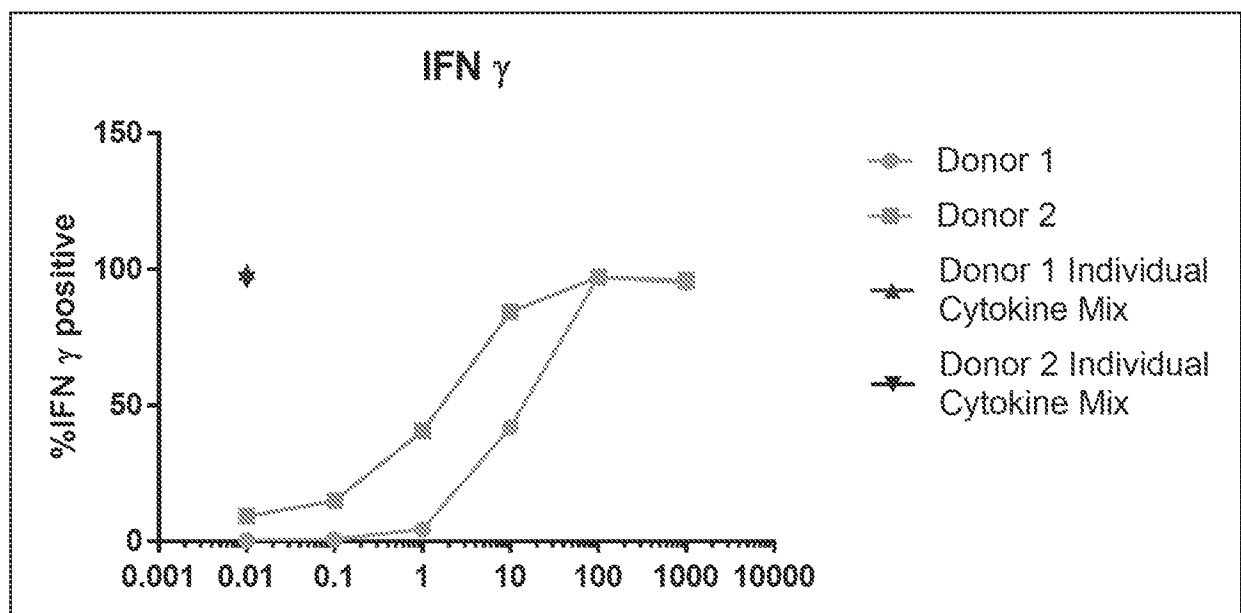


Figure 19

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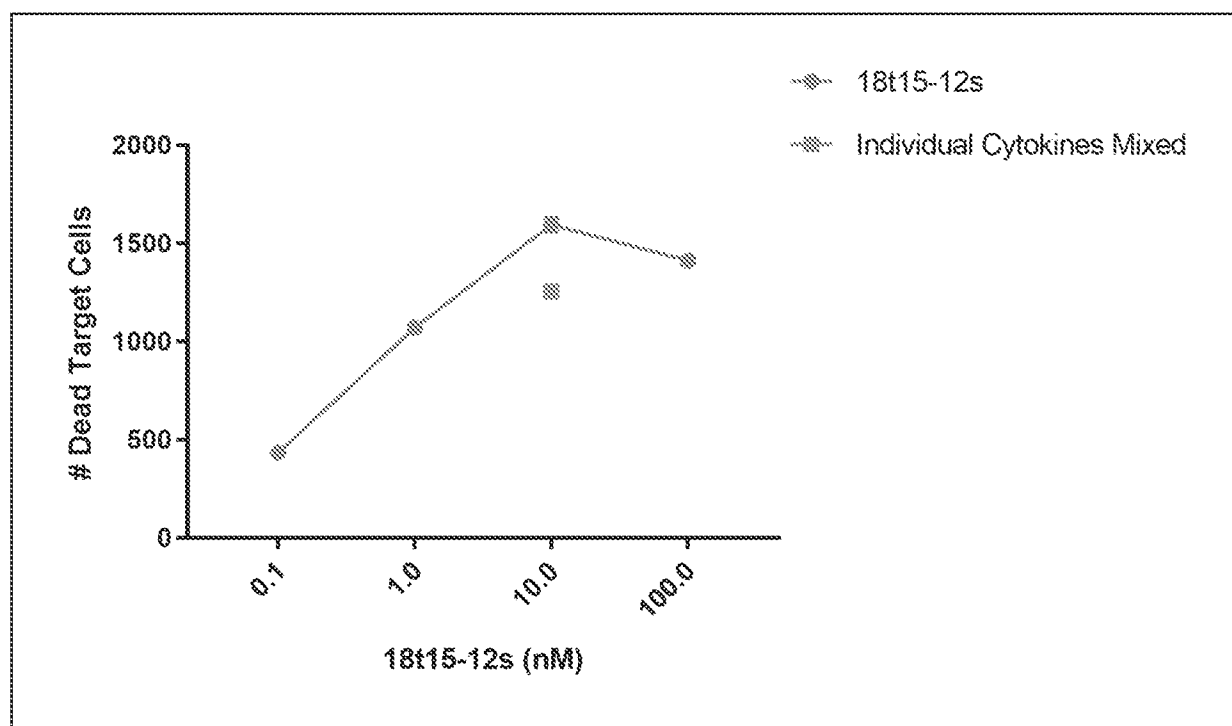


Figure 20



Figure 21



Figure 22

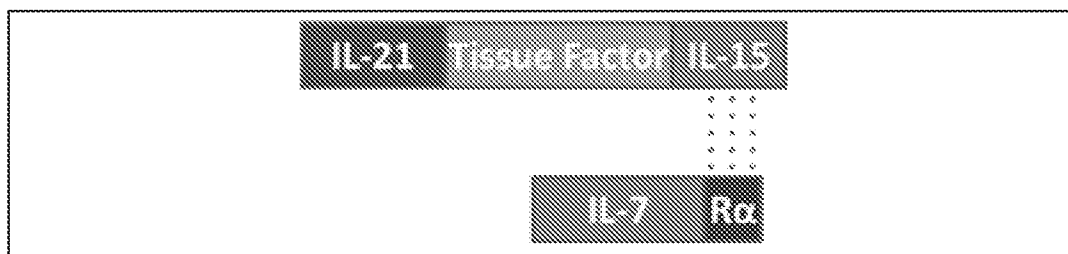


Figure 23

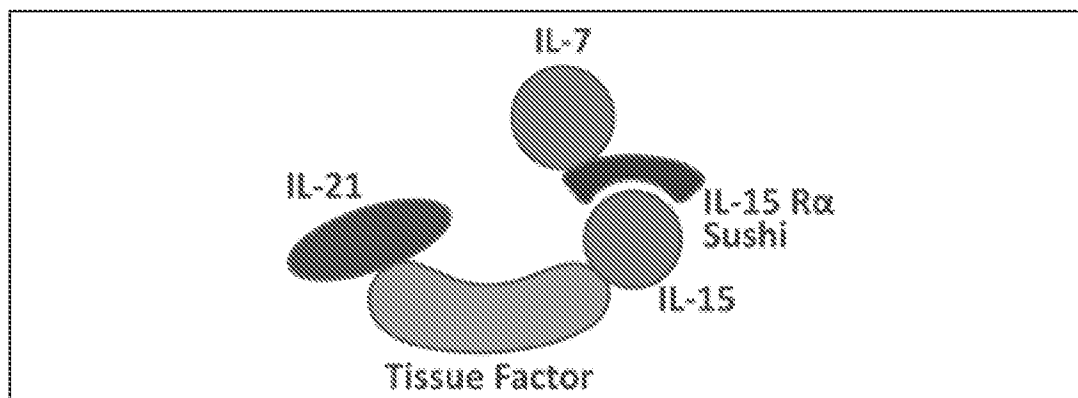


Figure 24

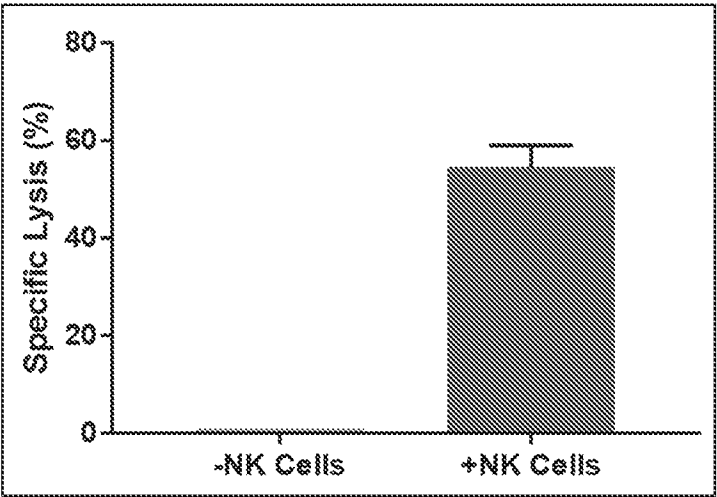


Figure 25



Figure 26



Figure 27

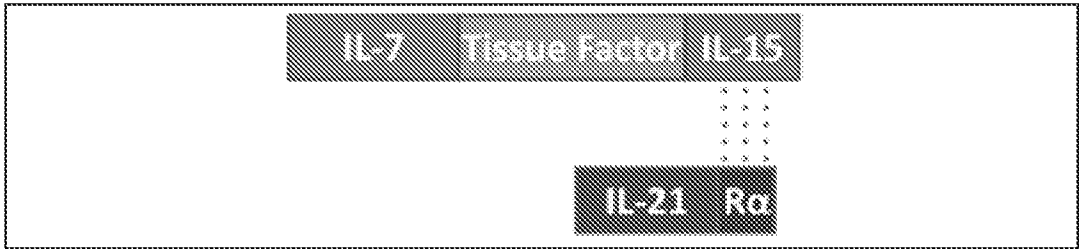


Figure 28

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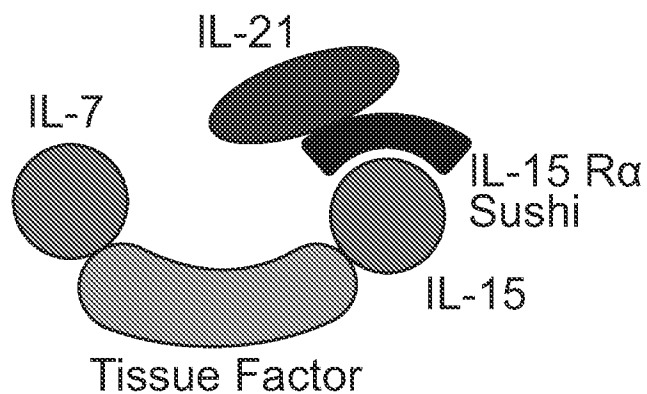


Figure 29

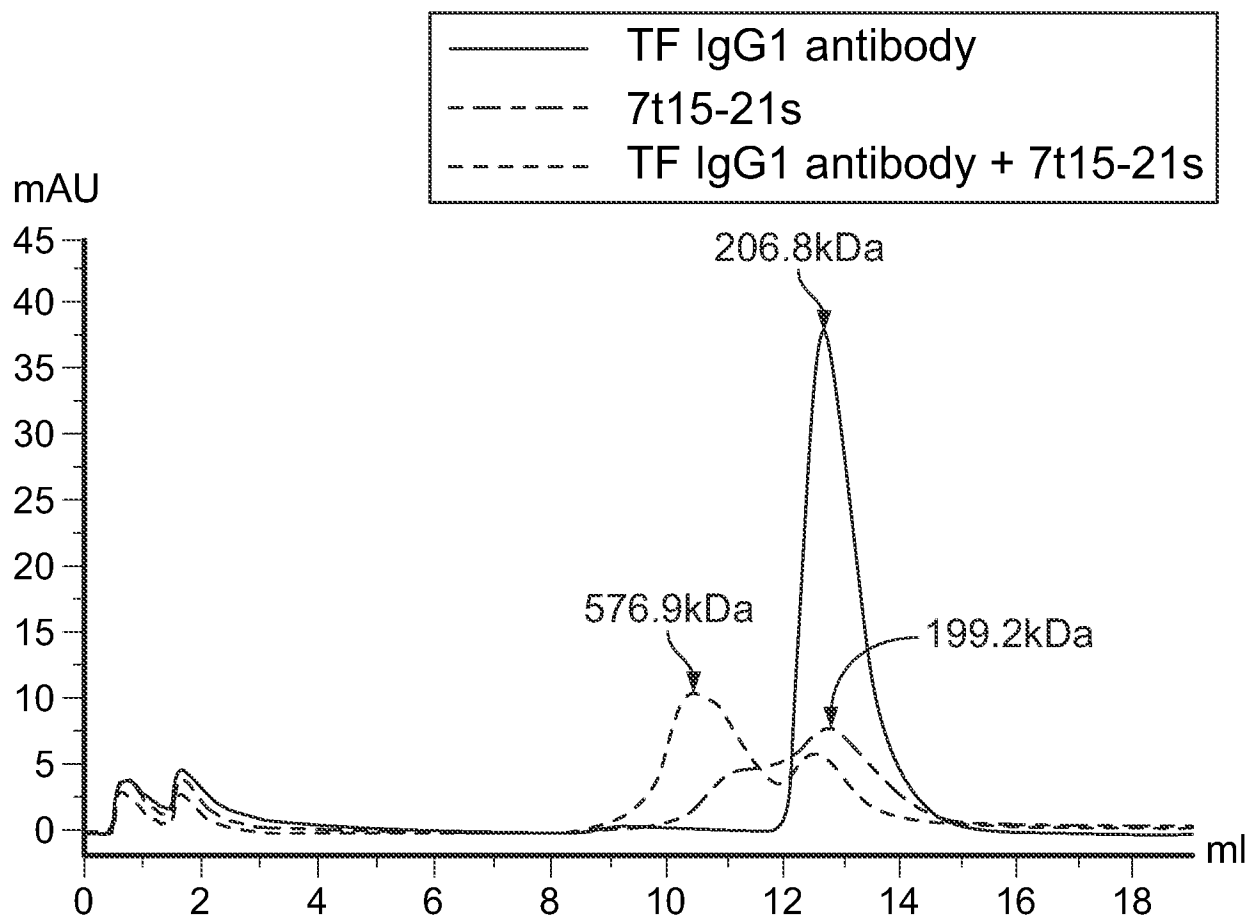


Figure 30

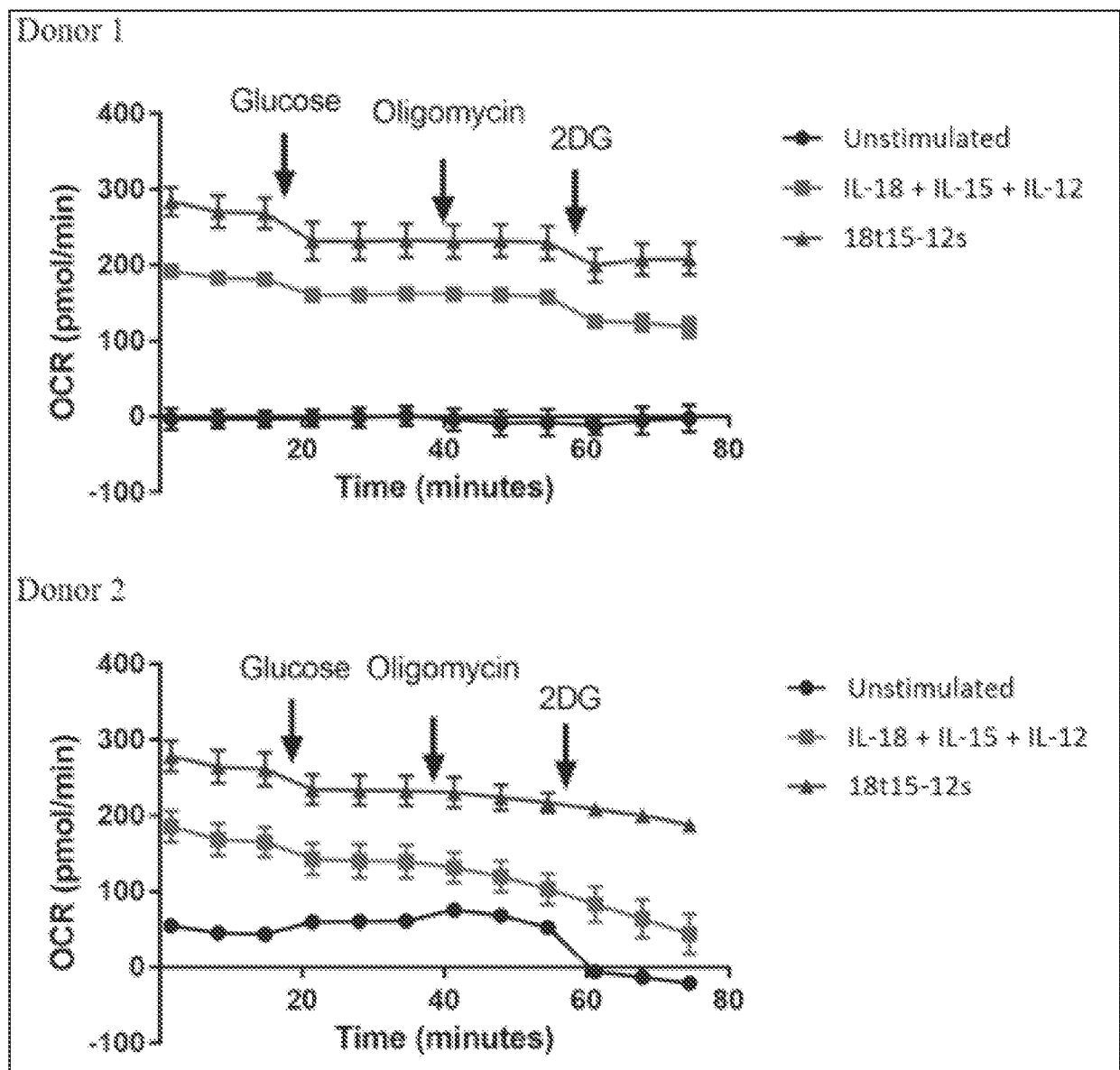


Figure 31

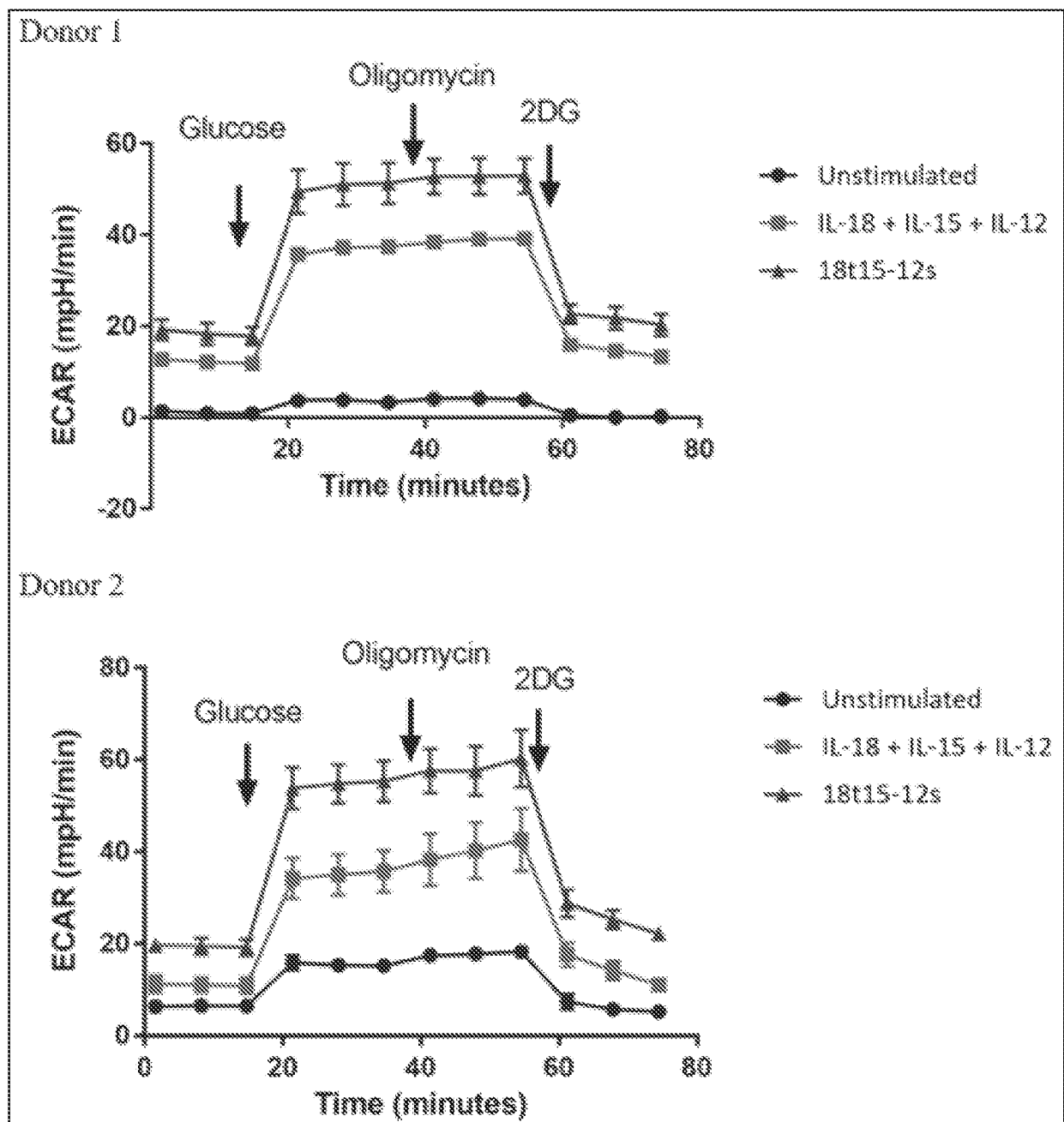


Figure 32

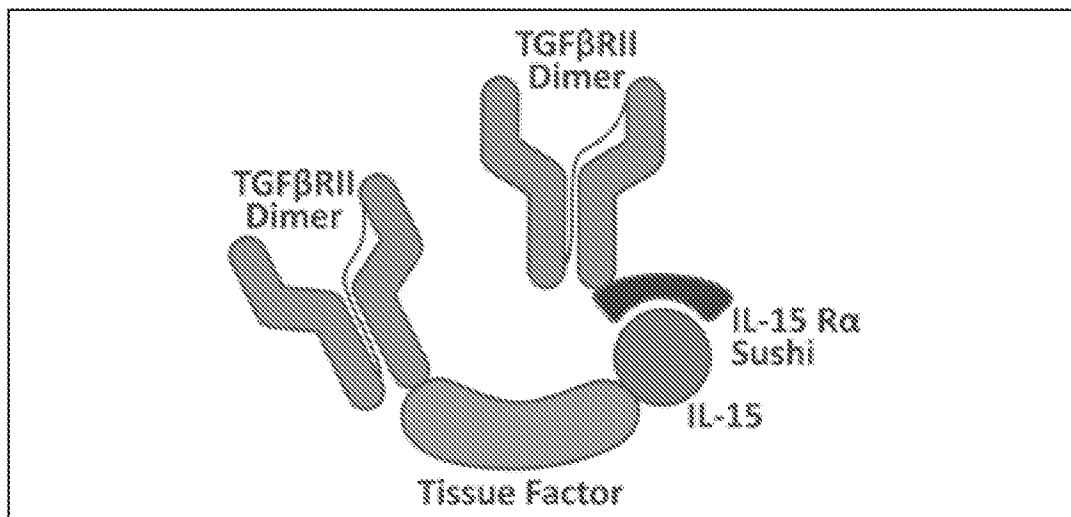


Figure 33

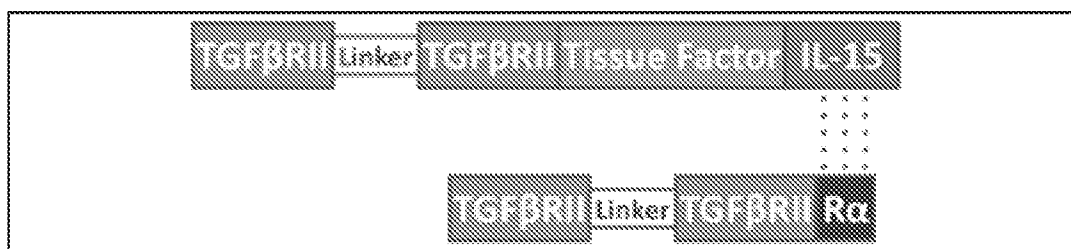


Figure 34

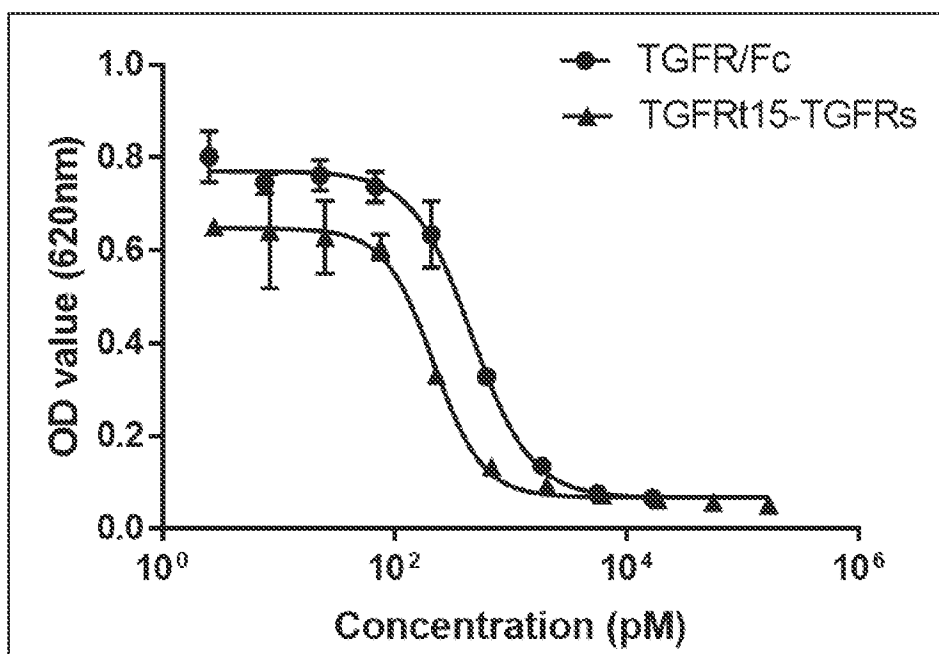


Figure 35

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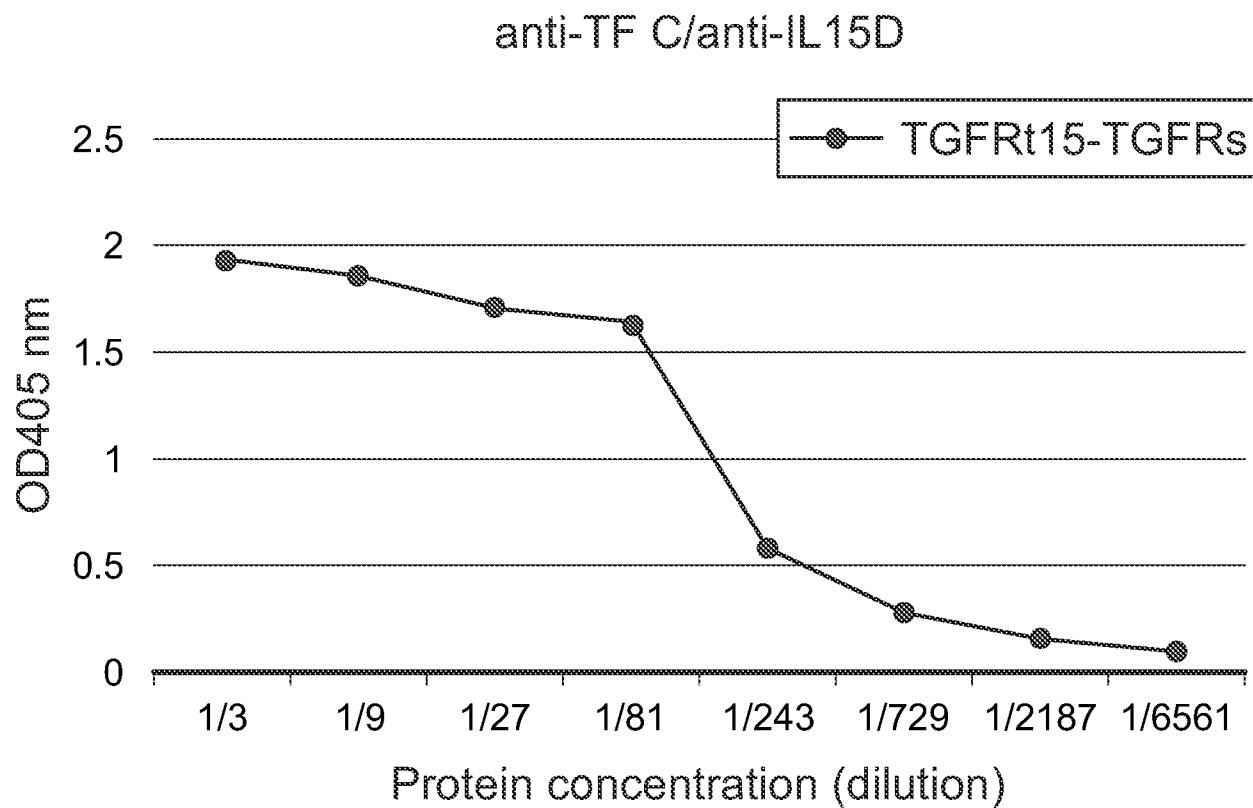


Figure 36A

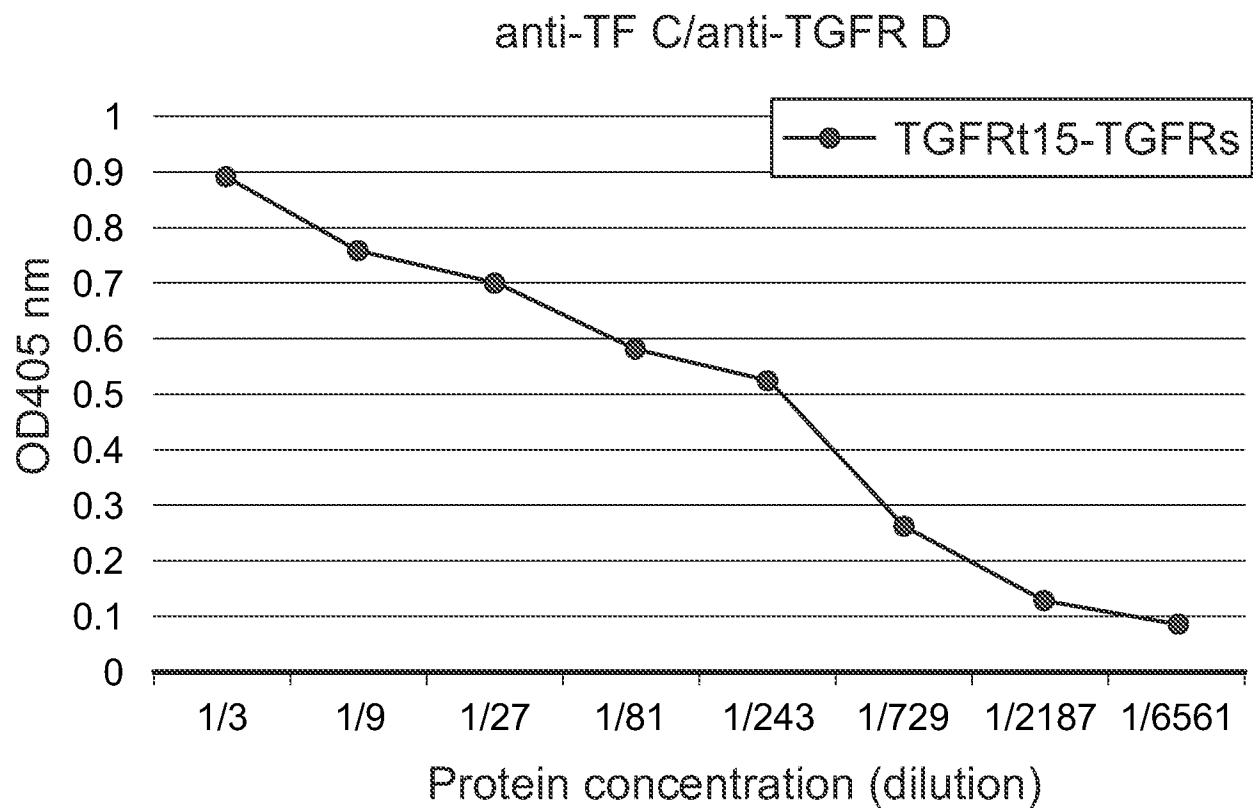


Figure 36B

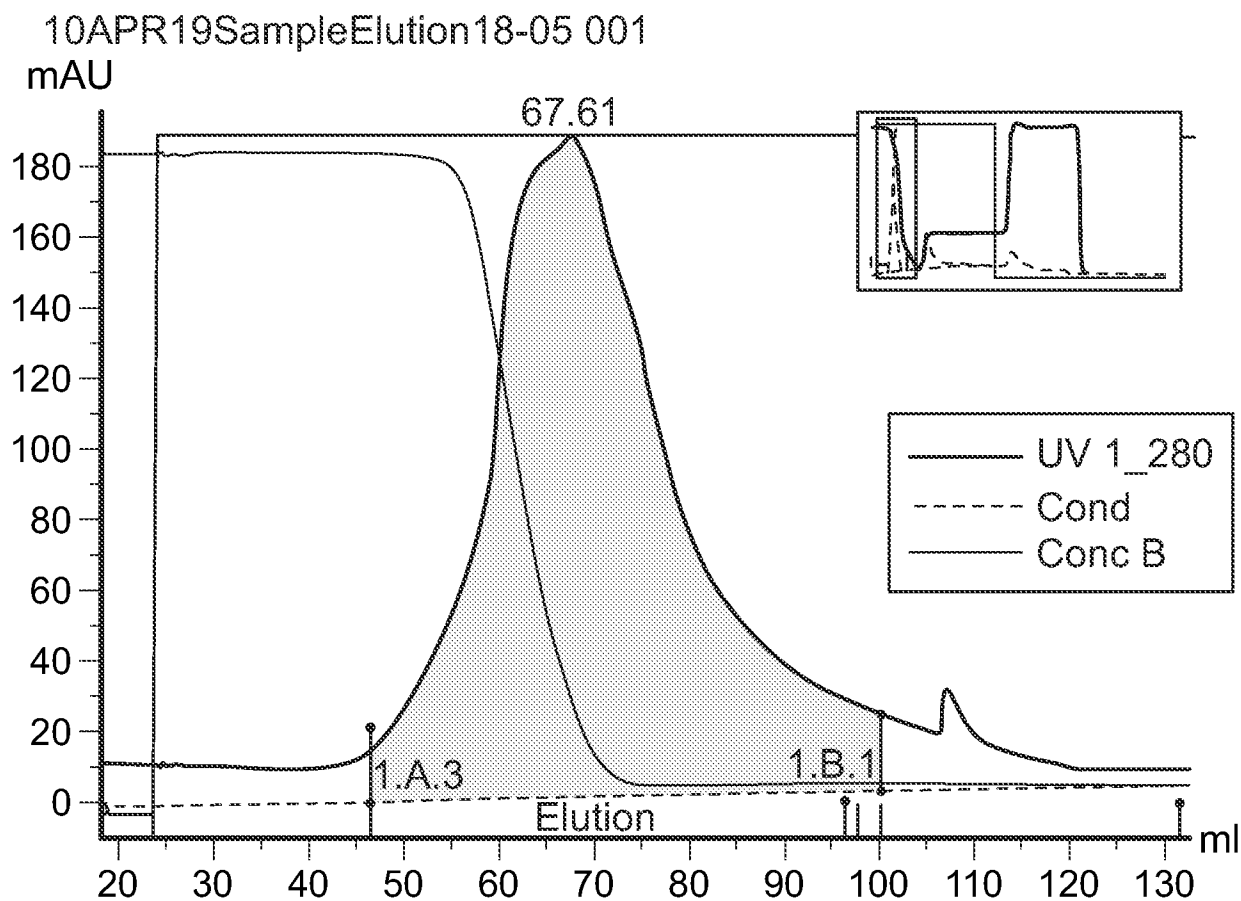


Figure 37

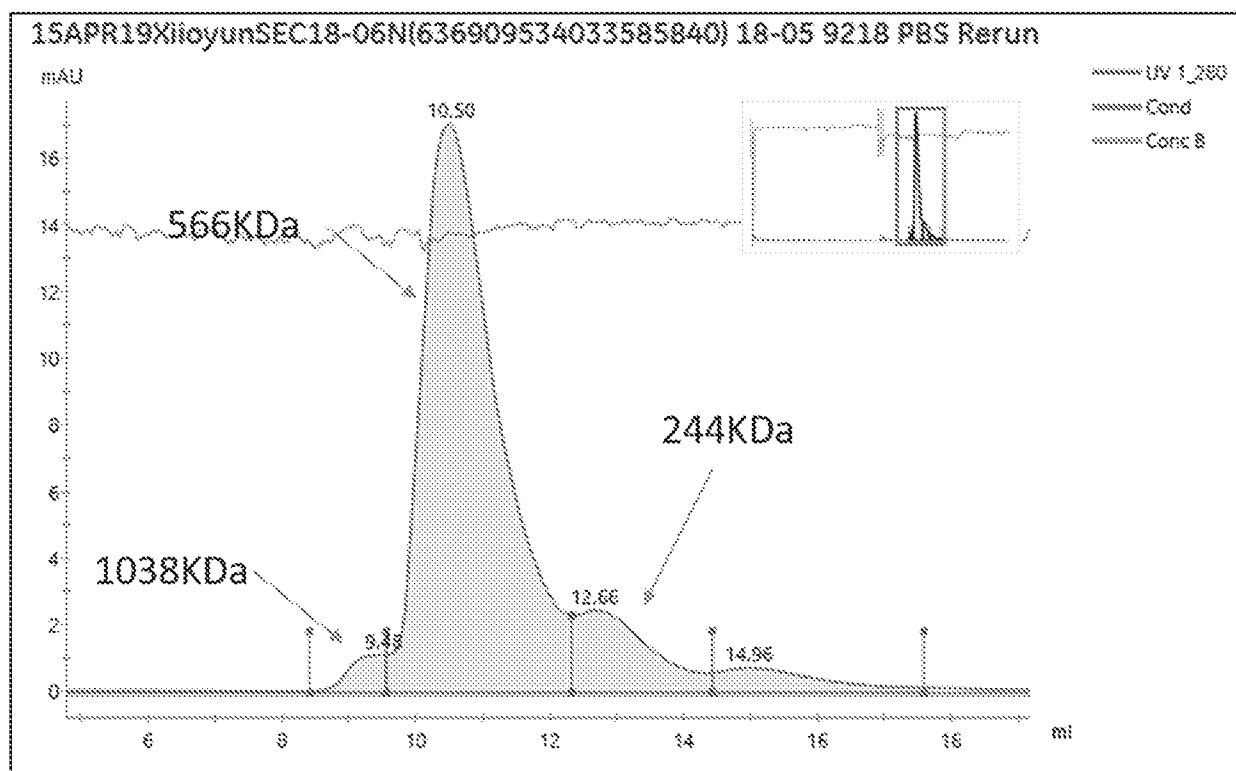


Figure 38

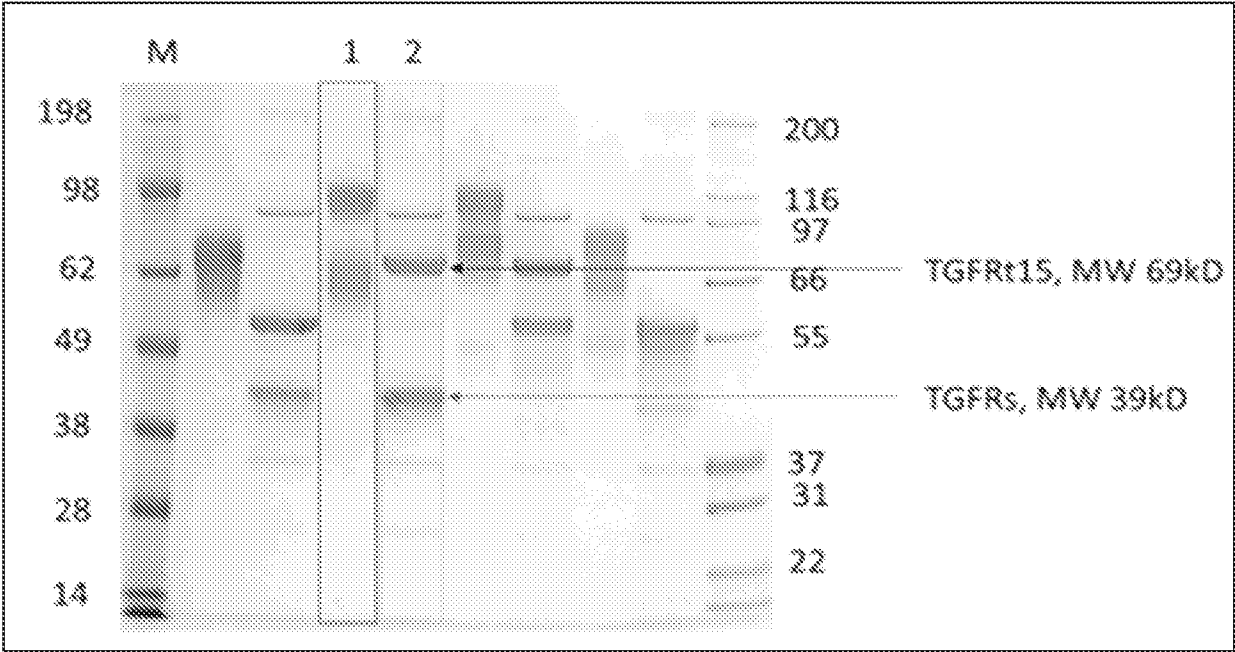


Figure 39

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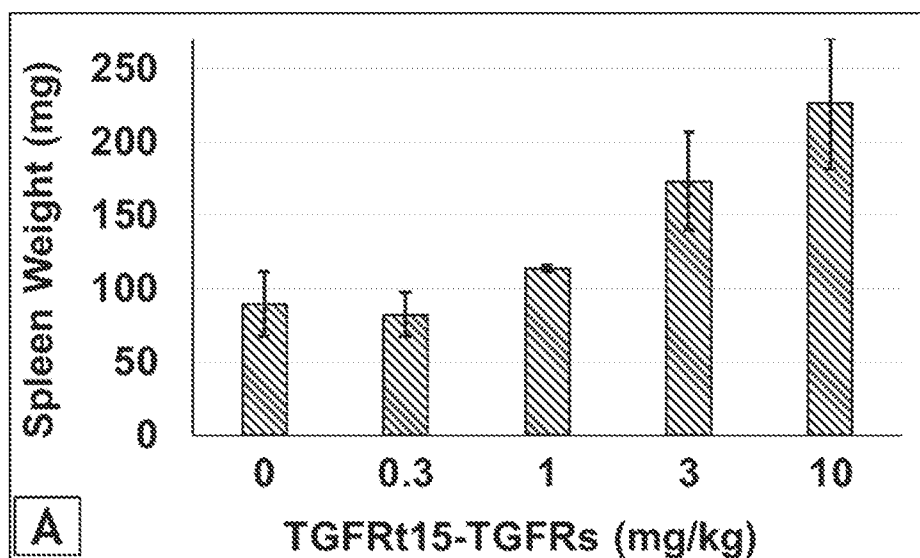


Figure 40A

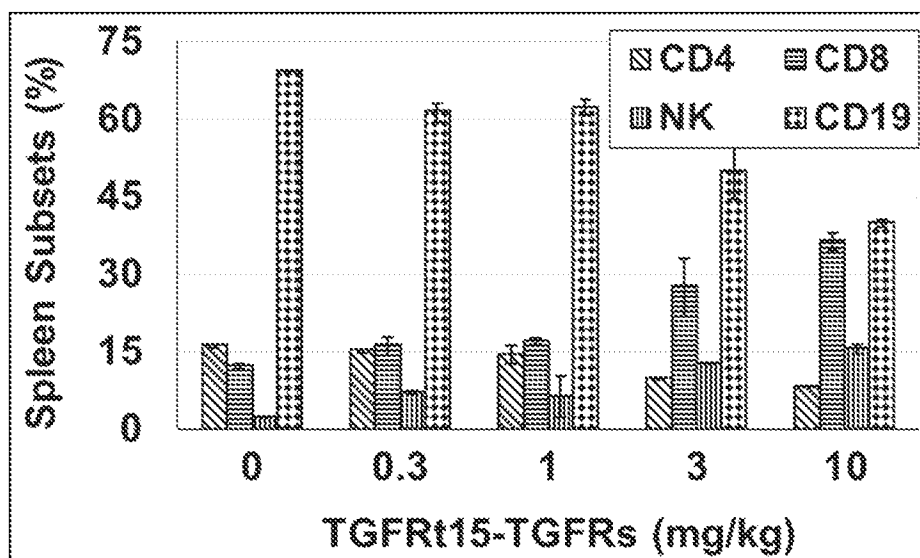


Figure 40B

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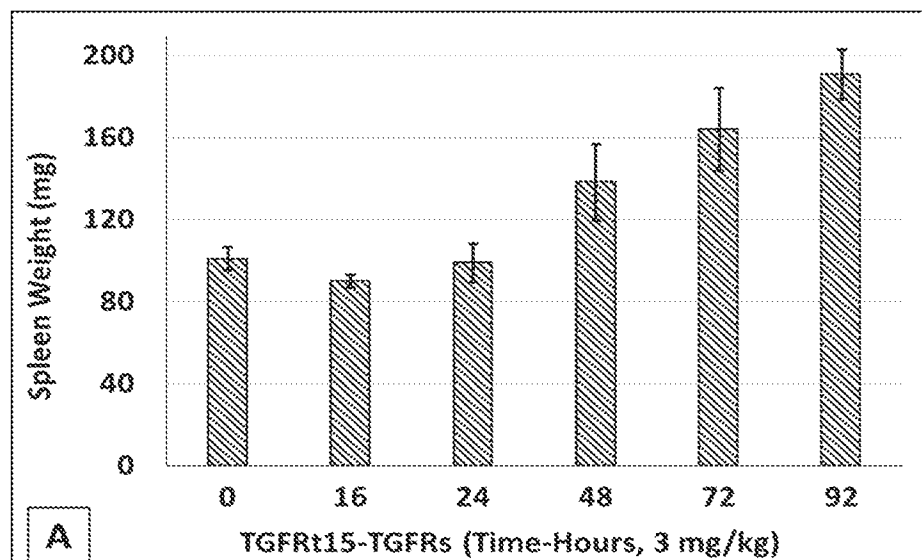


Figure 41A

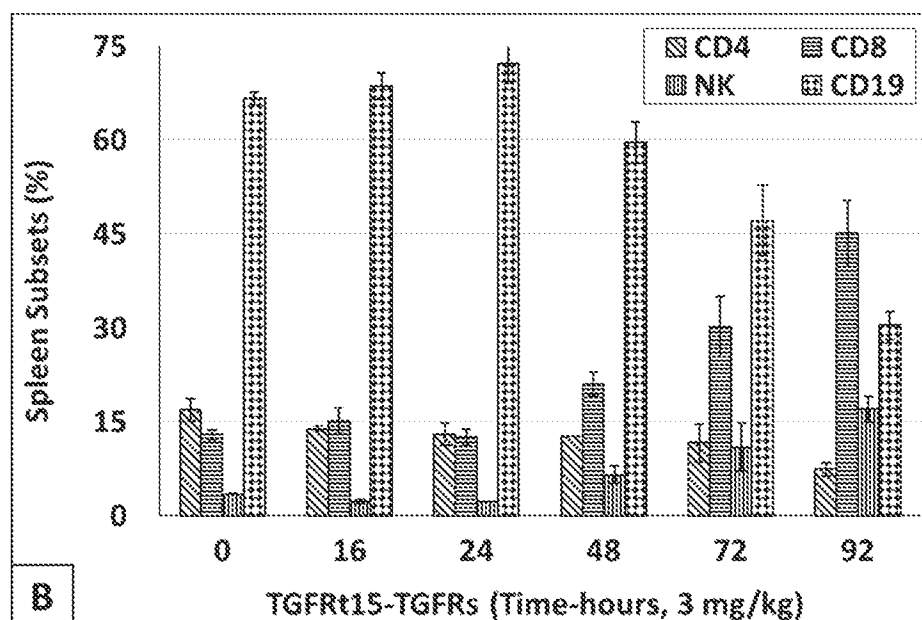


Figure 41B

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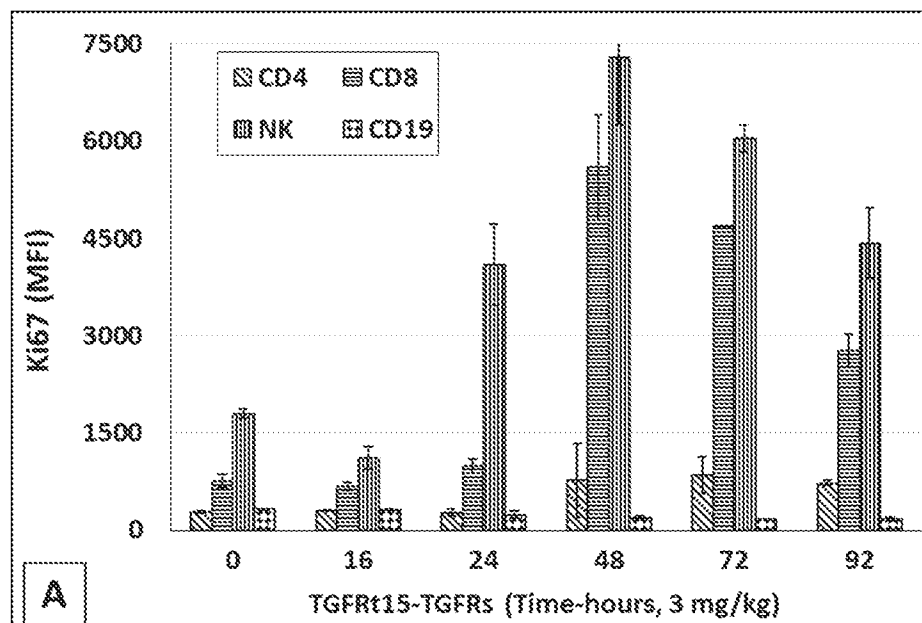


Figure 42A

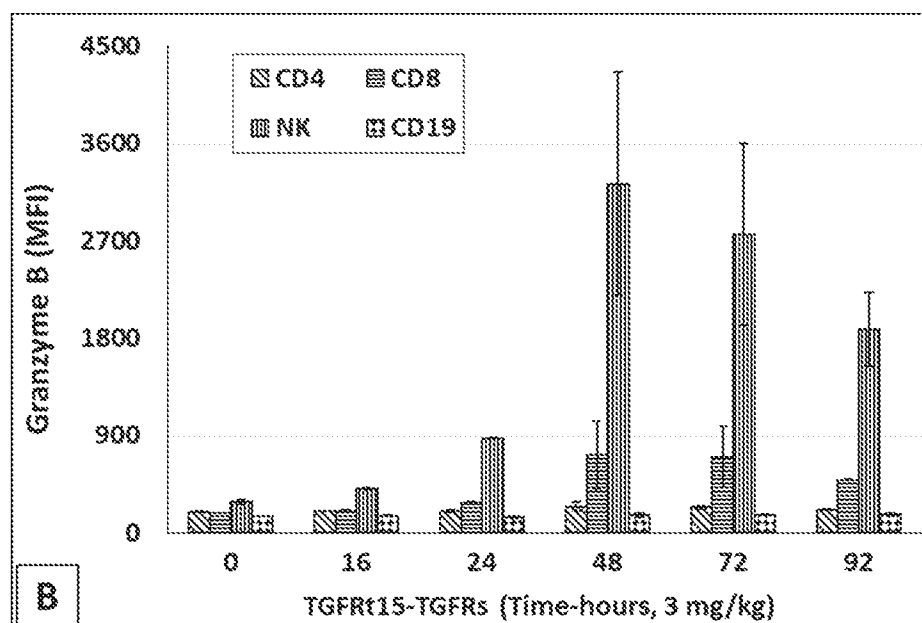


Figure 42B

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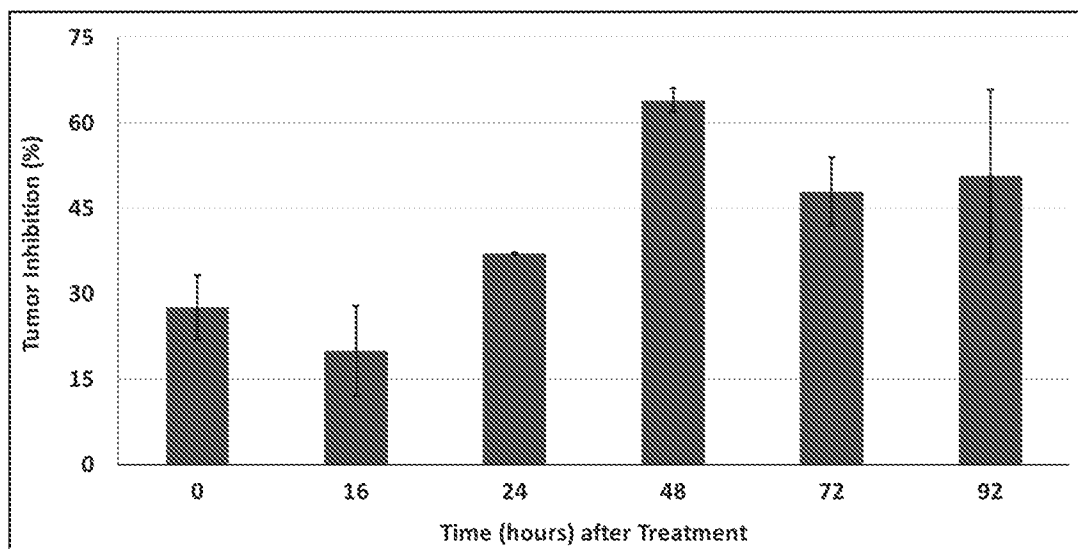


Figure 43

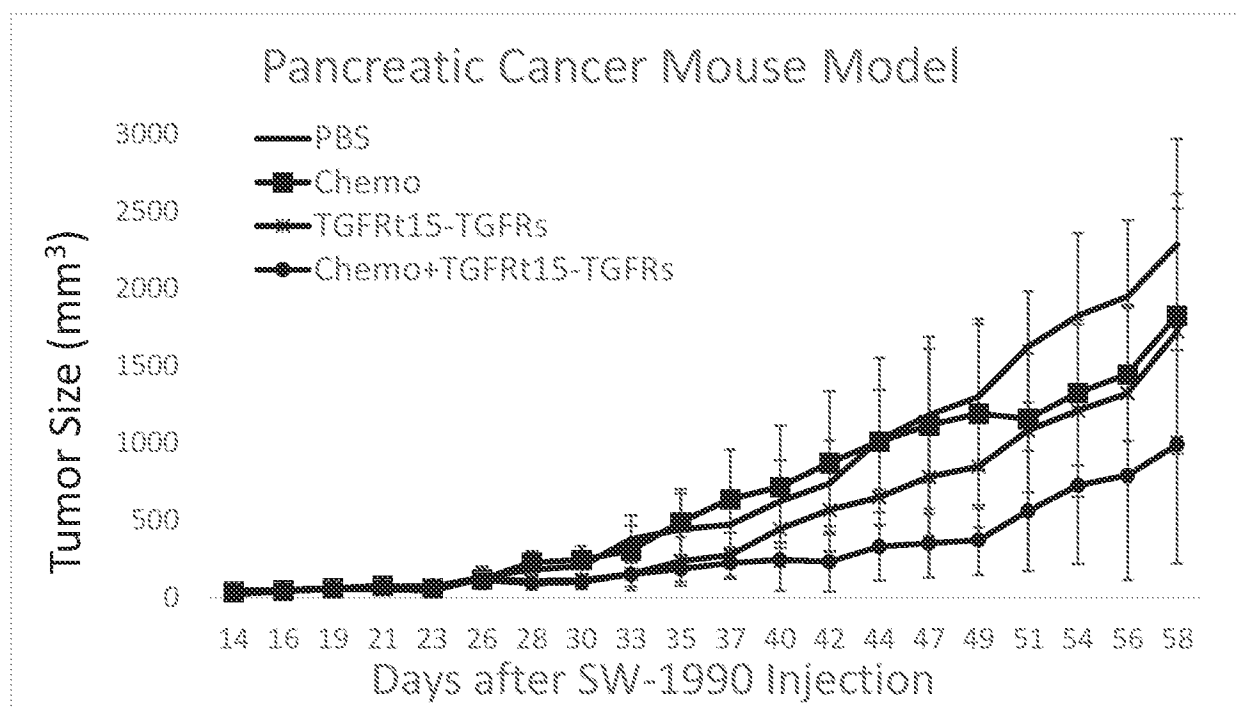


Figure 44

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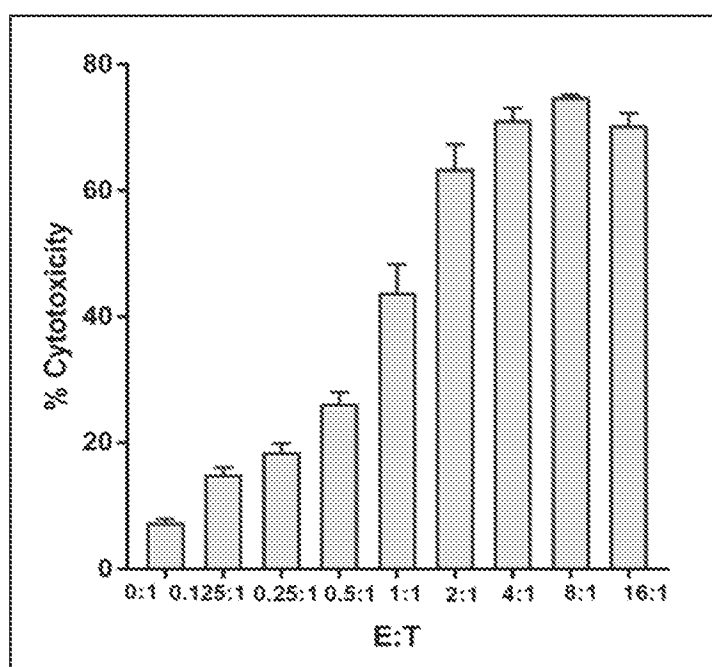


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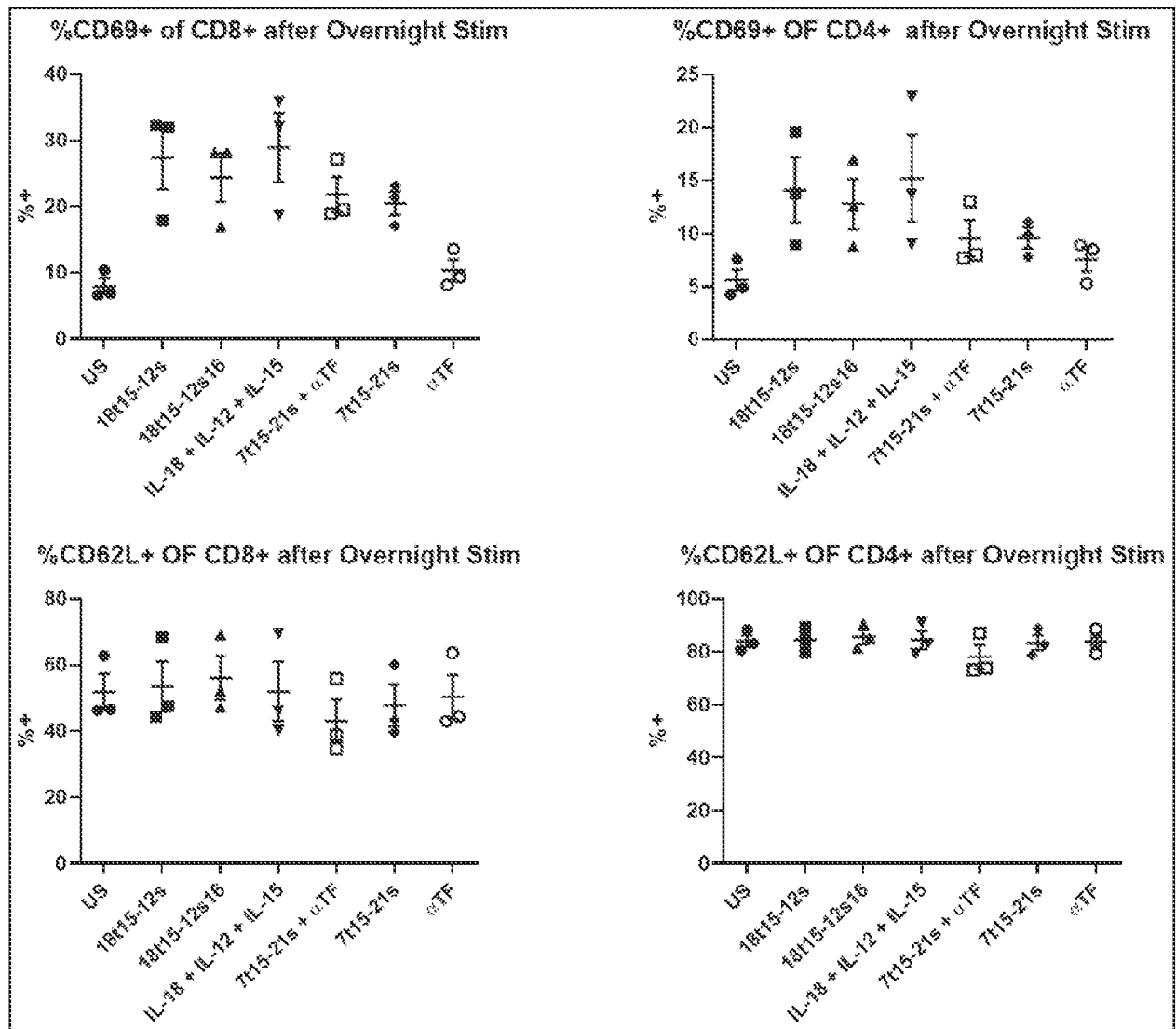


Figure 46

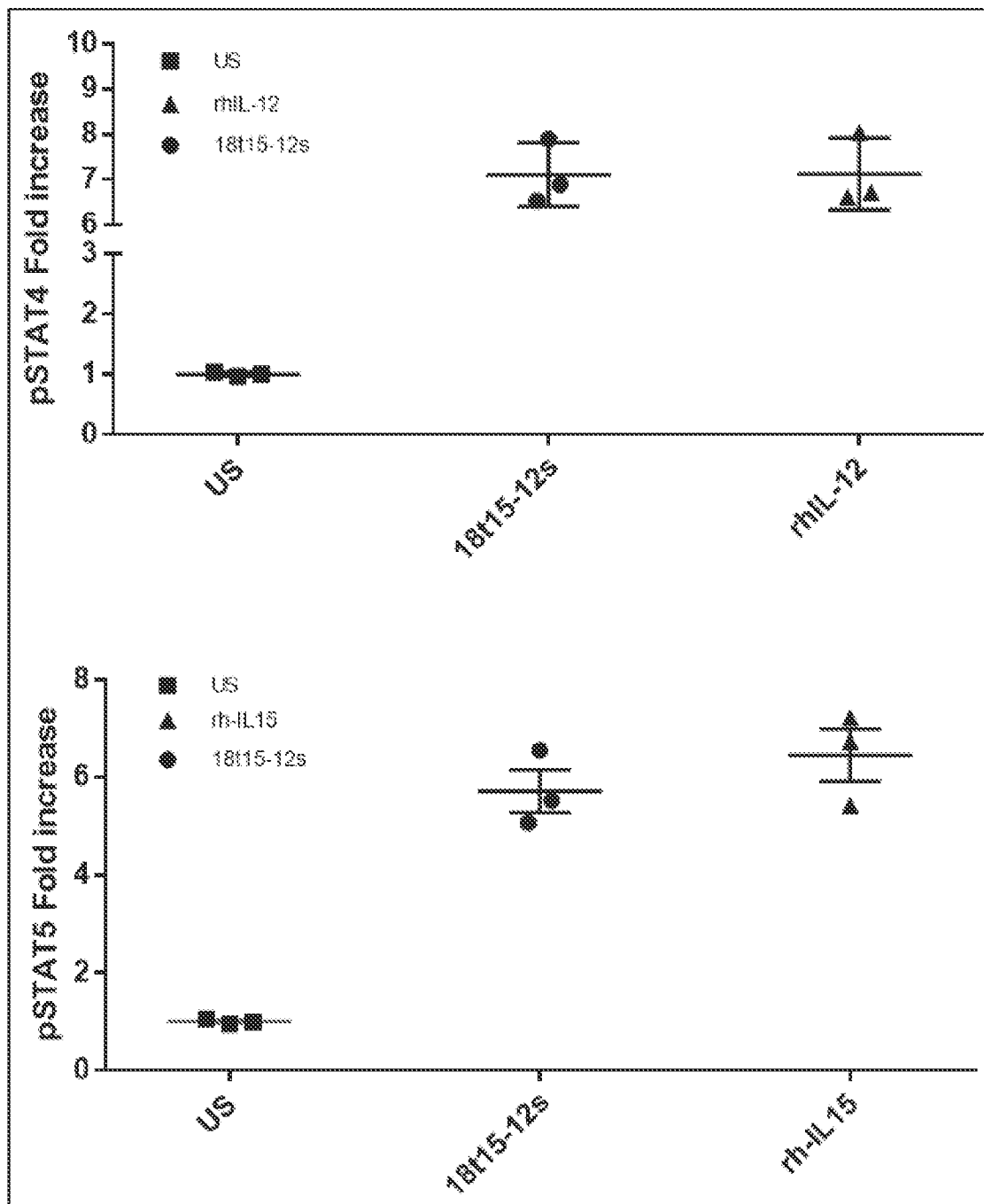


Figure 47

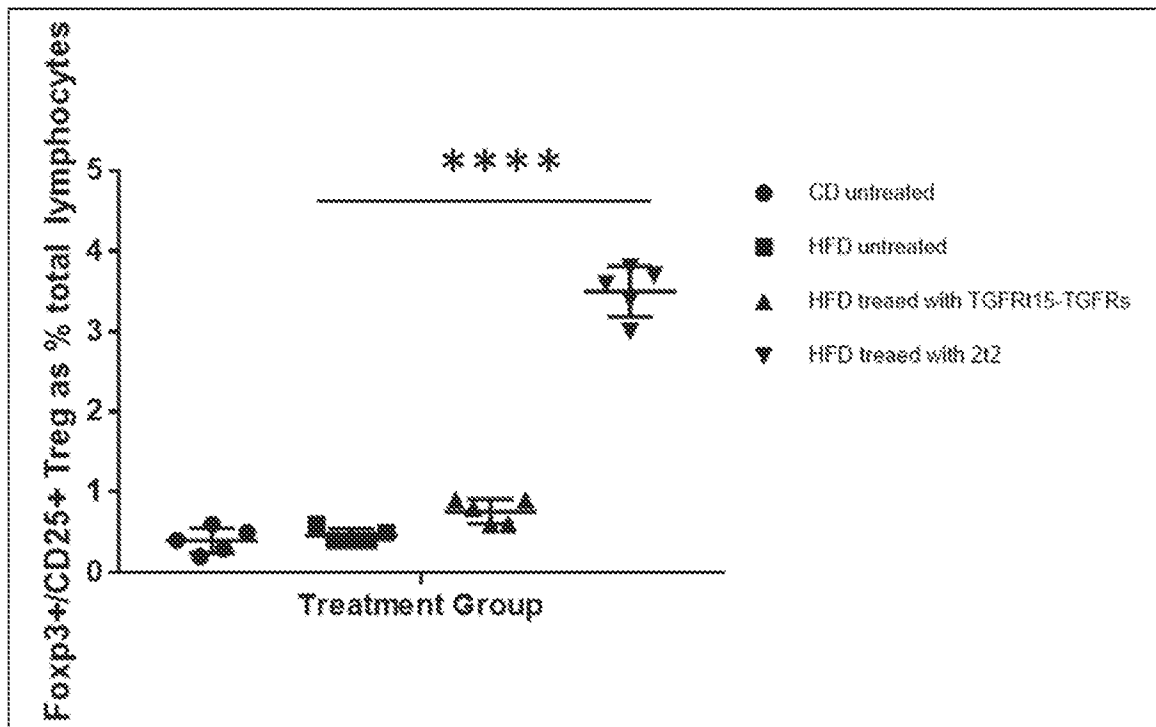


Figure 48A

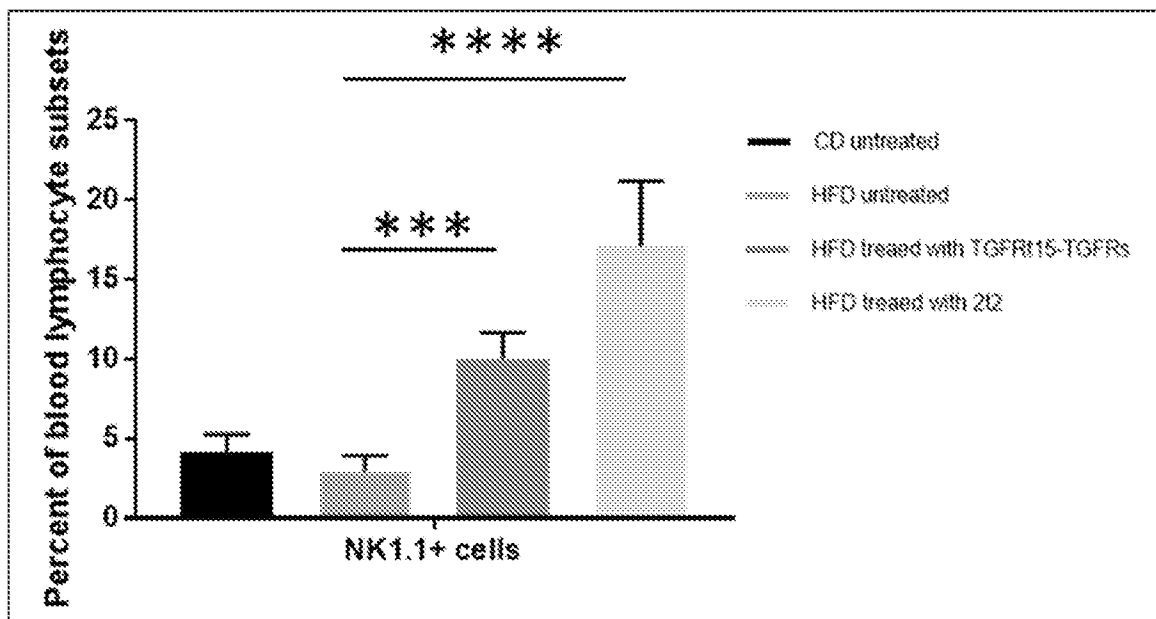


Figure 48B

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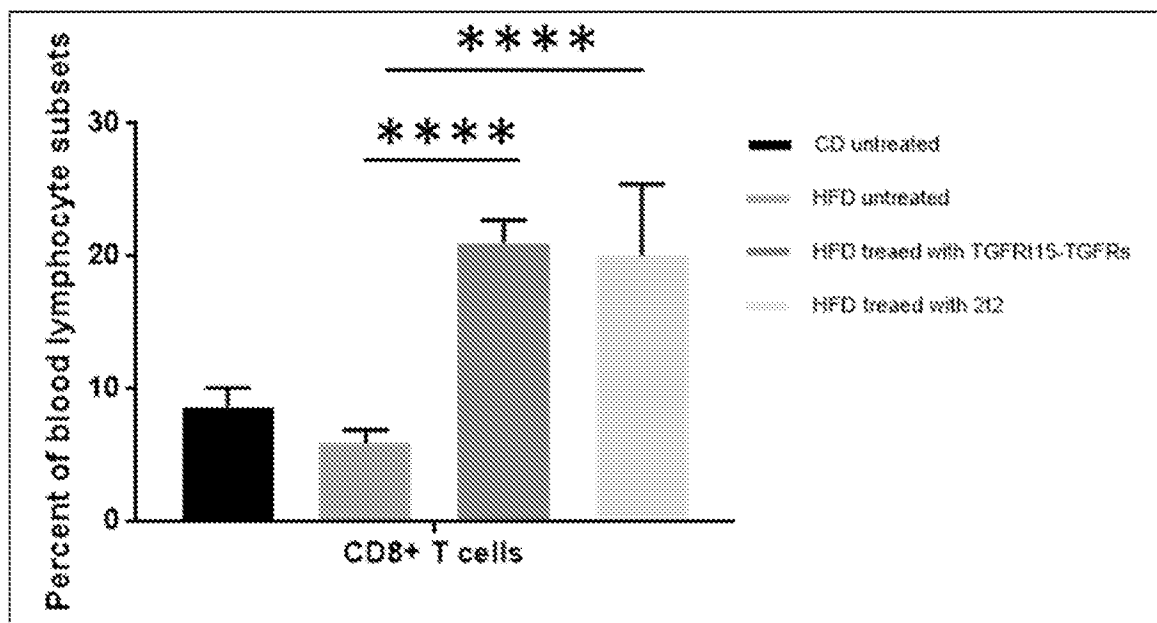


Figure 48C

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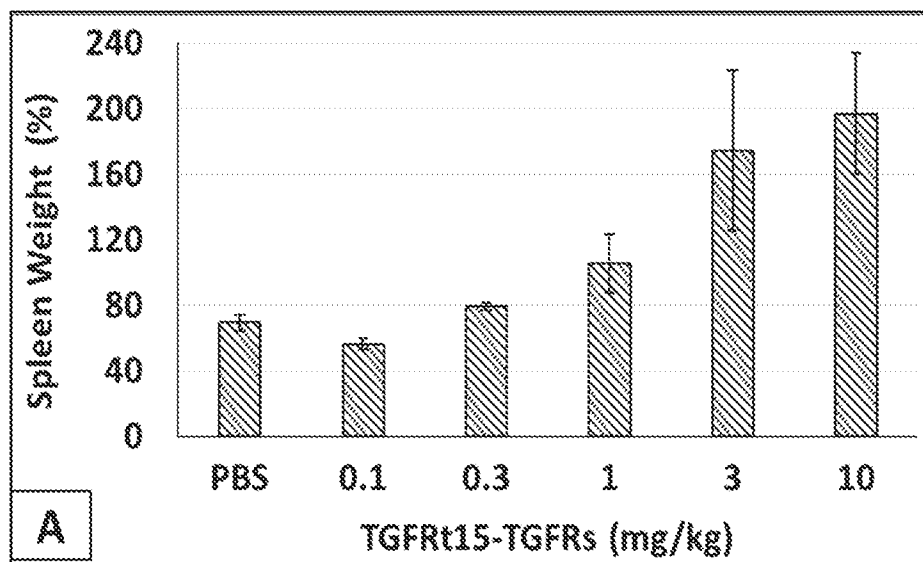


Figure 49A

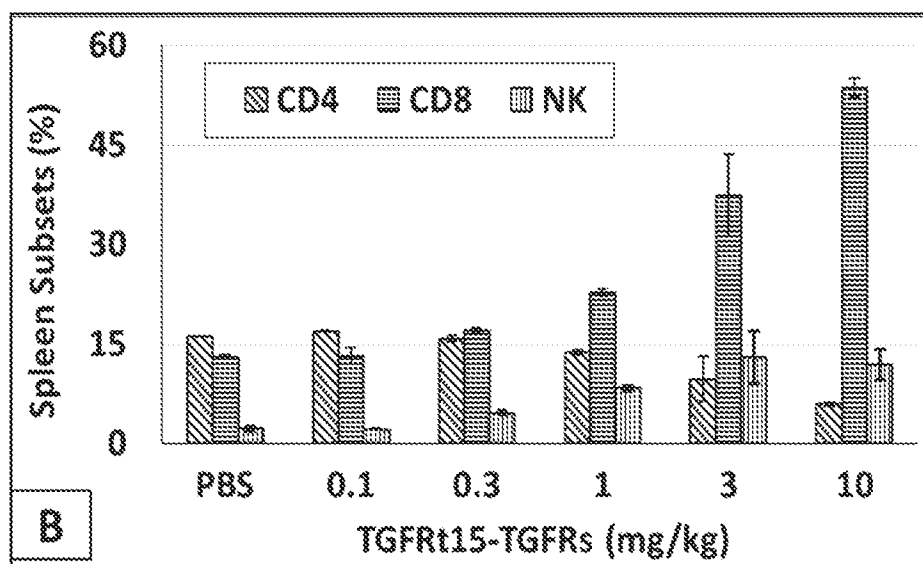


Figure 49B

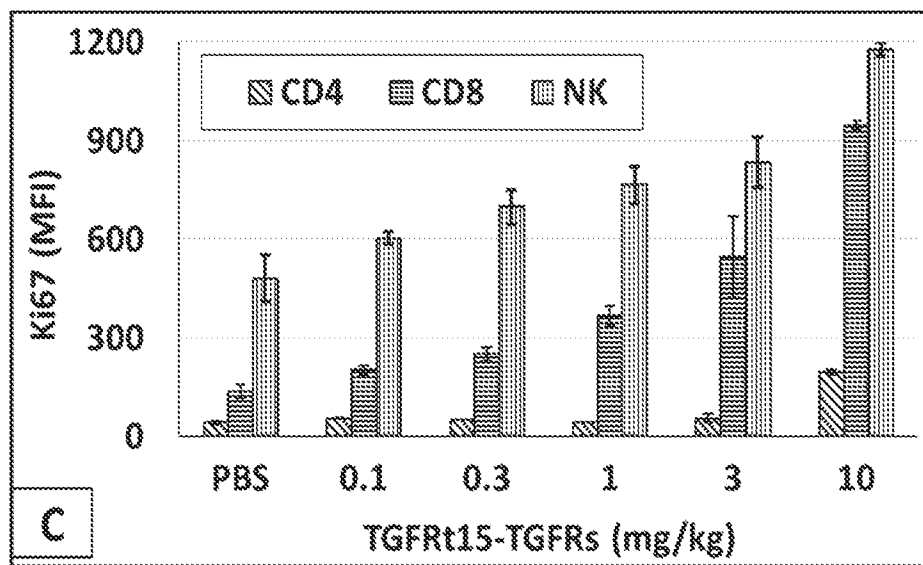


Figure 49C

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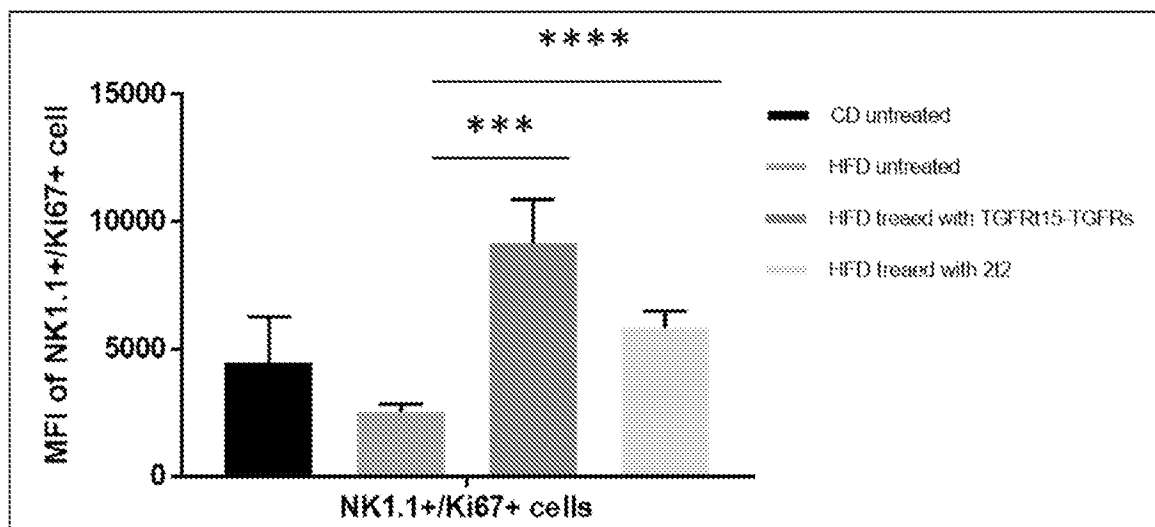


Figure 50A

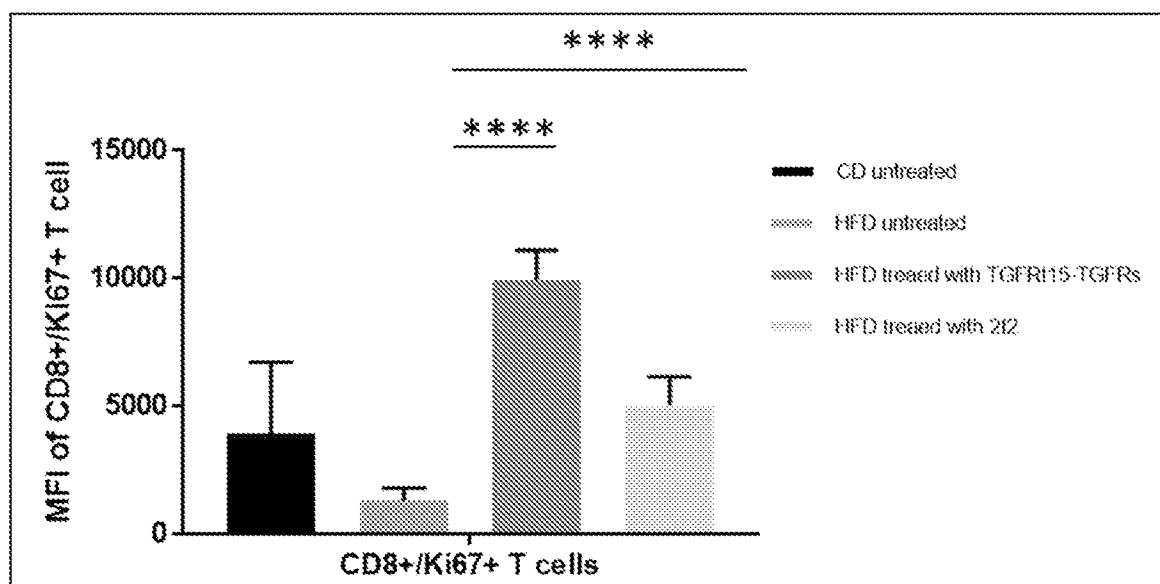


Figure 50B

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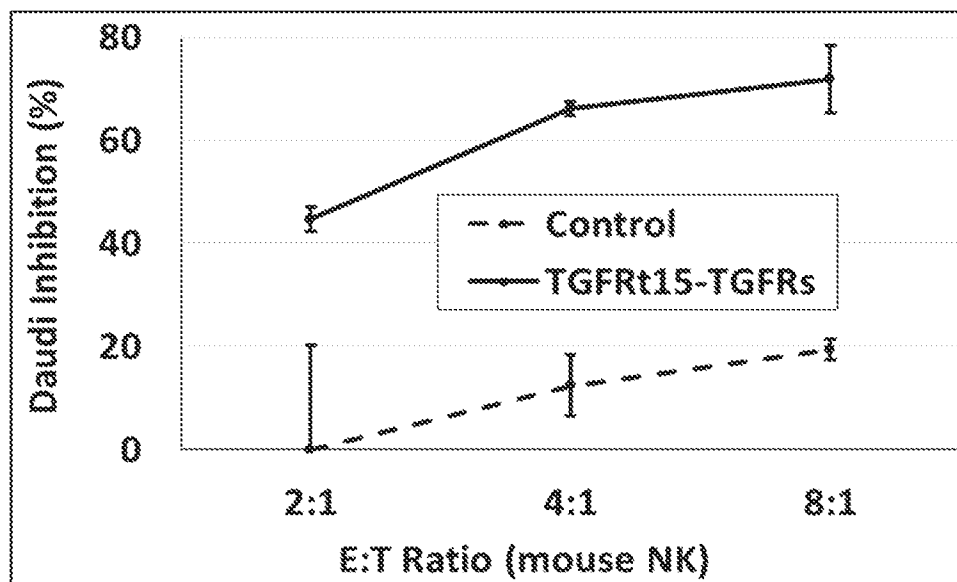


Figure 51A

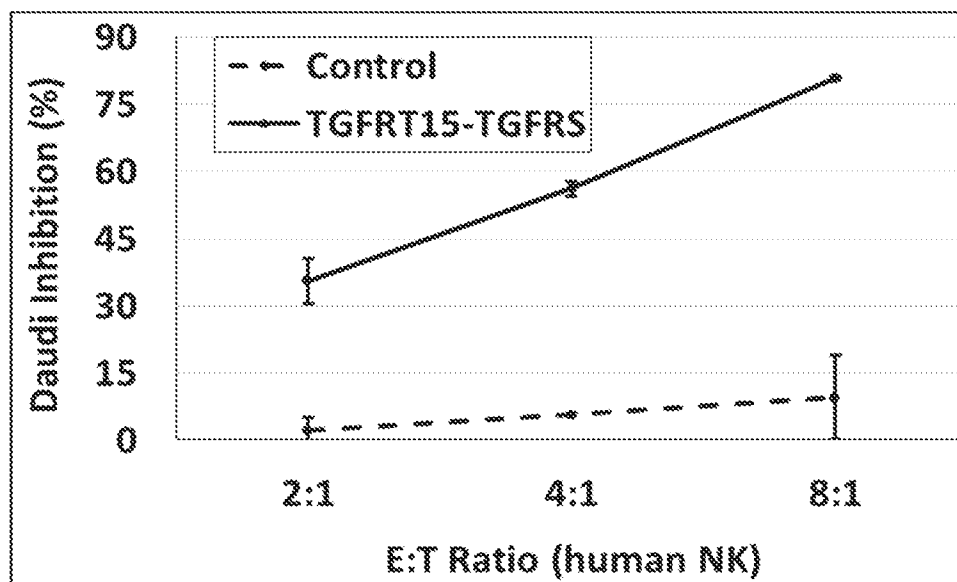


Figure 51B

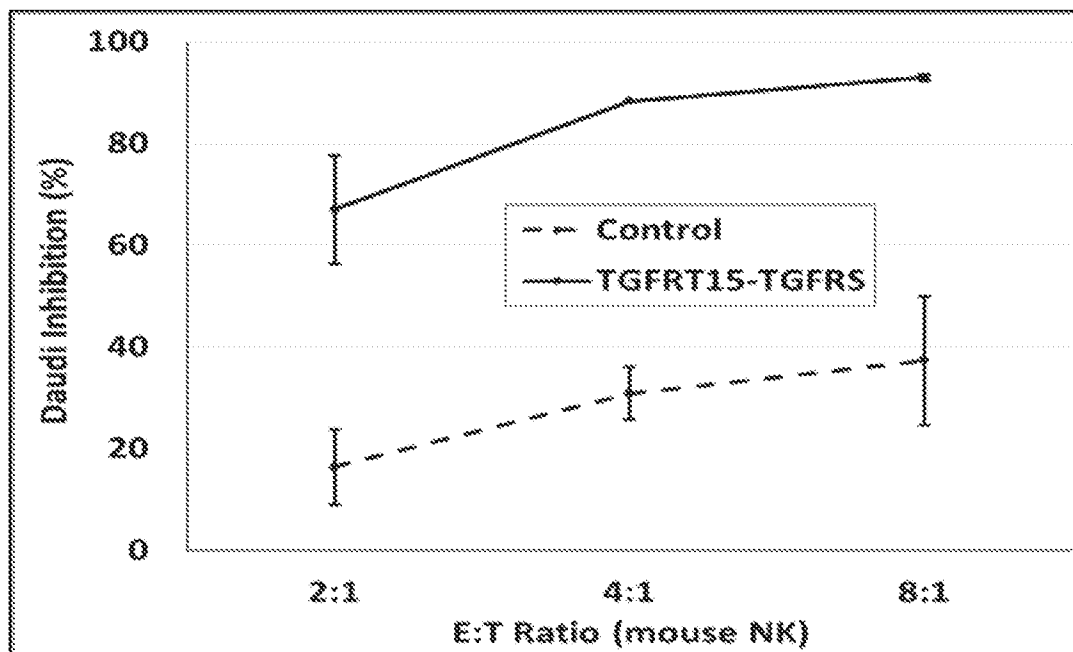


Figure 52A

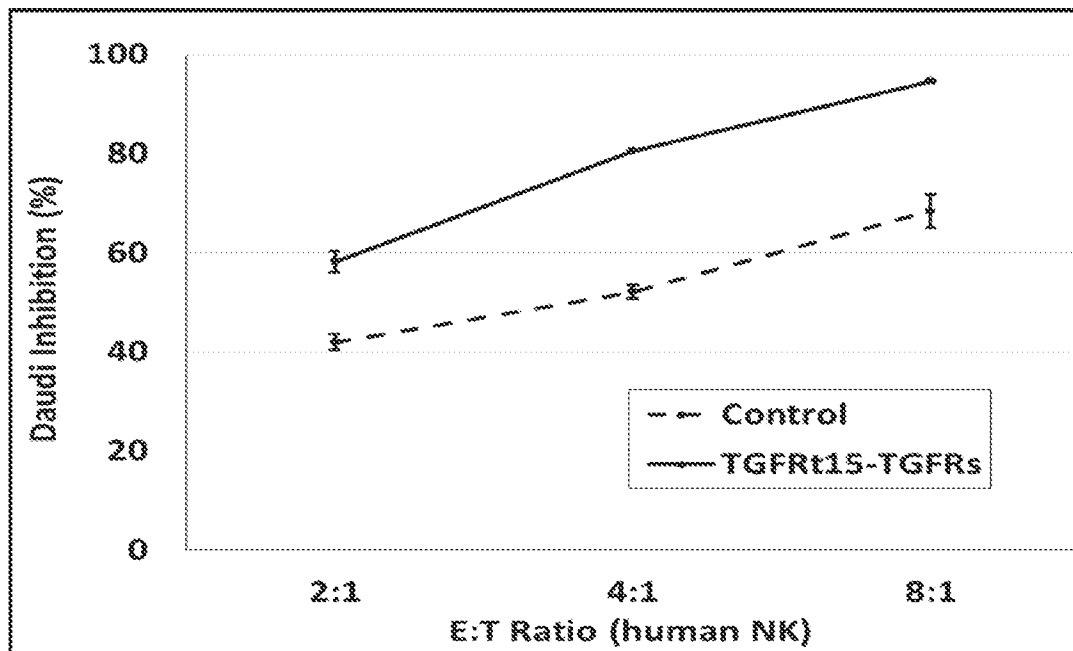


Figure 52B

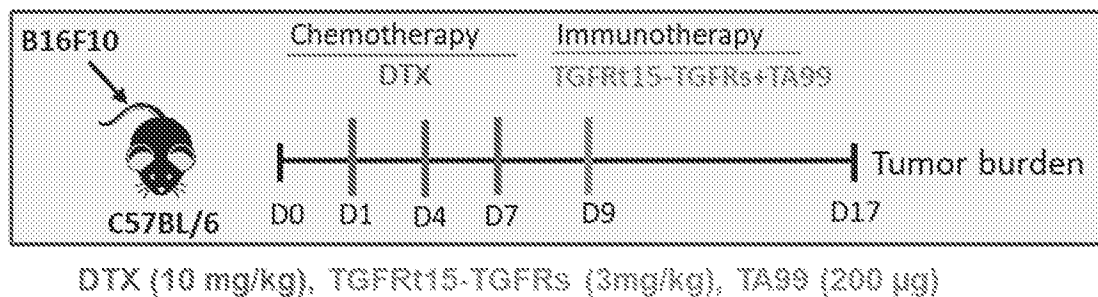


Figure 53A

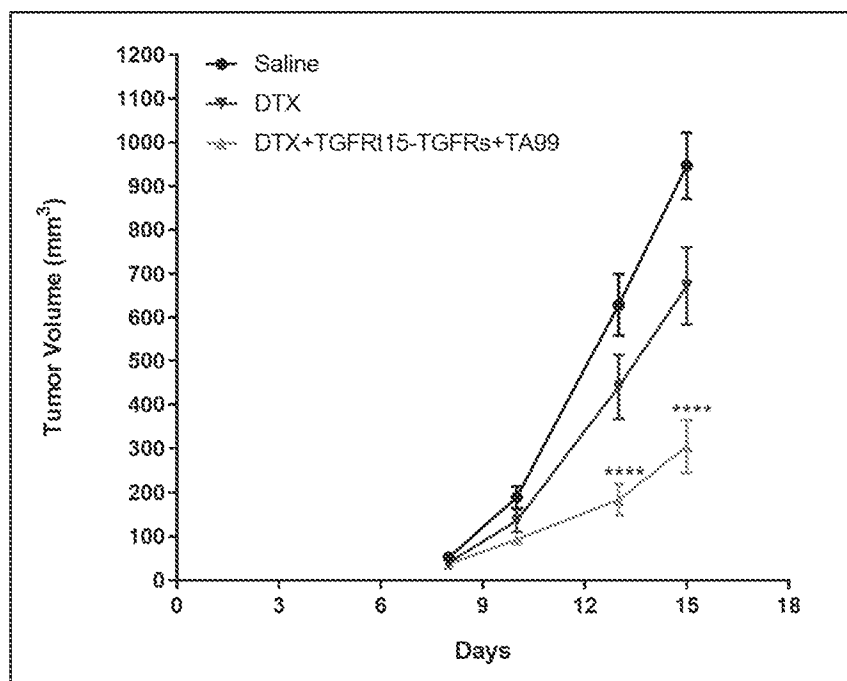


Figure 53B

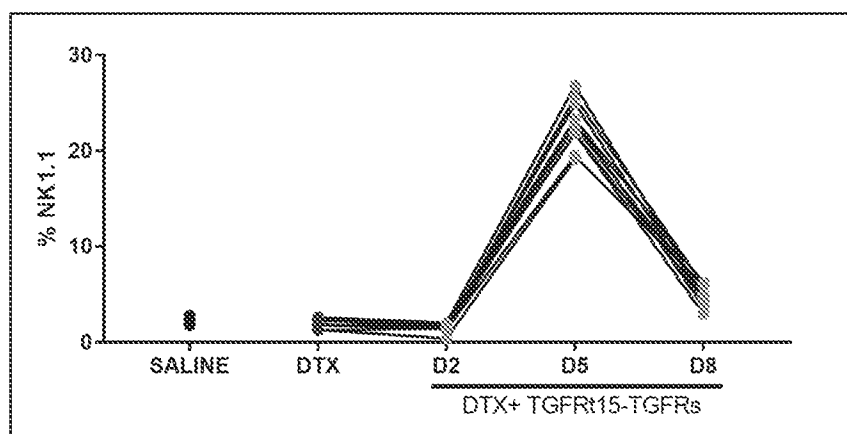


Figure 53C

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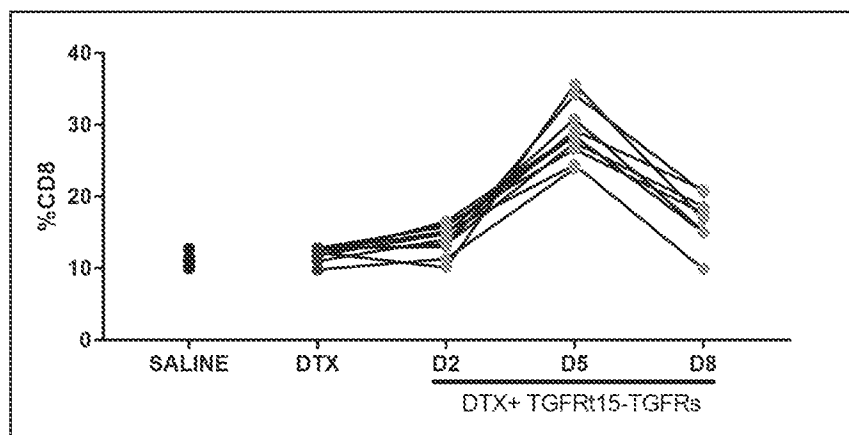


Figure 53D

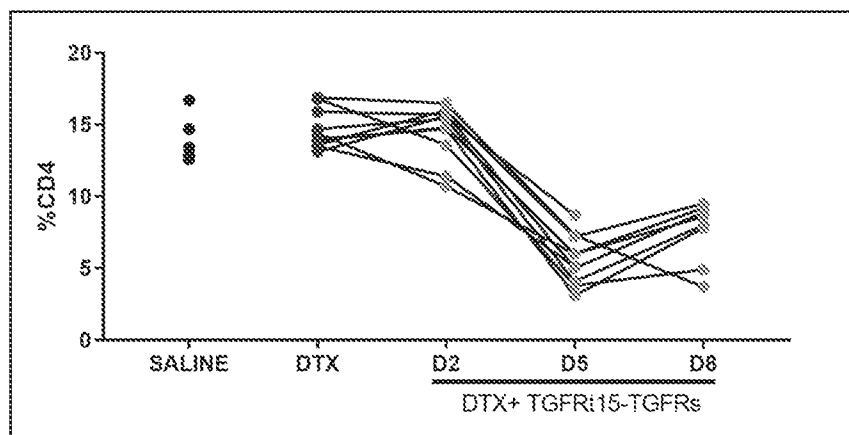


Figure 53E

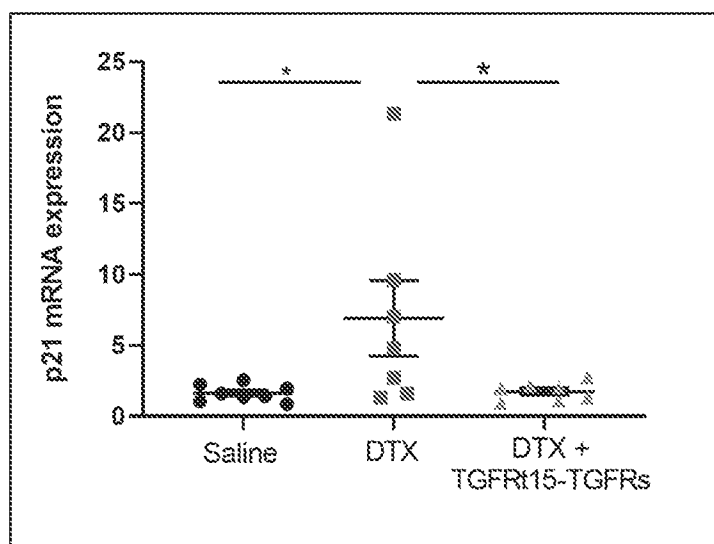


Figure 53F

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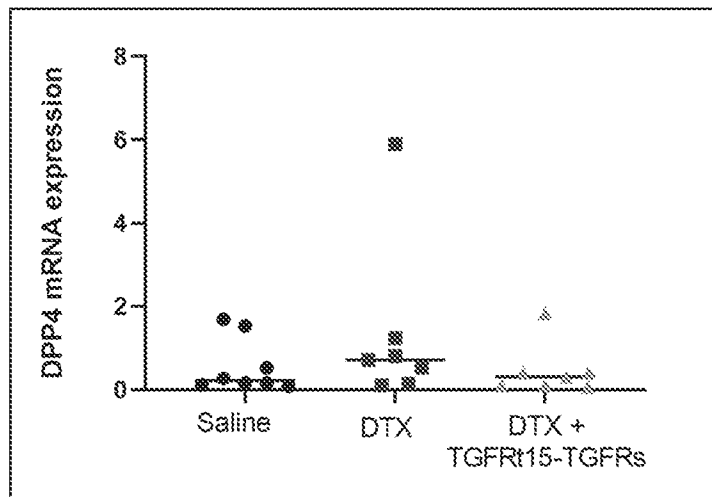


Figure 53G

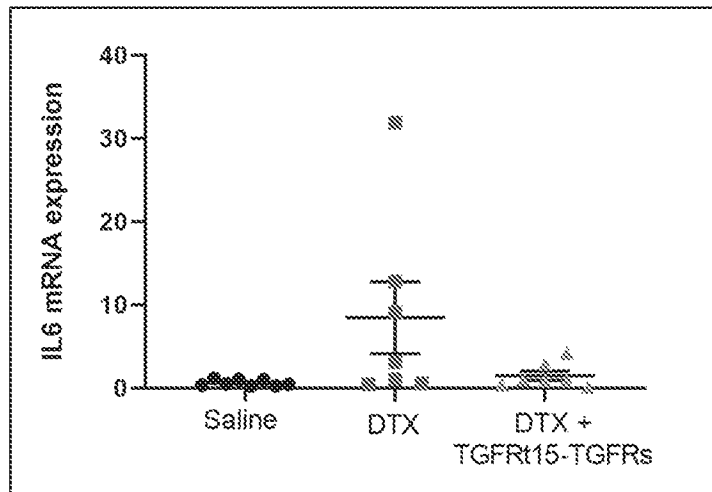


Figure 53H

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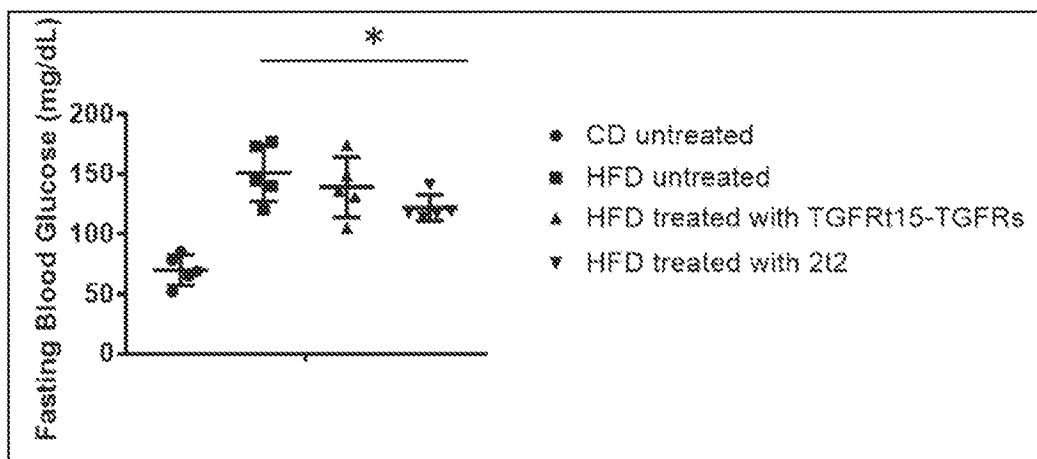


Figure 54A

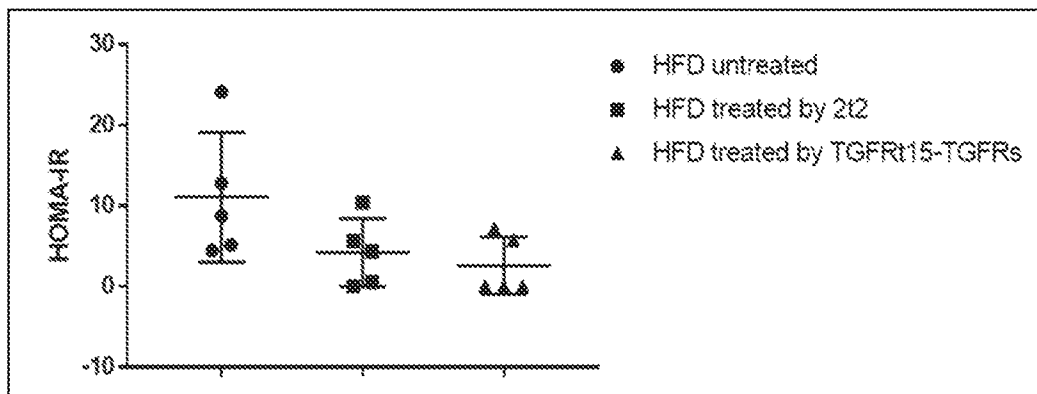


Figure 54B

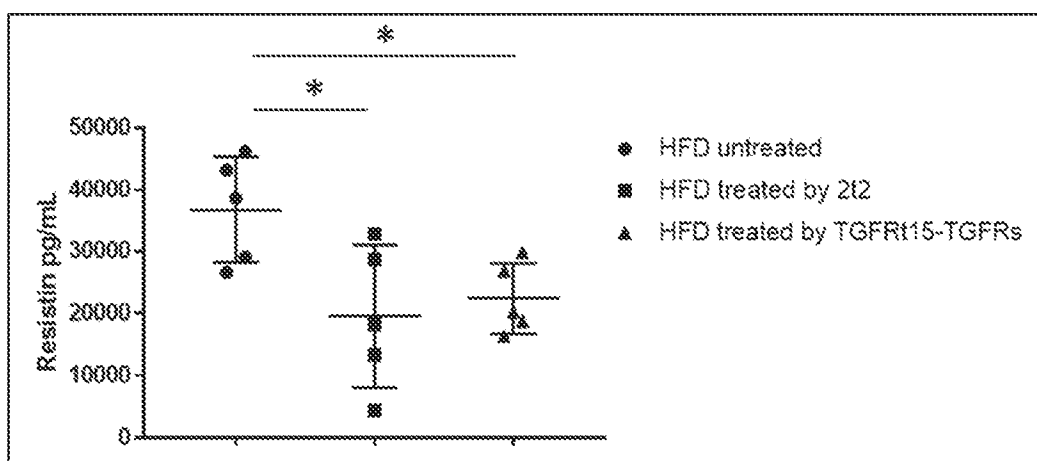


Figure 54C

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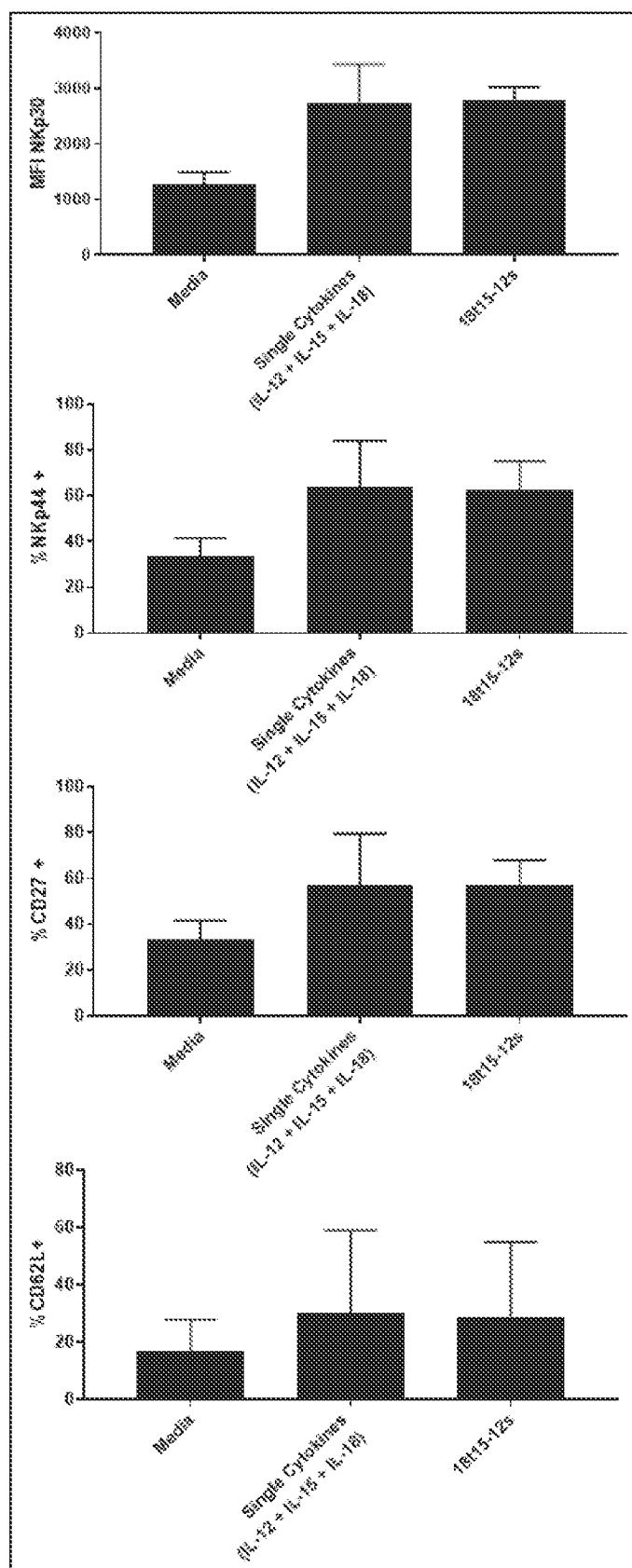


Figure 55

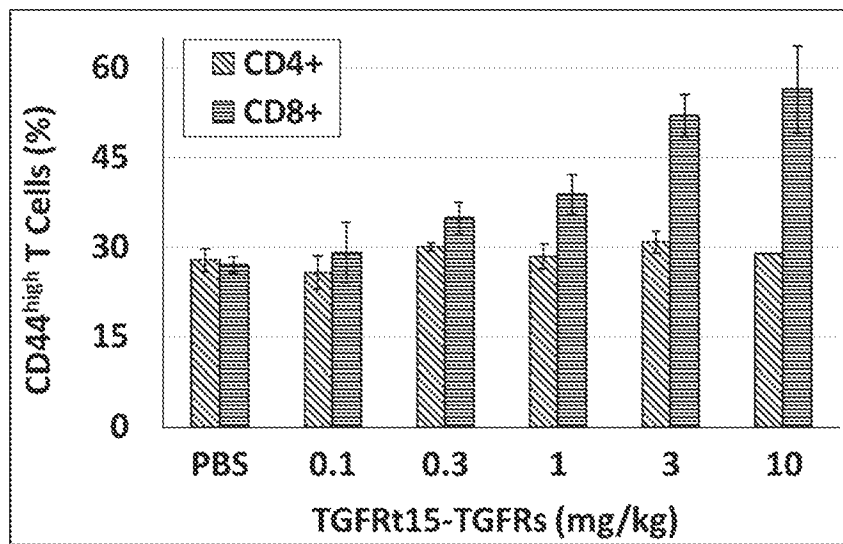


Figure 56

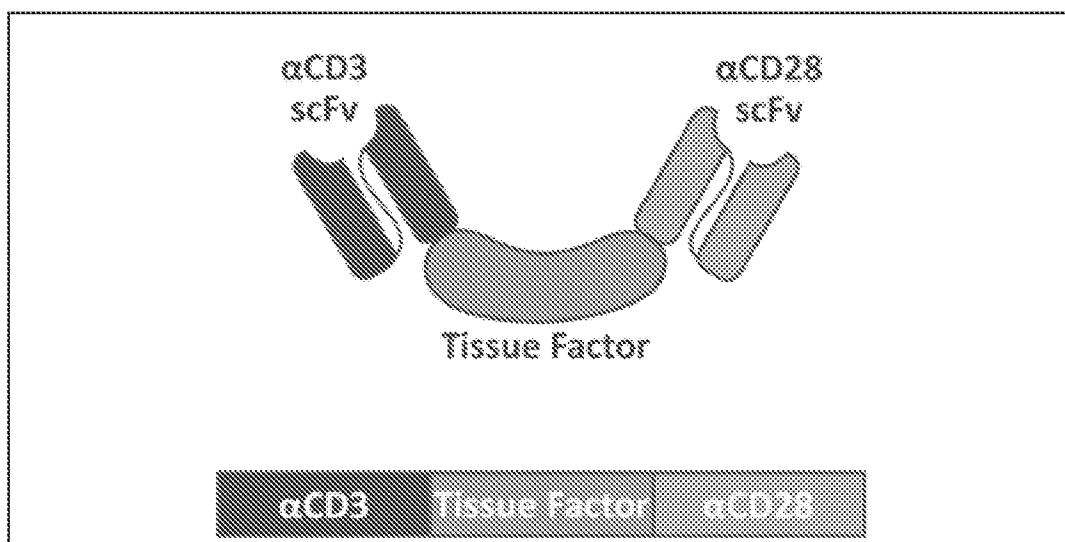


Figure 57

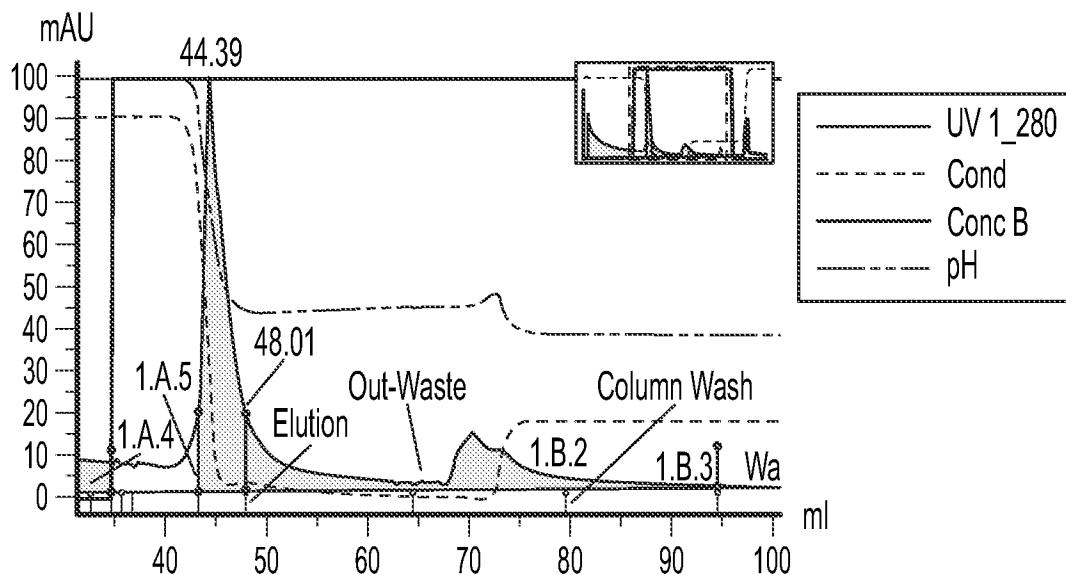


Figure 58

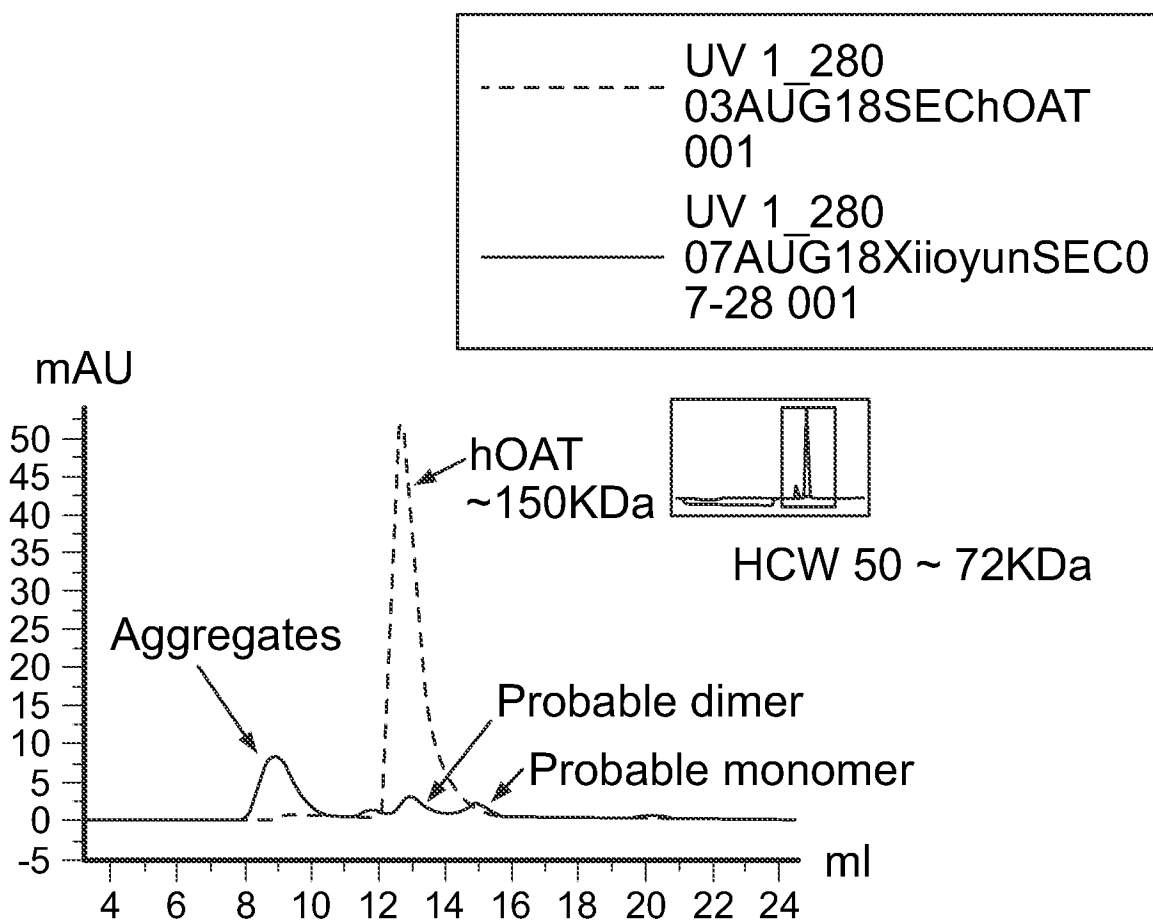


Figure 59

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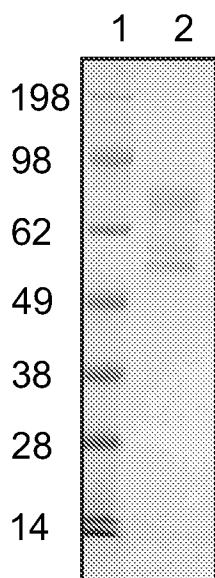


Figure 60

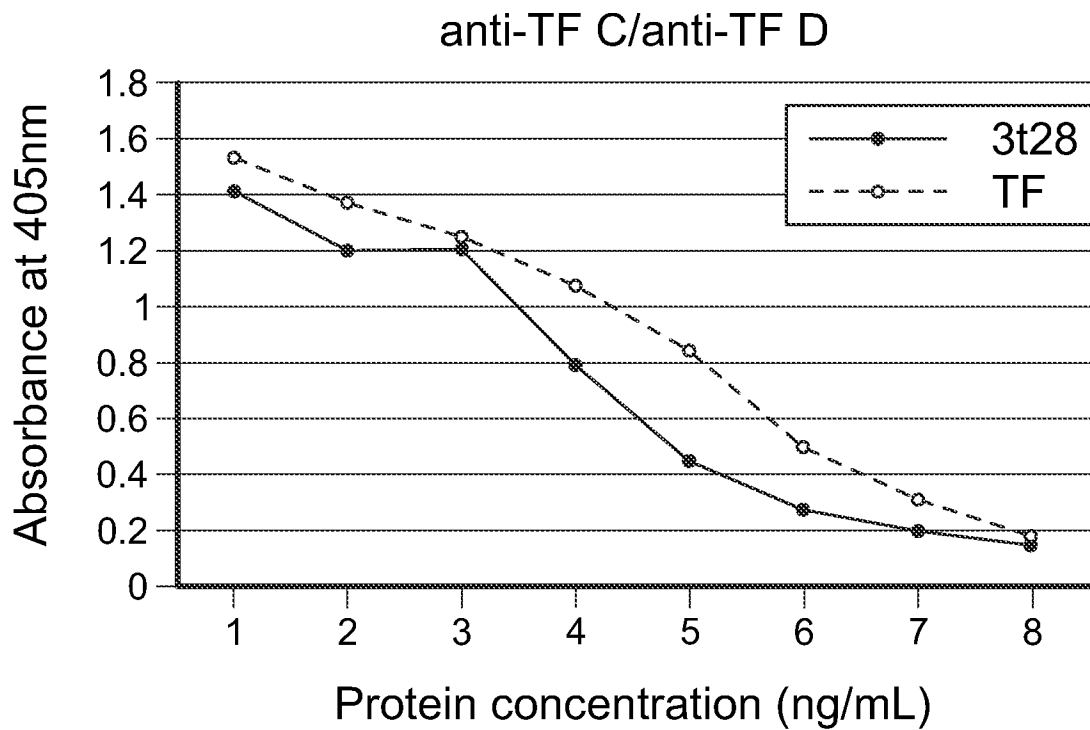


Figure 61

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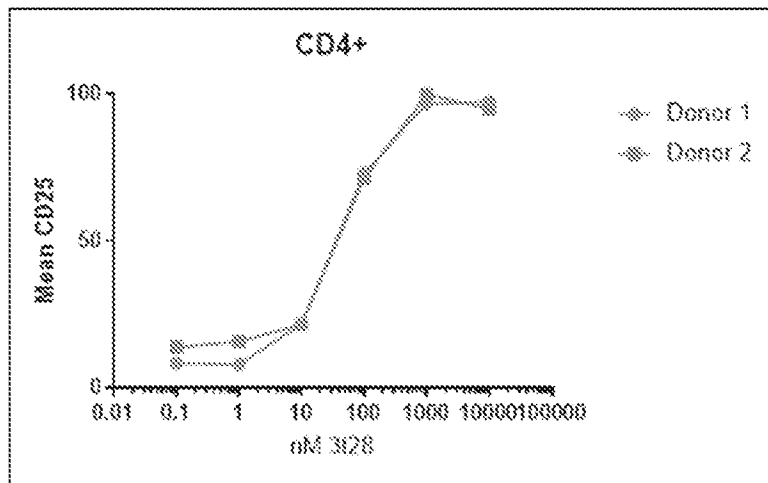


Figure 62

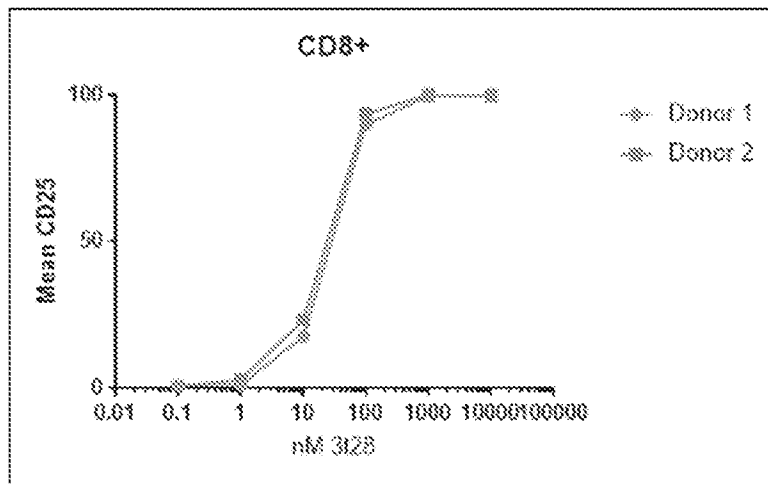


Figure 63

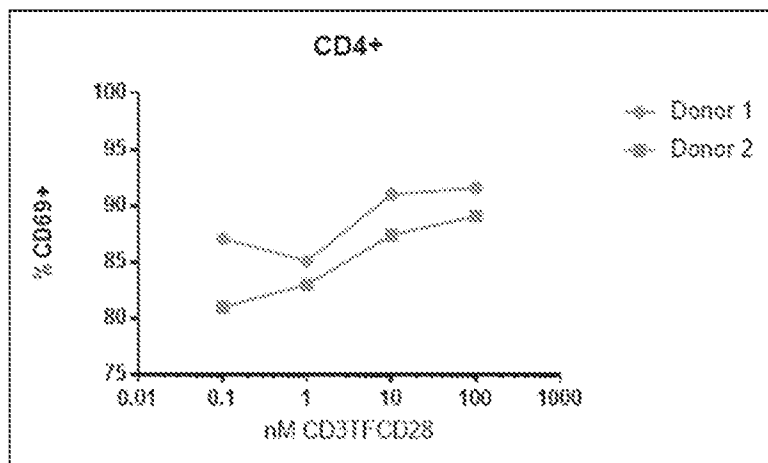


Figure 64

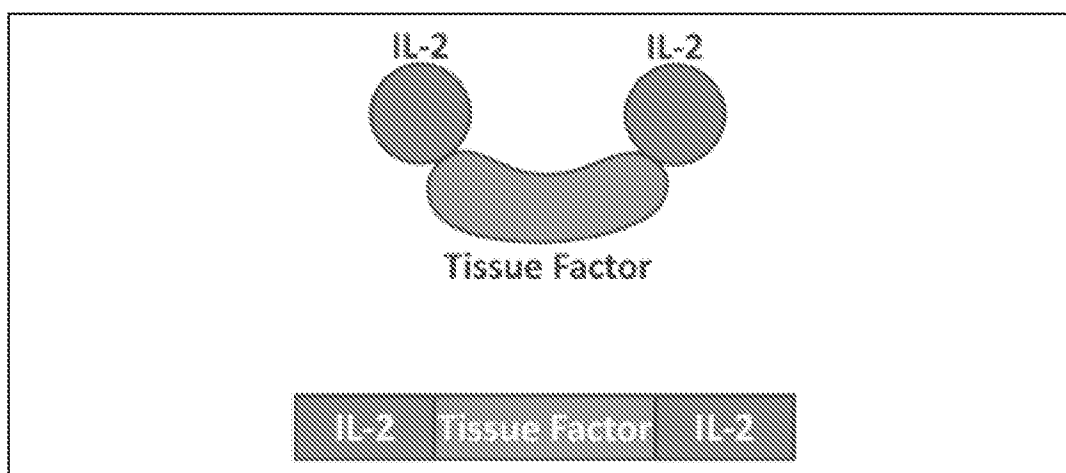


Figure 65

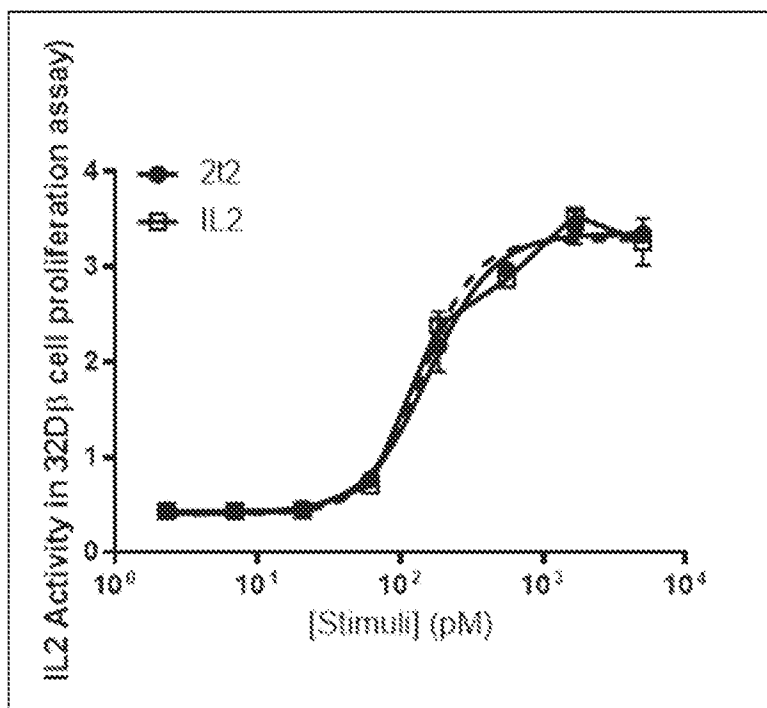


Figure 66

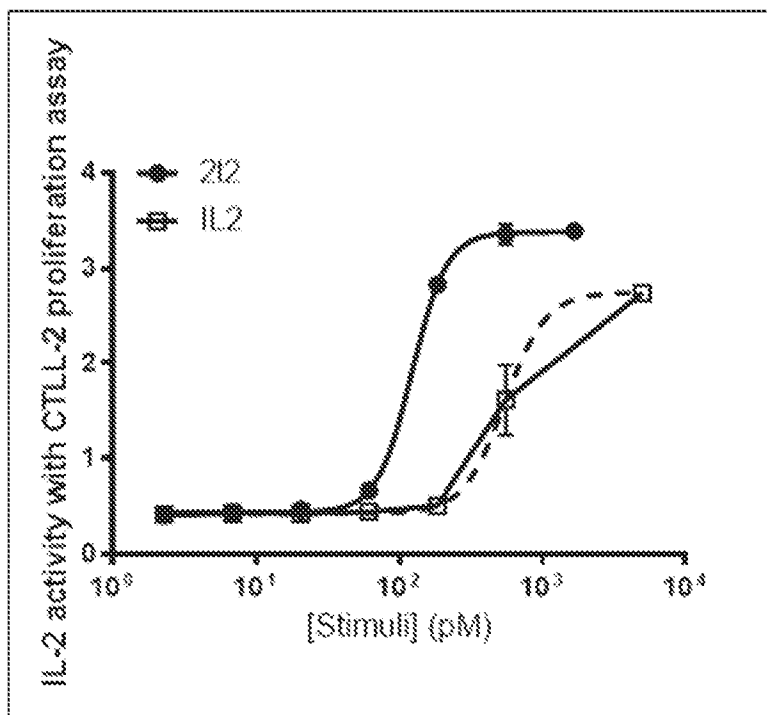


Figure 67

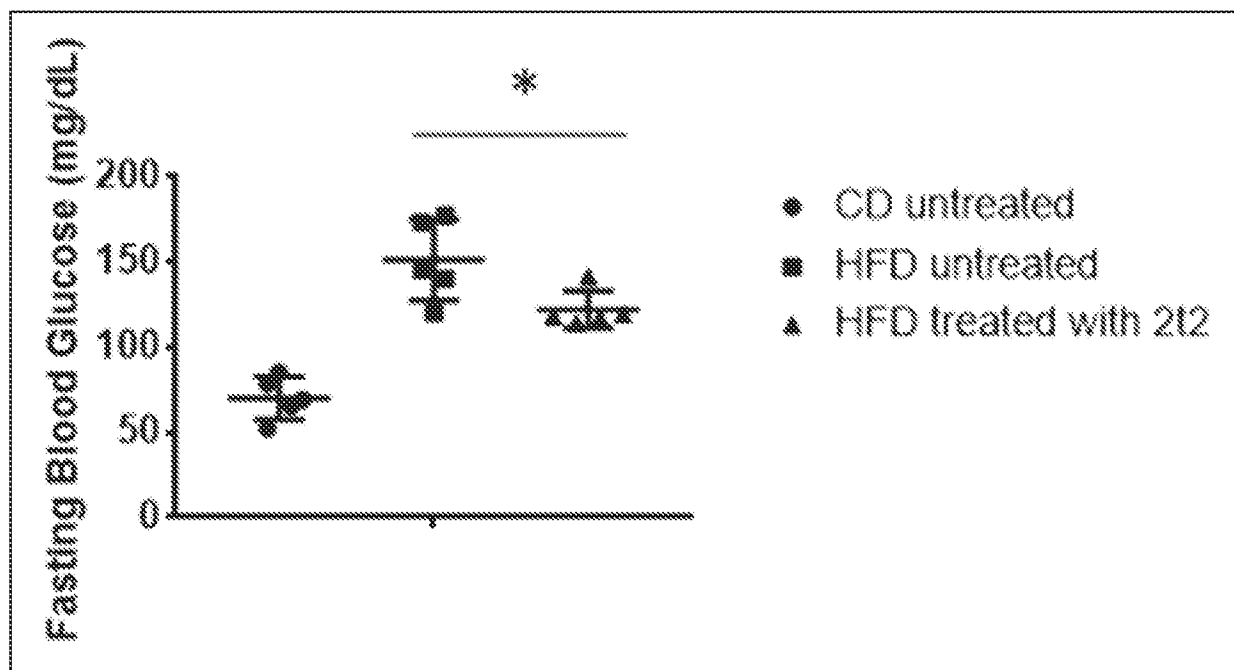


Figure 68

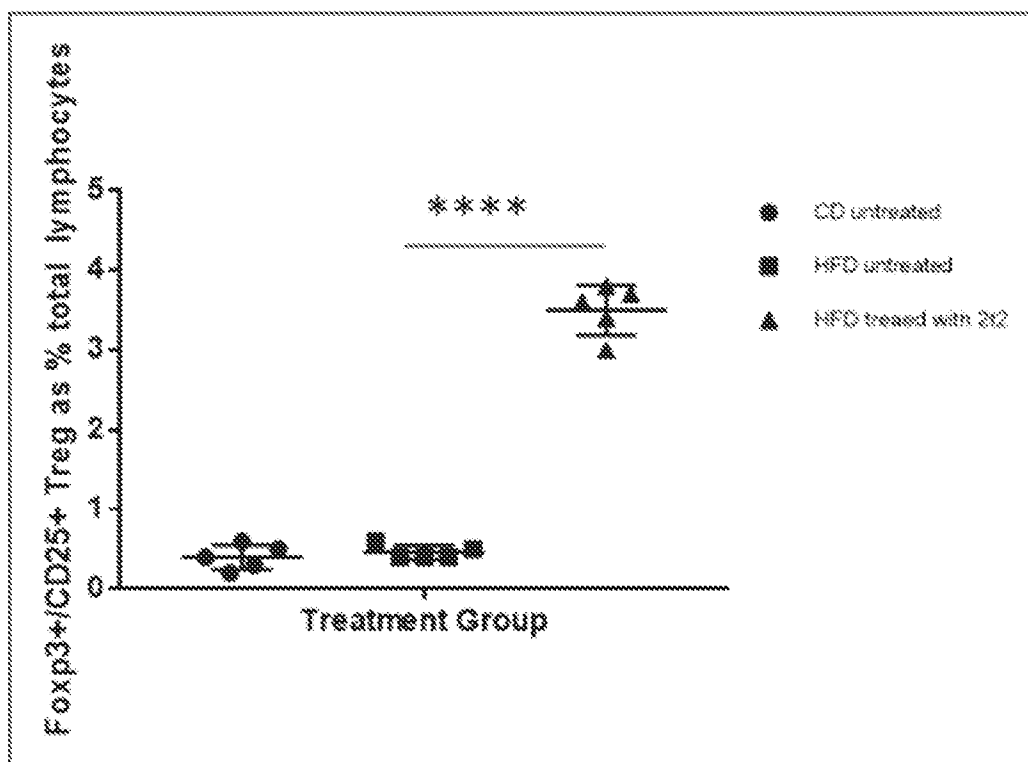


Figure 69

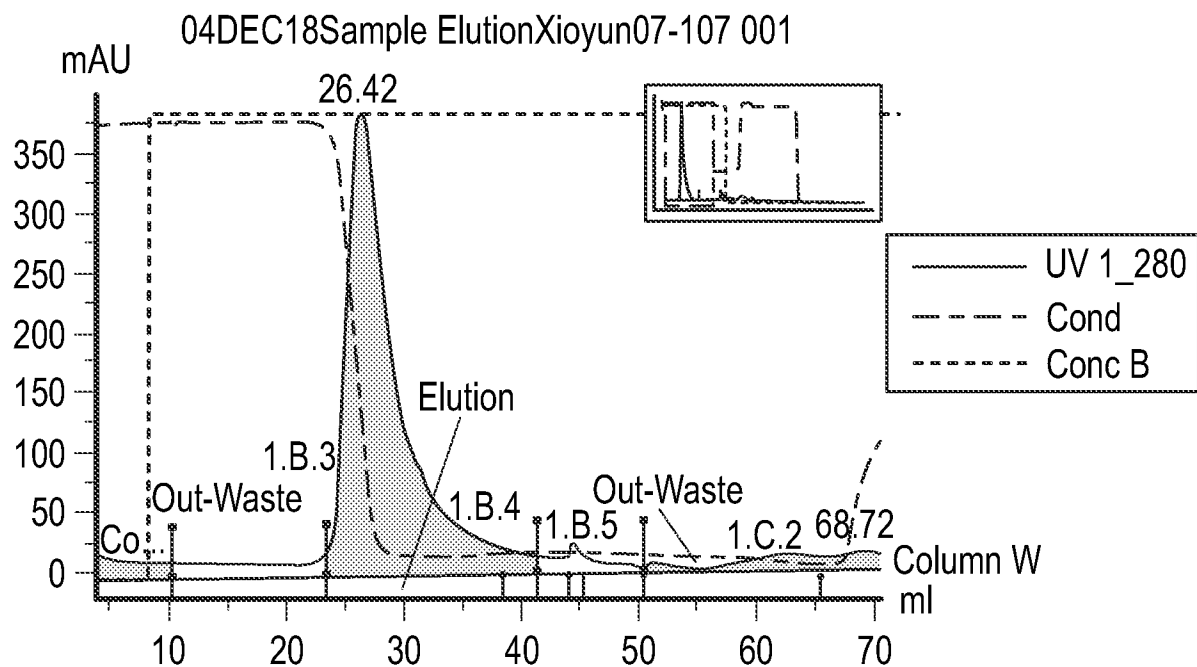


Figure 70

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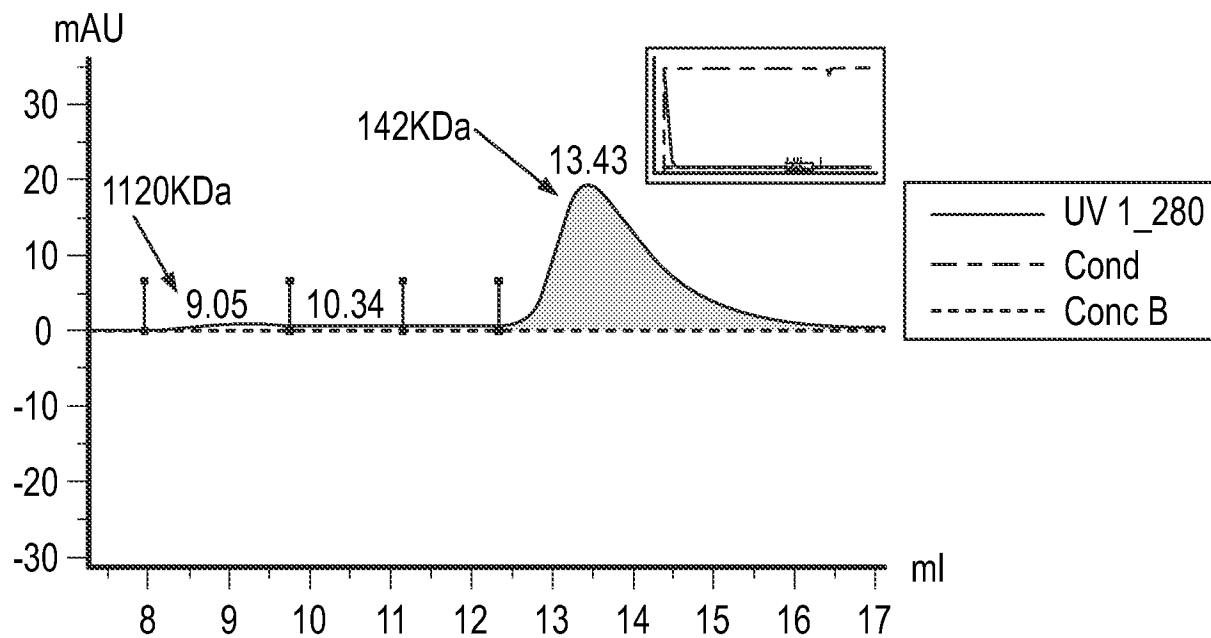


Figure 71

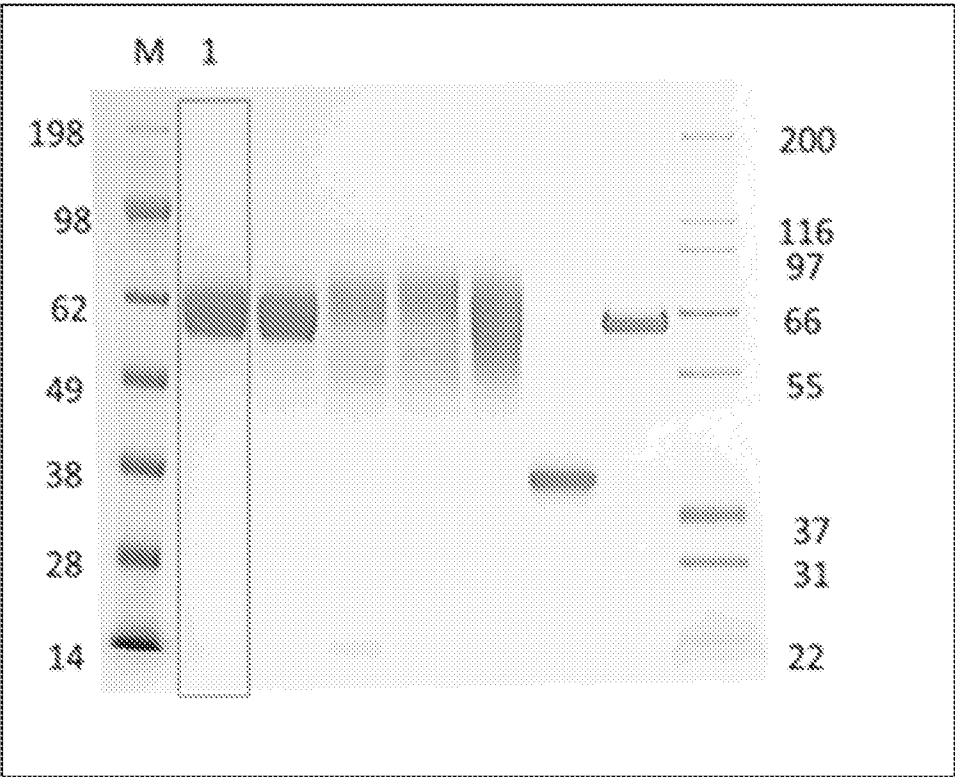


Figure 72A

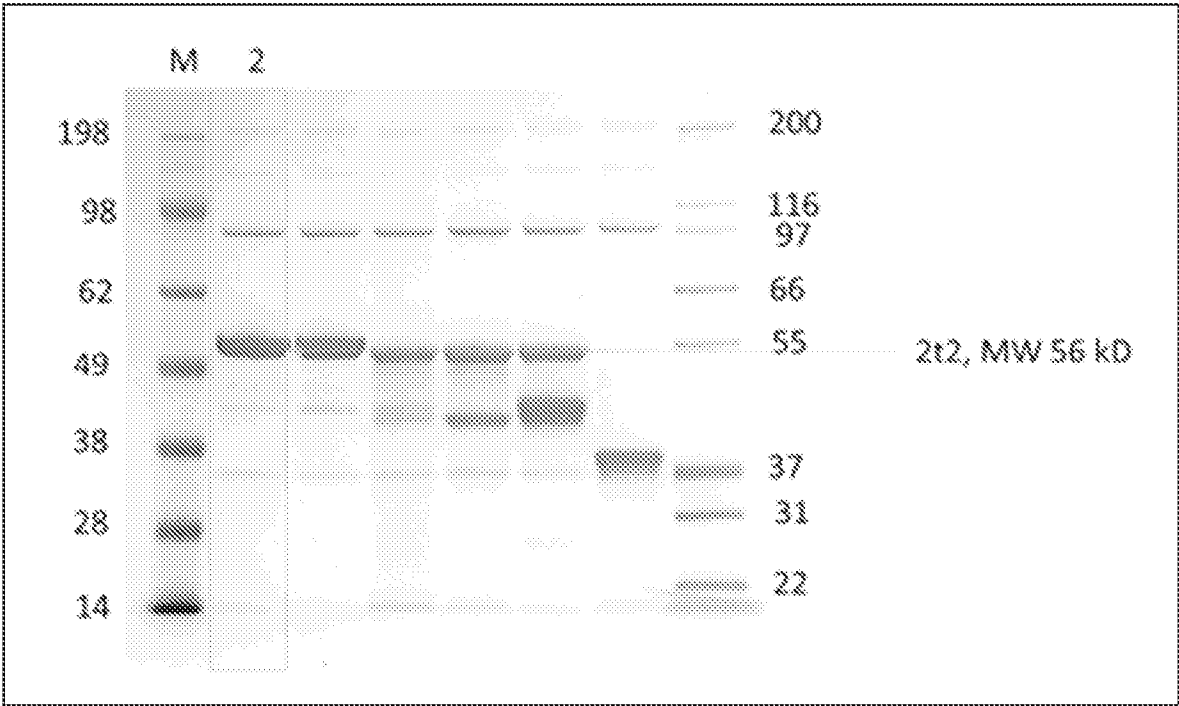


Figure 72B

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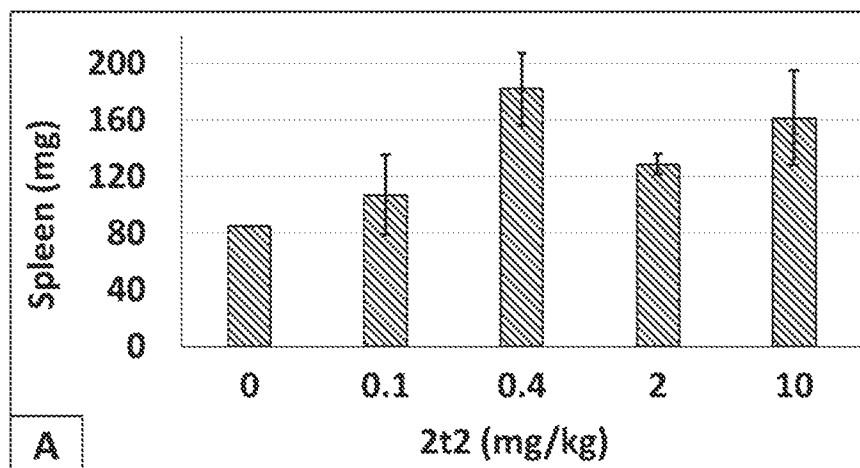


Figure 73A

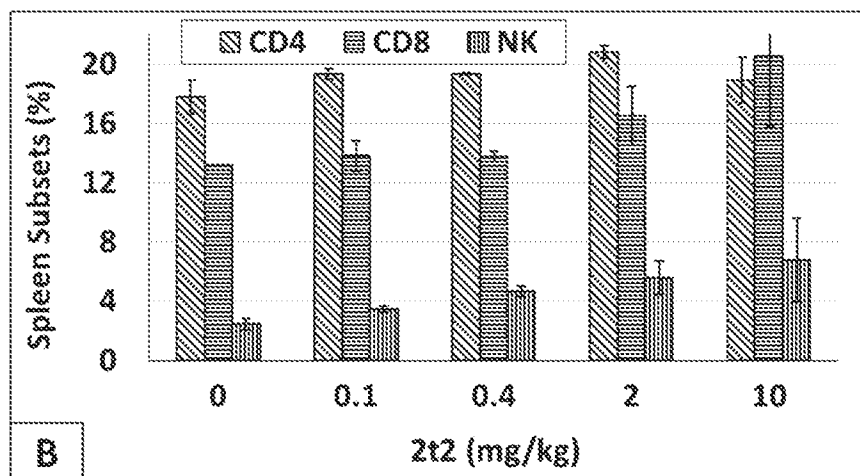


Figure 73B

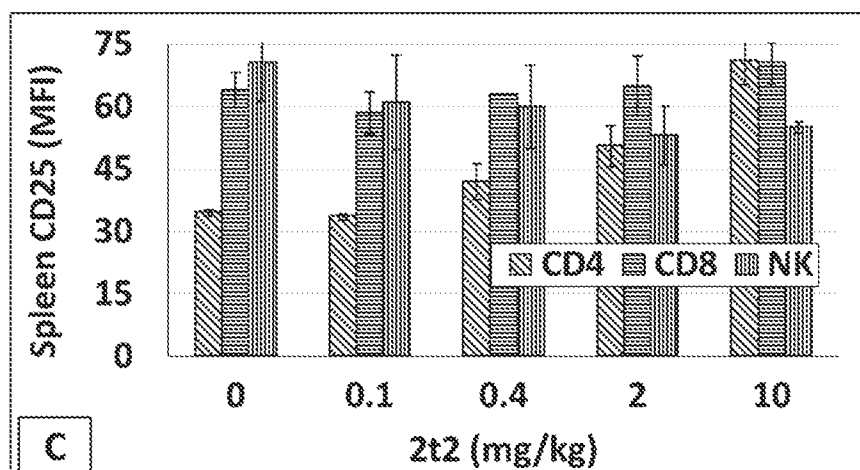


Figure 74

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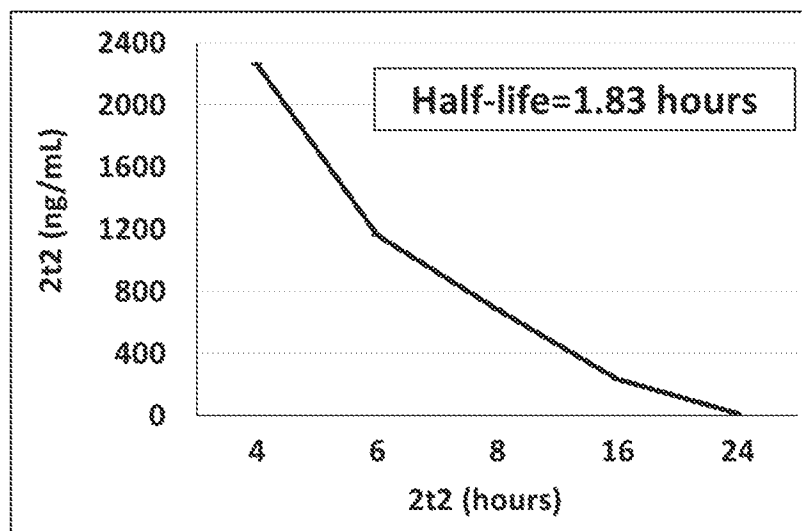


Figure 75

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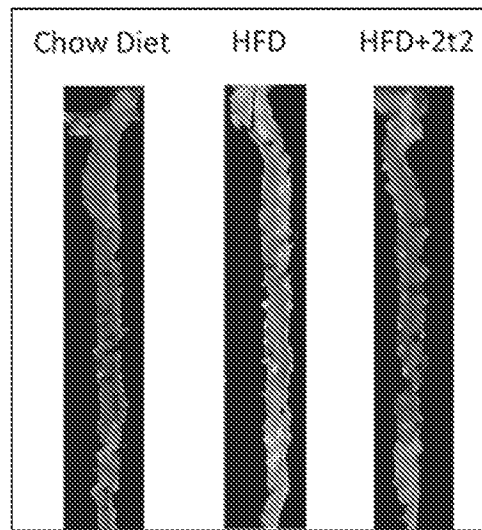


Figure 76A

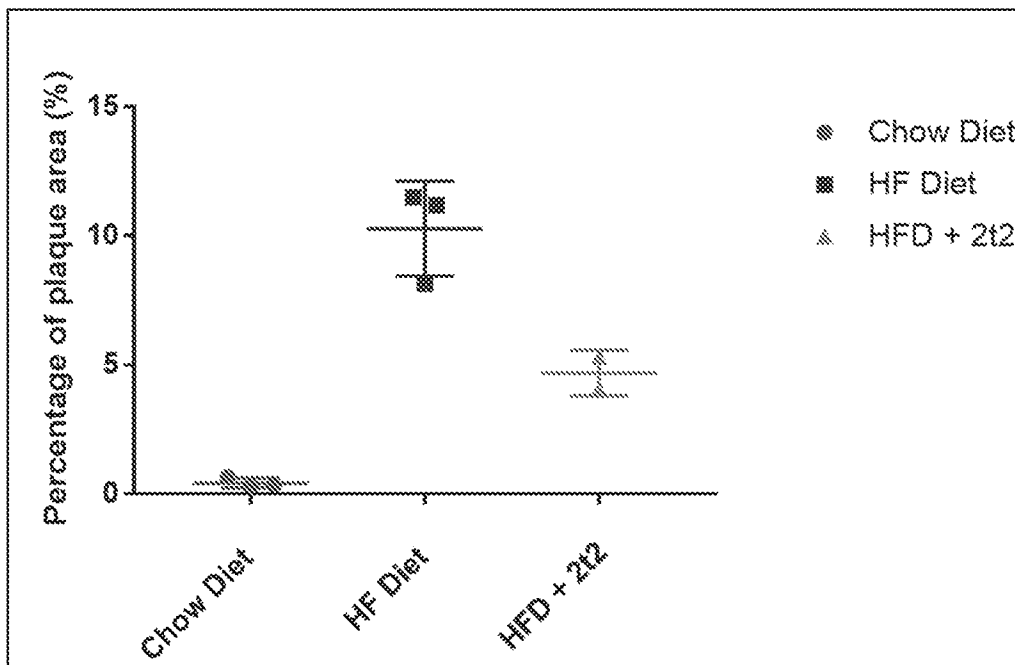


Figure 76B

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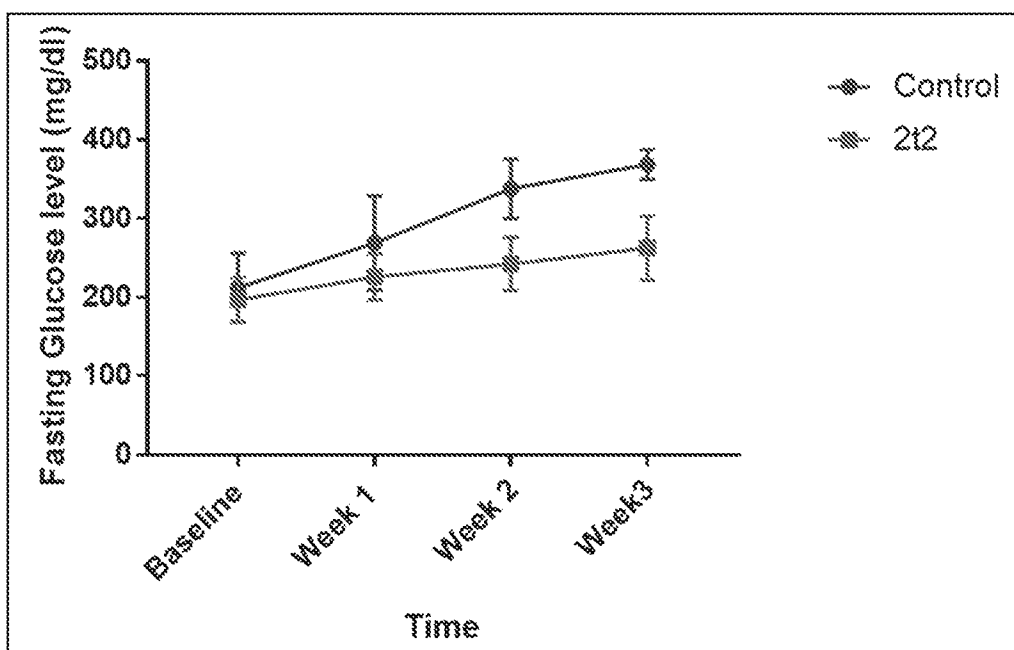


Figure 77

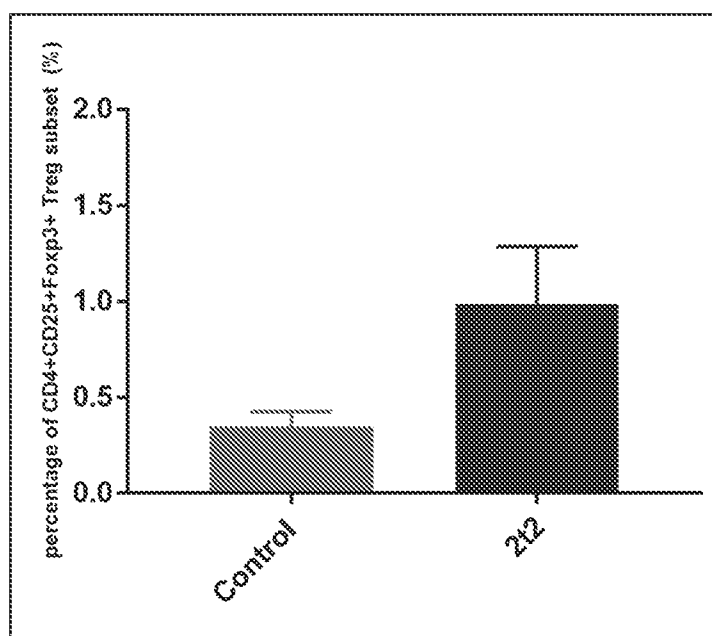


Figure 78

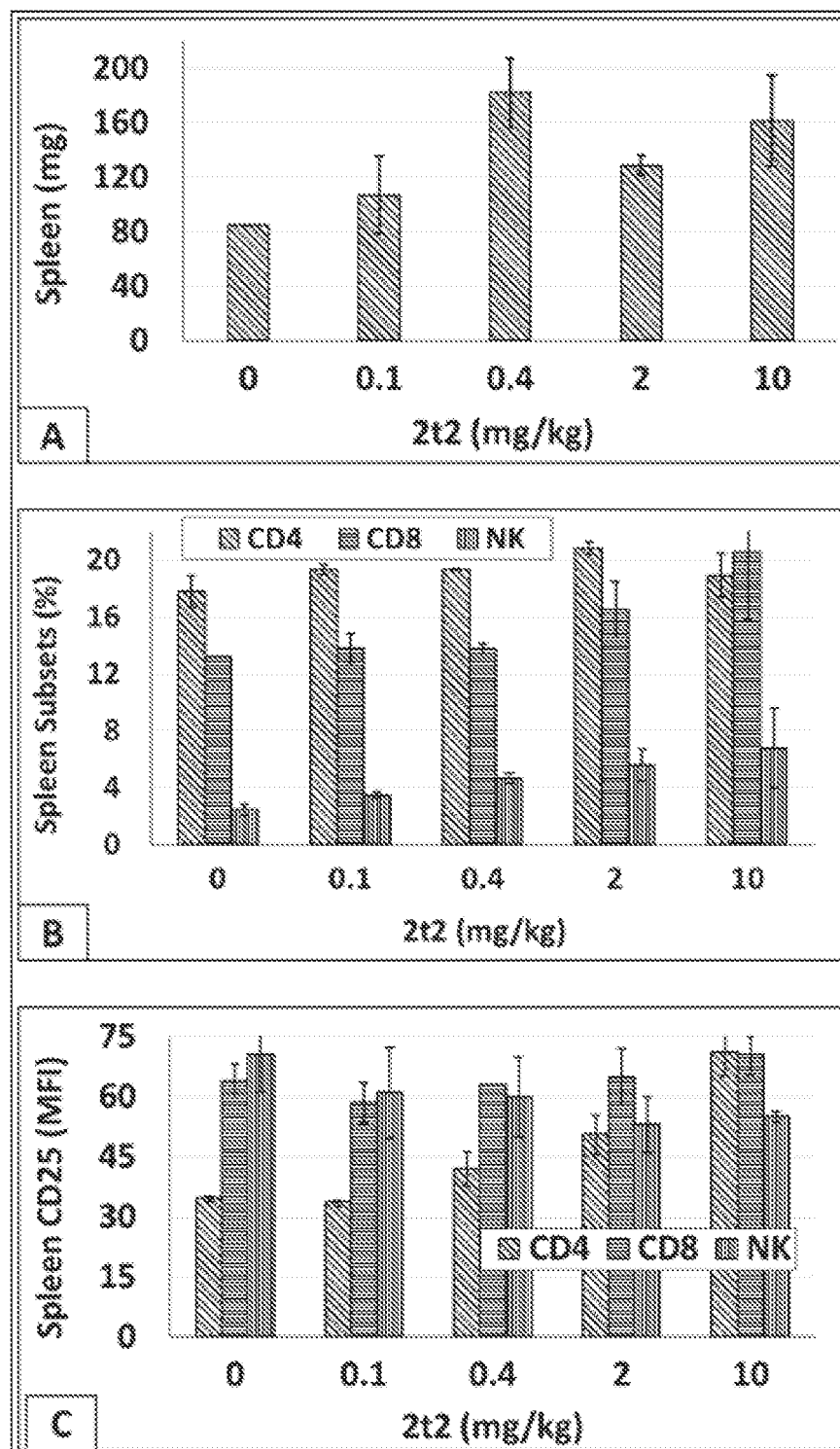


Figure 79

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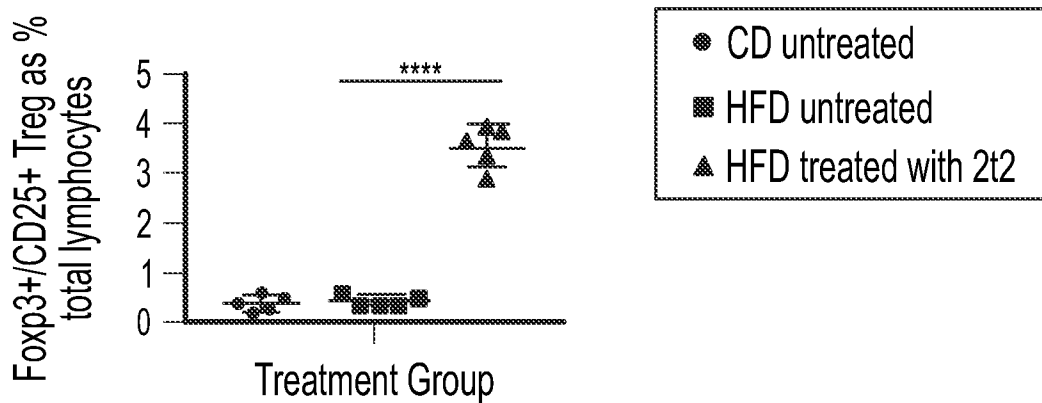


Figure 80A

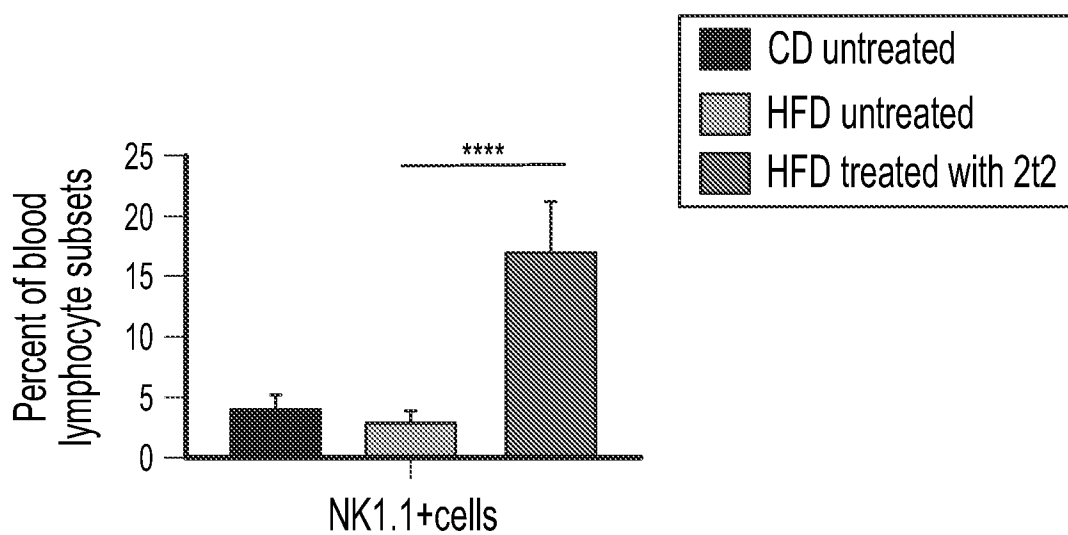


Figure 80B

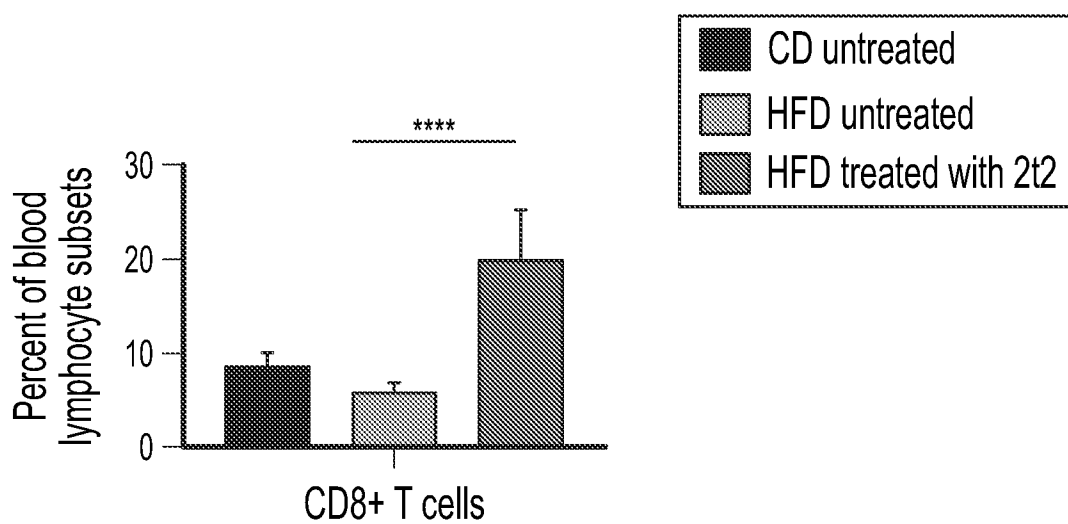


Figure 80C

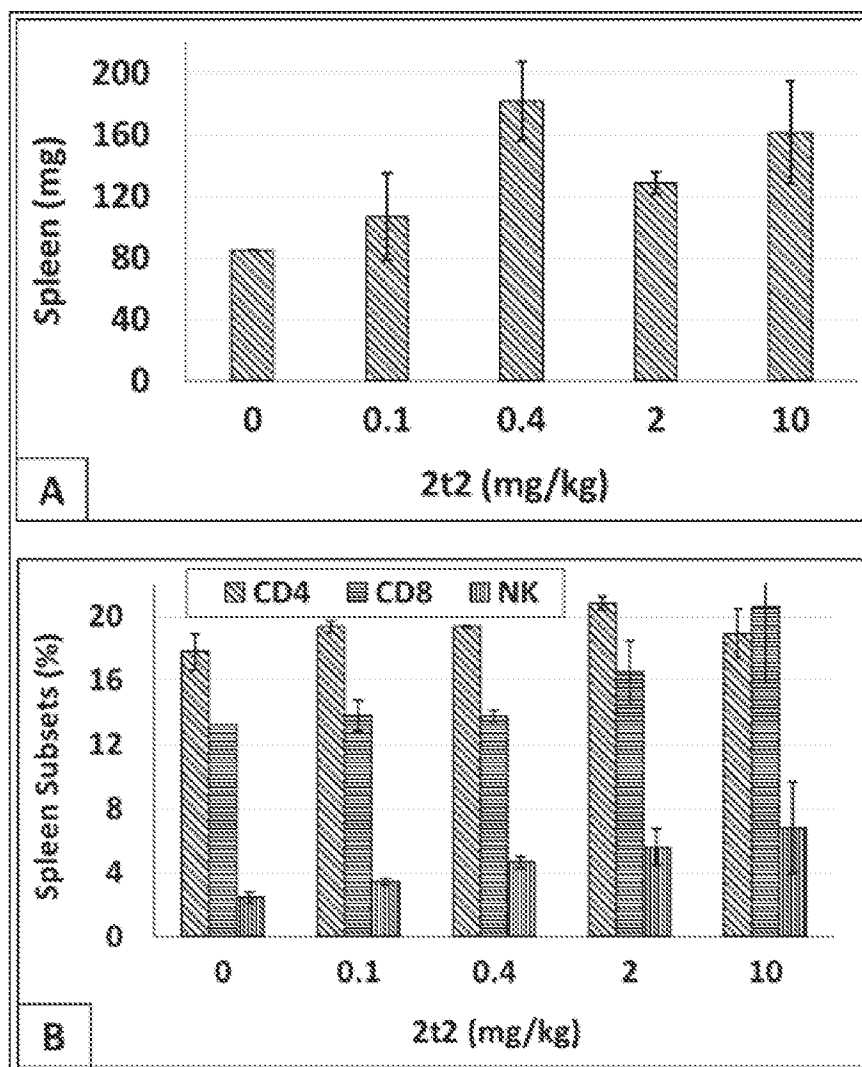


Figure 81

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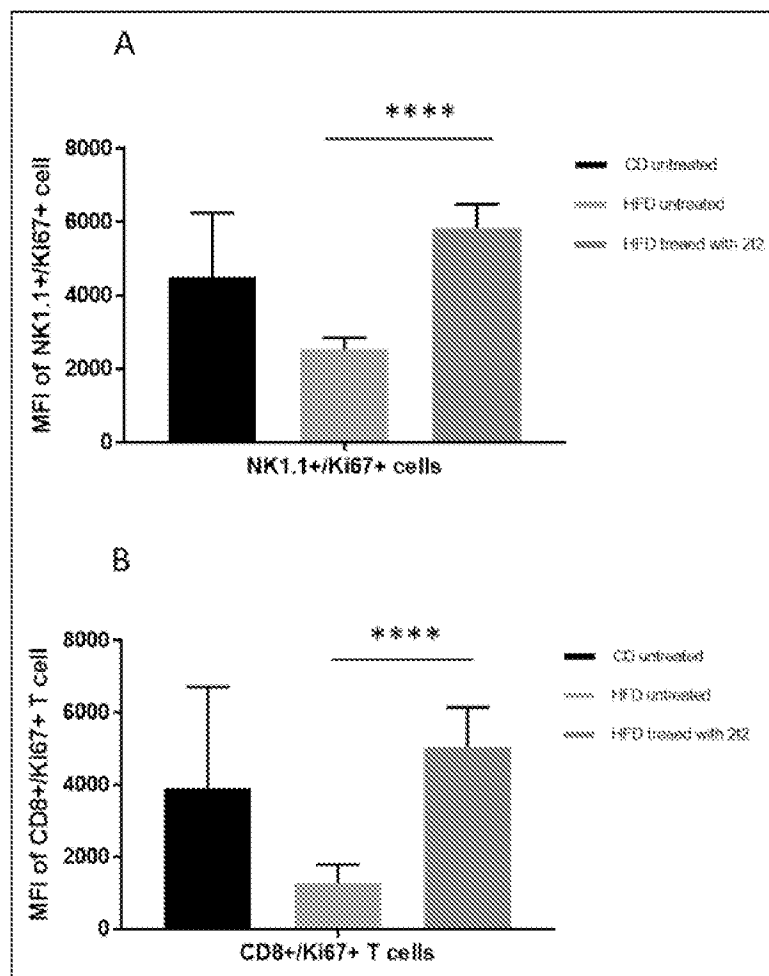


Figure 82

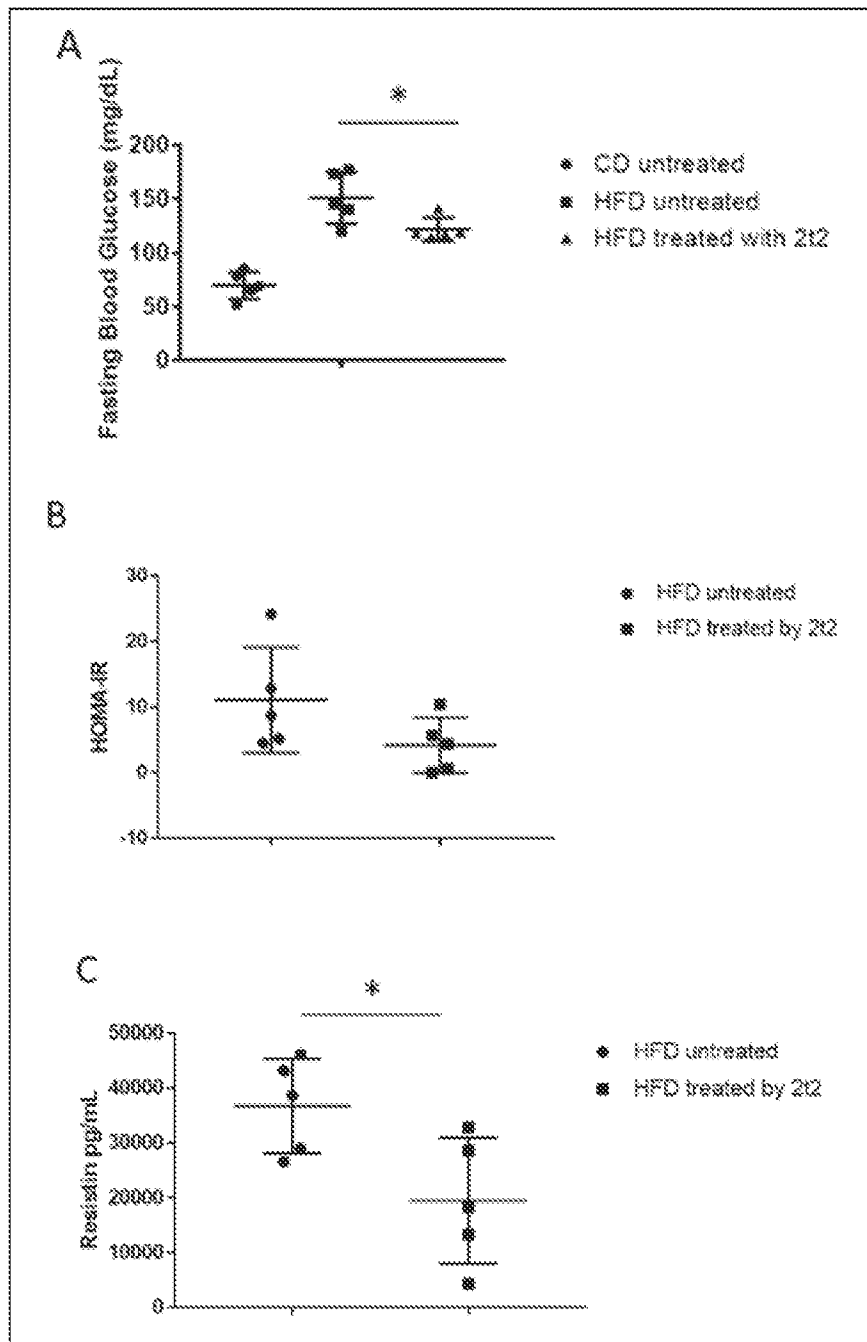


Figure 83

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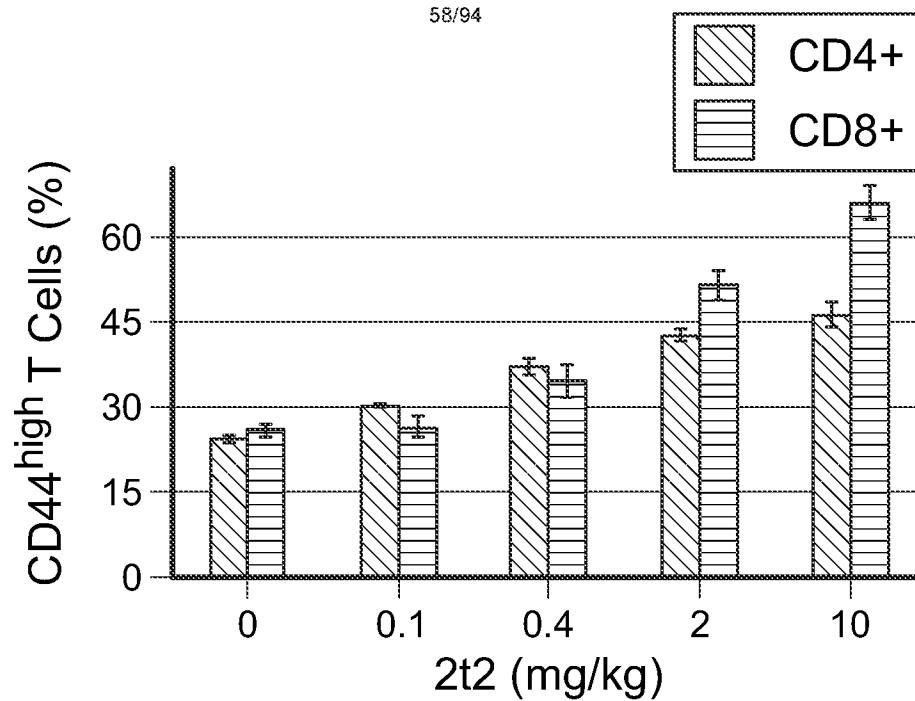


Figure 84

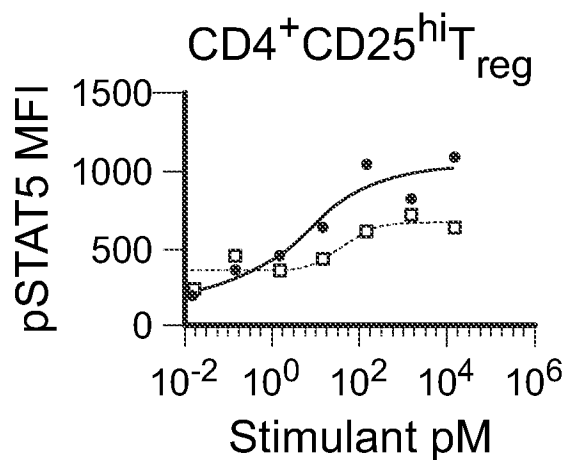
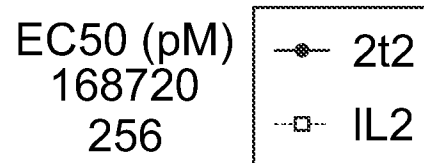
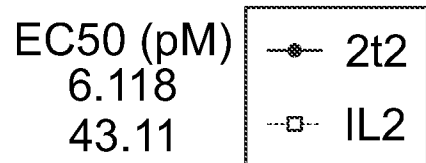


Figure 85A

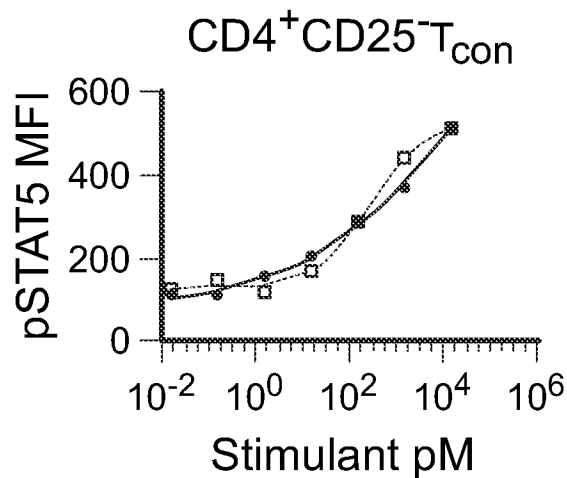


Figure 85B

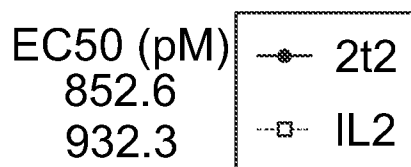
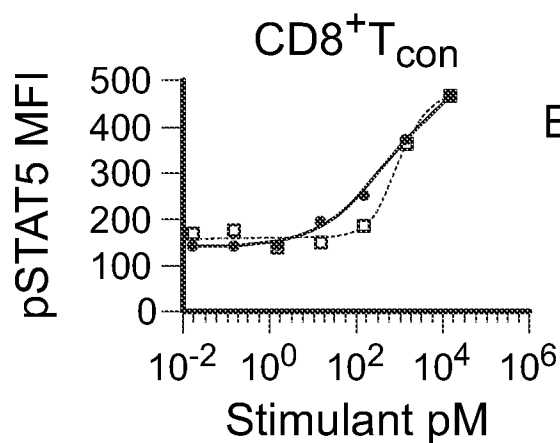


Figure 85C

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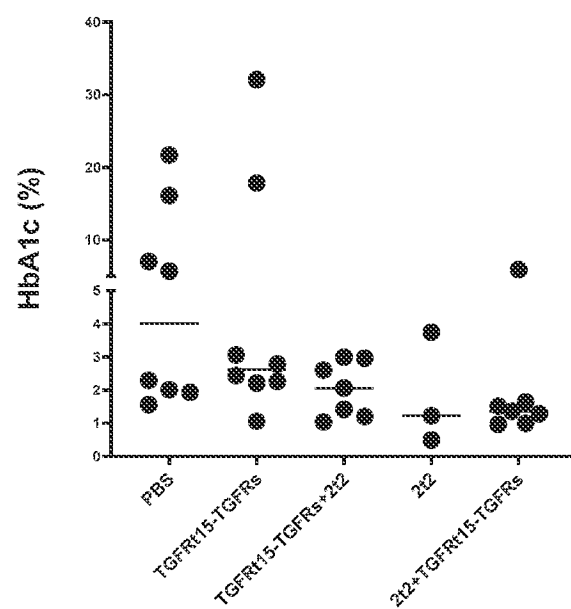


Figure 86

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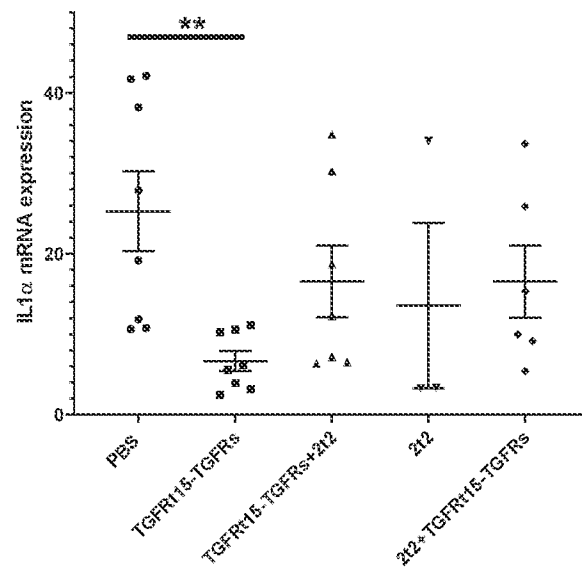


Figure 87A

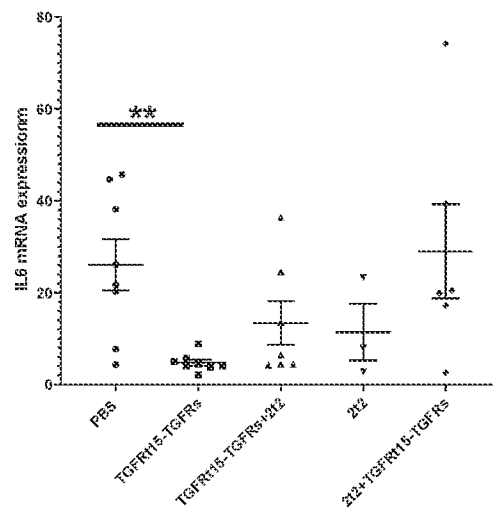


Figure 87B

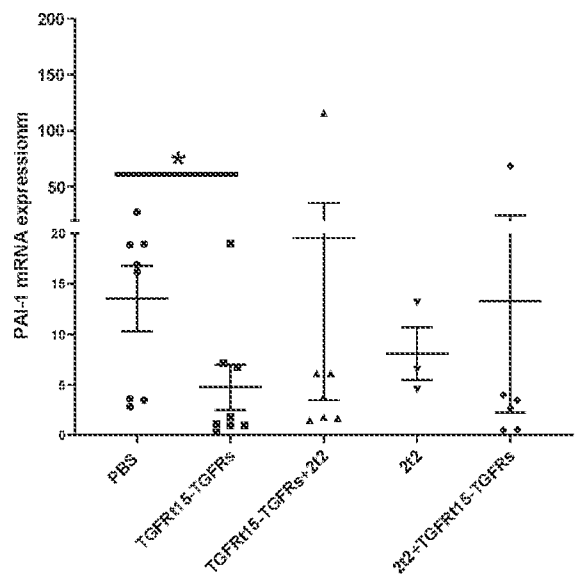


Figure 87C

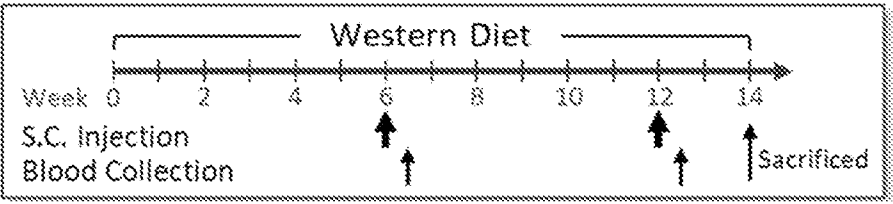


Figure 88A

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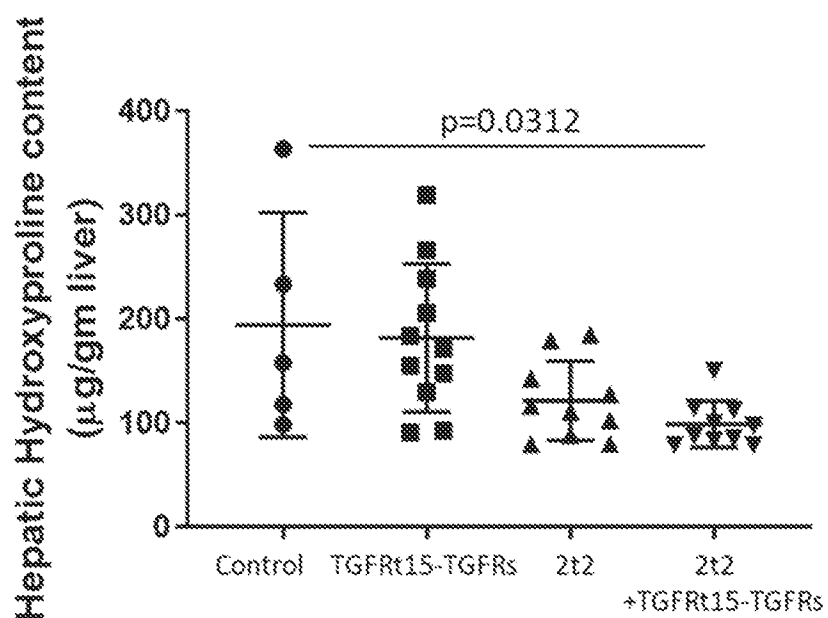


Figure 88B

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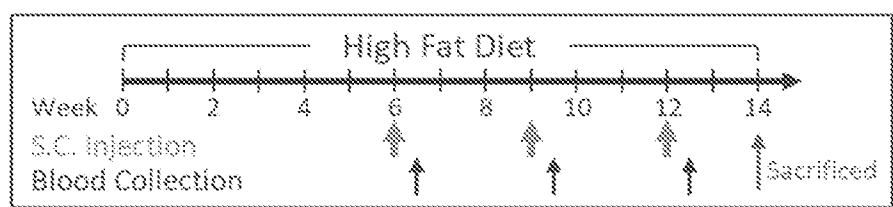


Figure 89A

Plasma Cytokine	Untreated Plasma Level (pg/ml) (M±SEM)	2t2 Treated Plasma Level (pg/ml) (M±SEM)	P value
IL-1β	21.75 ± 5.73	4.94 ± 2.02	0.03
GM-CSF	16.62 ± 3.5	29.88 ± 5.86	0.07
IL-6	26.33 ± 8.52	14.15 ± 2.60	0.25
IL-10	57.55 ± 28.29	19.68 ± 9.10	0.24
MCP-1	210.8 ± 33.42	109.2 ± 24.46	0.03
TNF-α	31.25 ± 12.65	13.56 ± 1.735	0.24

Figure 89B

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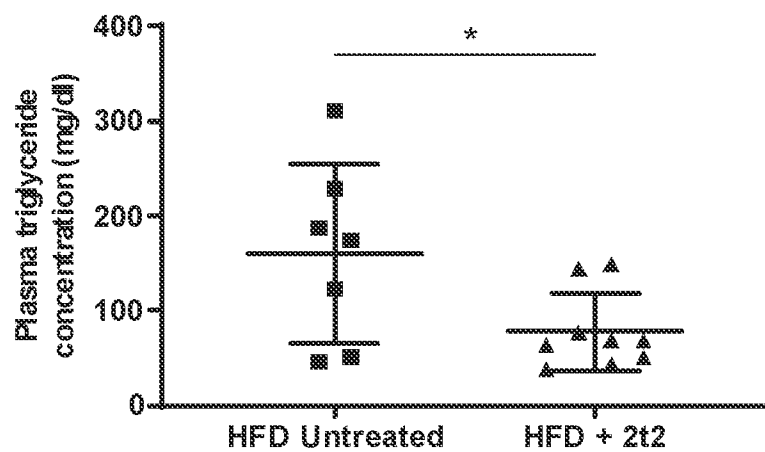


Figure 90

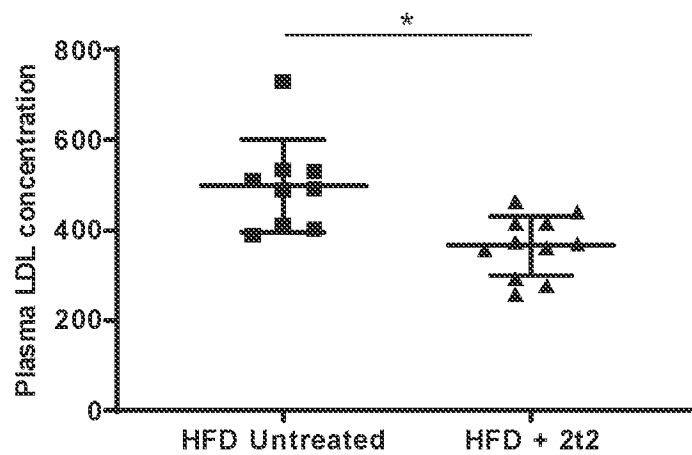


Figure 91

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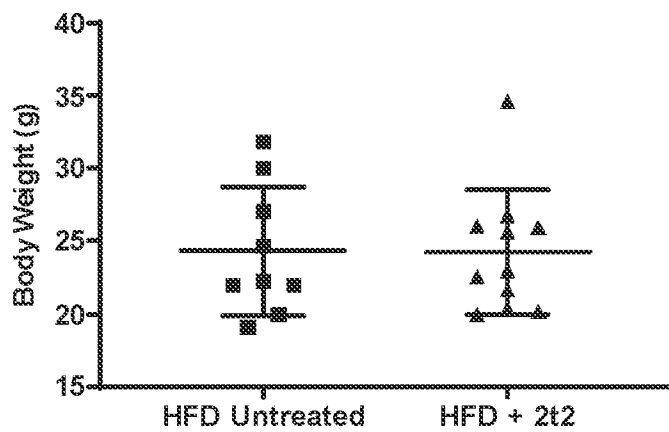
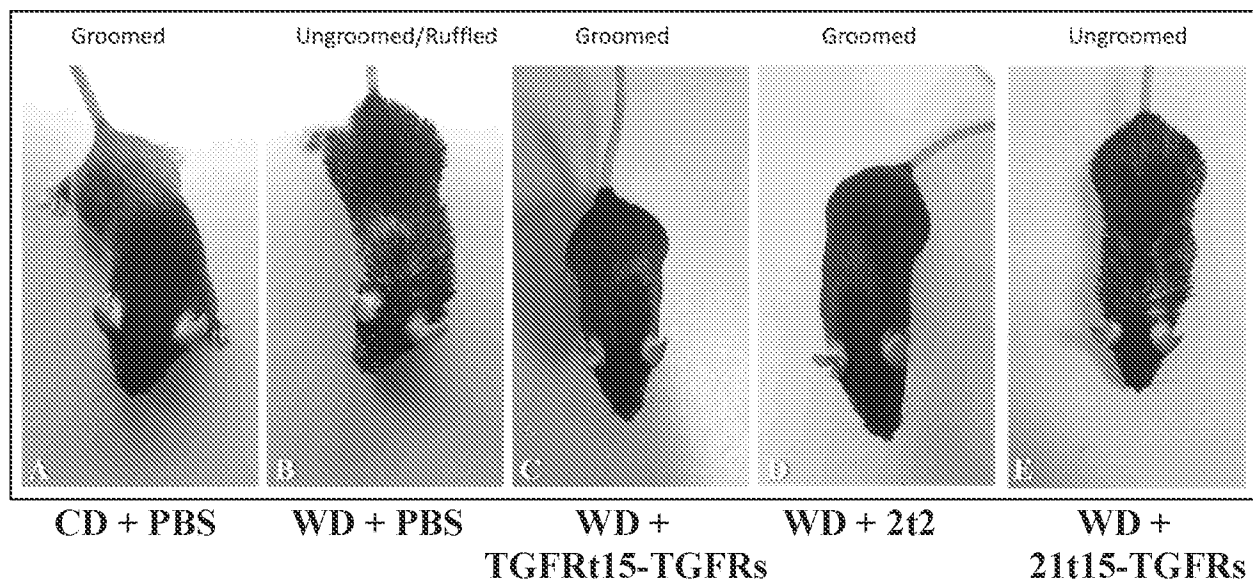
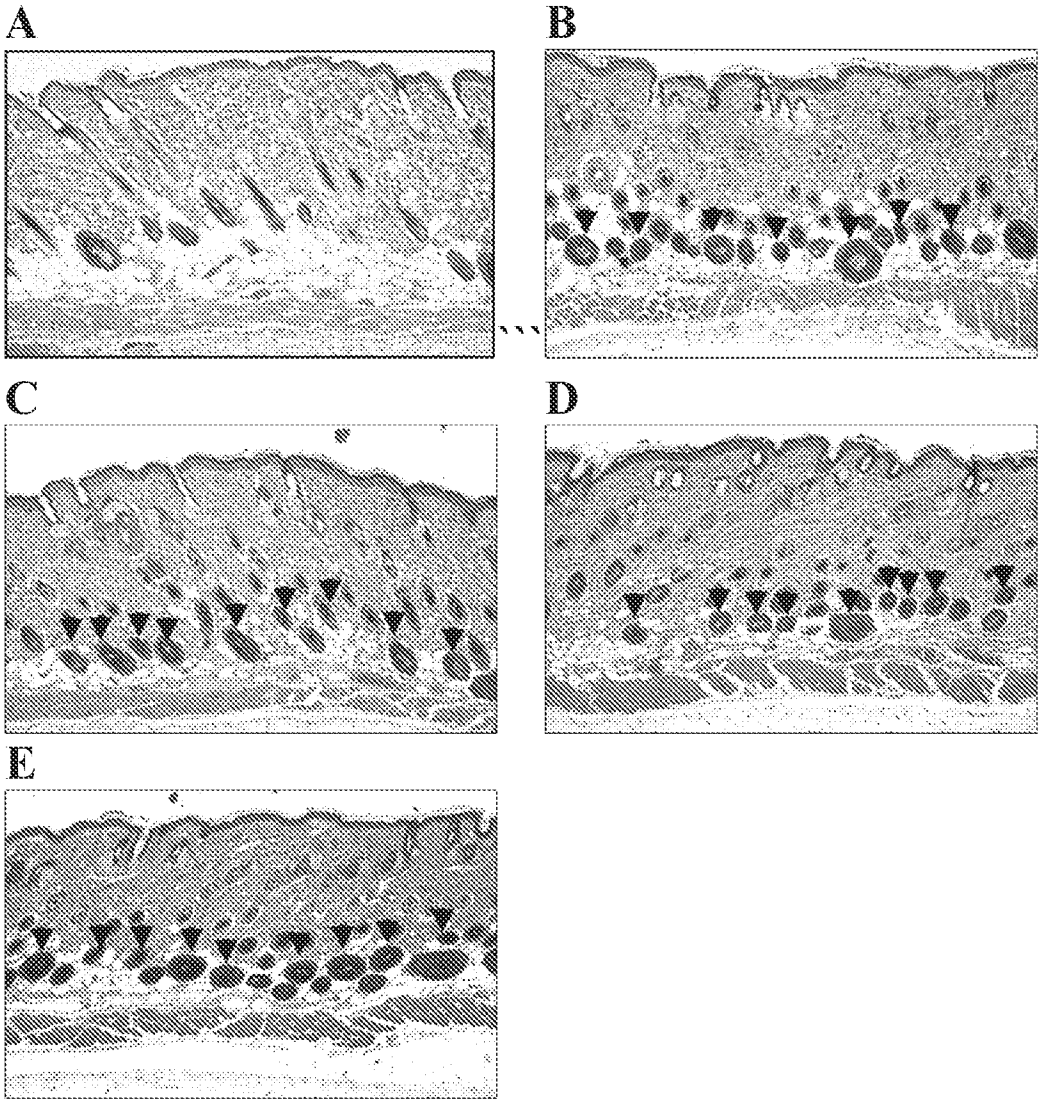


Figure 92



Figures 93A-93E



Figures 94A-94E

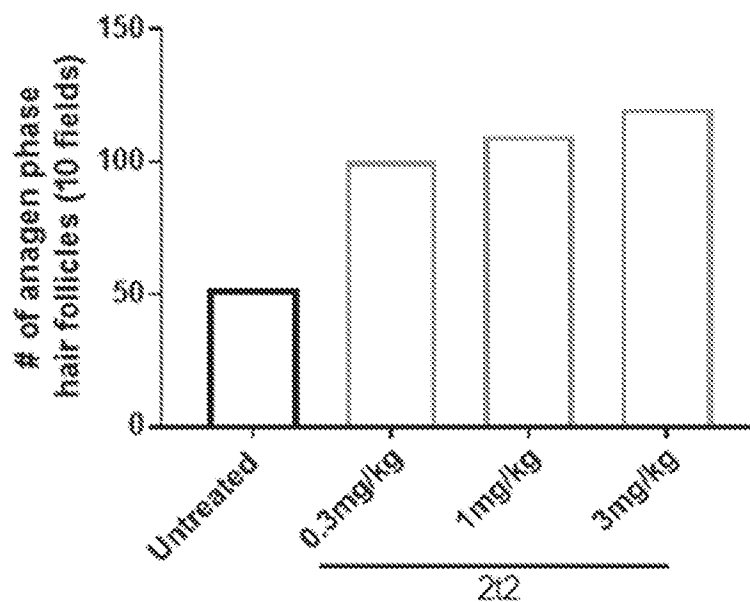


Figure 95

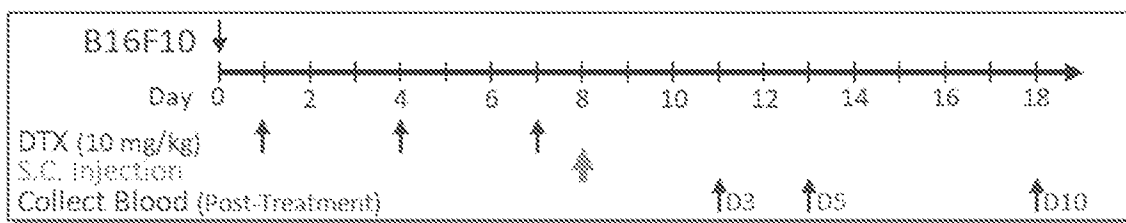


Figure 96

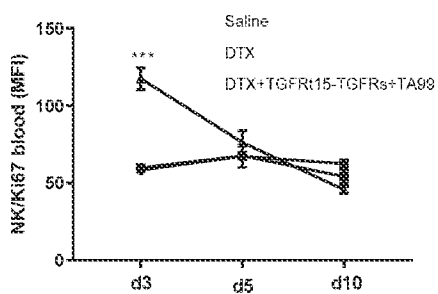


Figure 97A

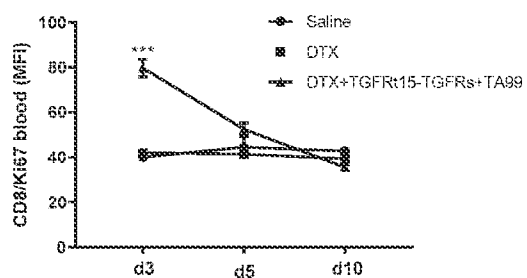


Figure 97B

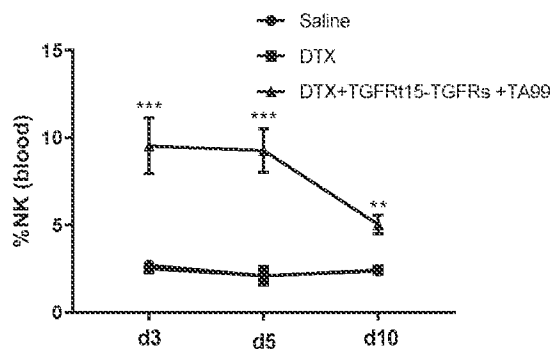


Figure 97C

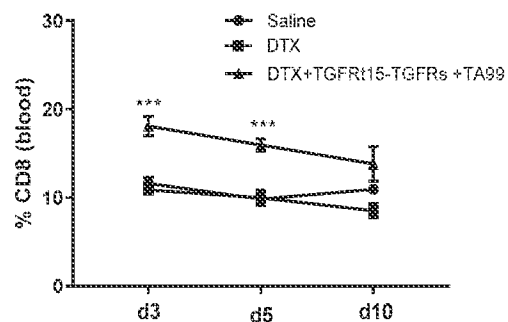


Figure 97D

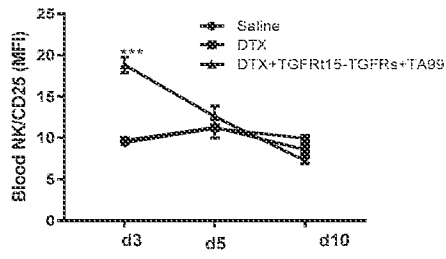


Figure 97E

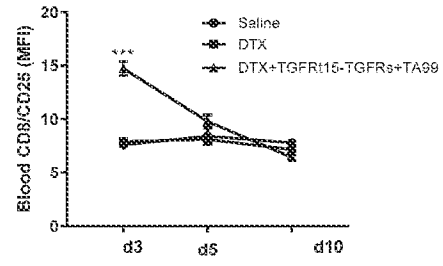


Figure 97F

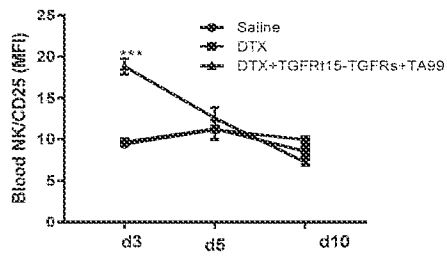


Figure 97G

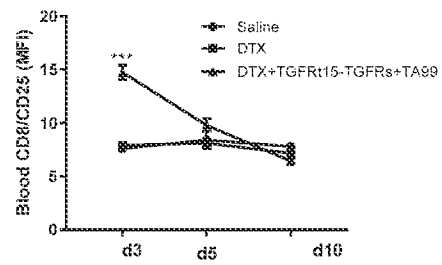


Figure 97H

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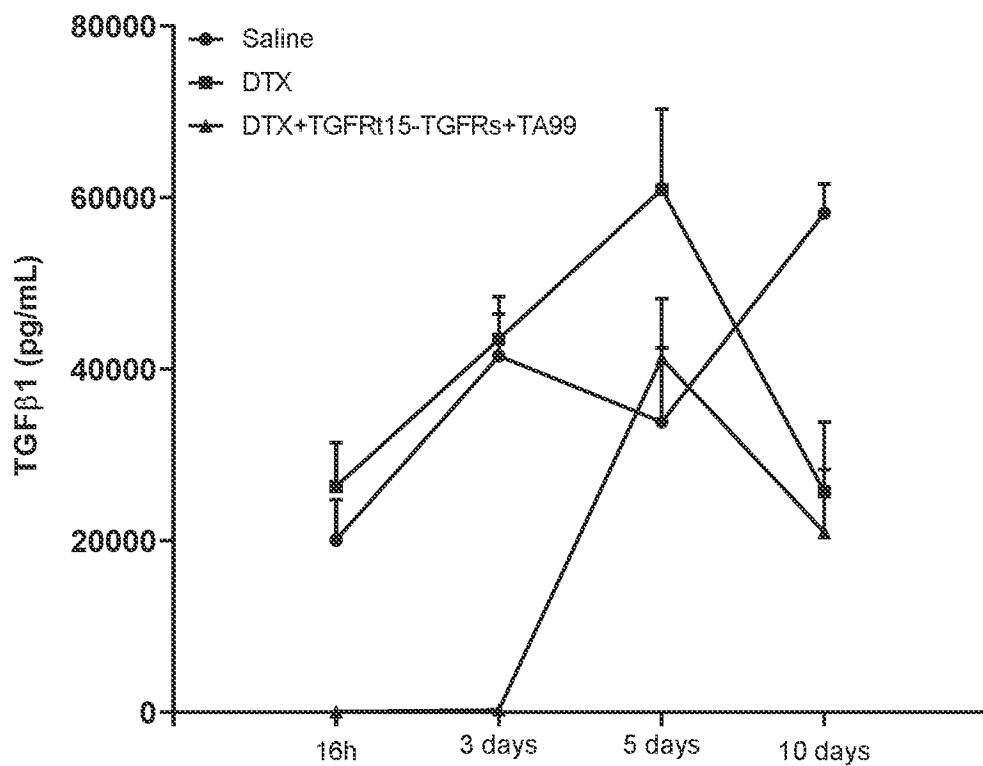


Figure 98A

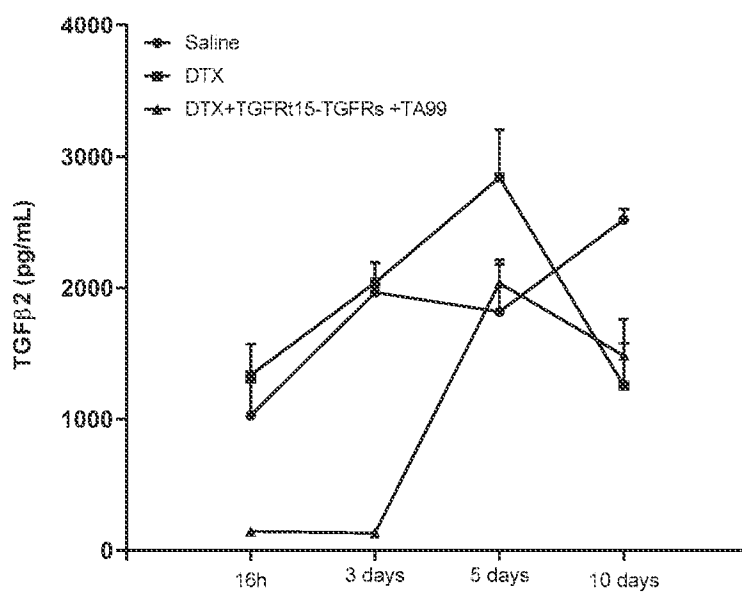


Figure 98B

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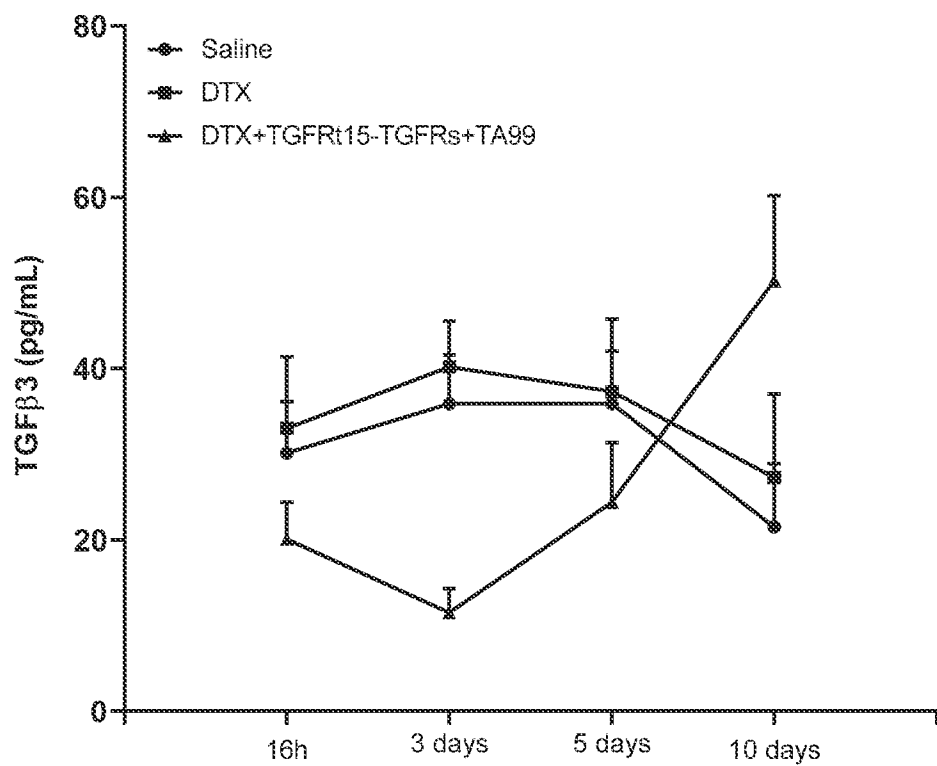


Figure 98C

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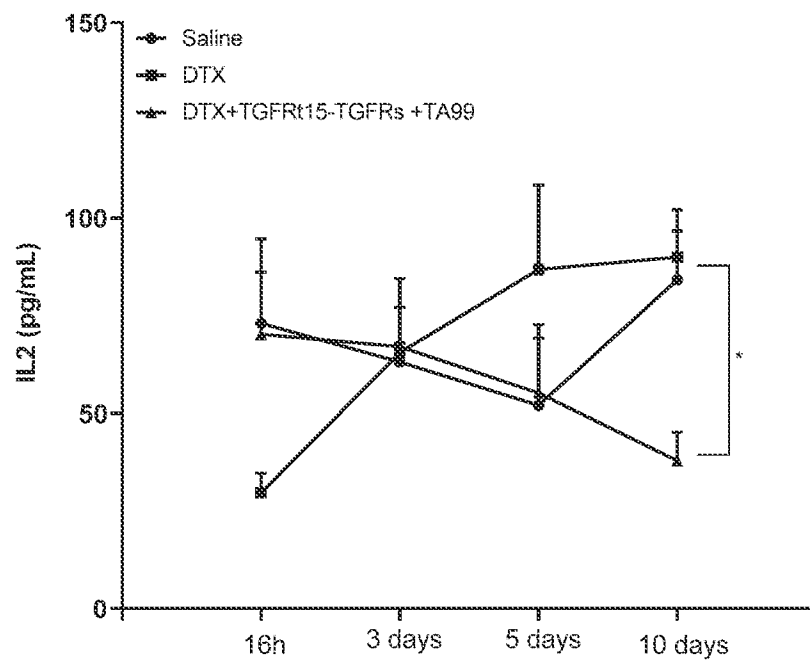


Figure 99A

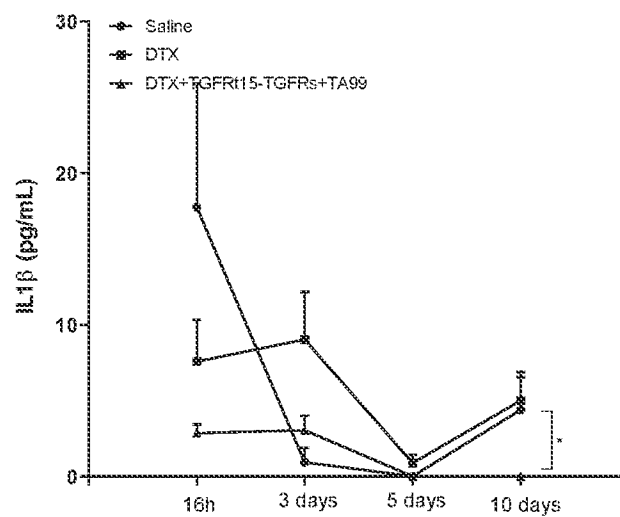


Figure 99B

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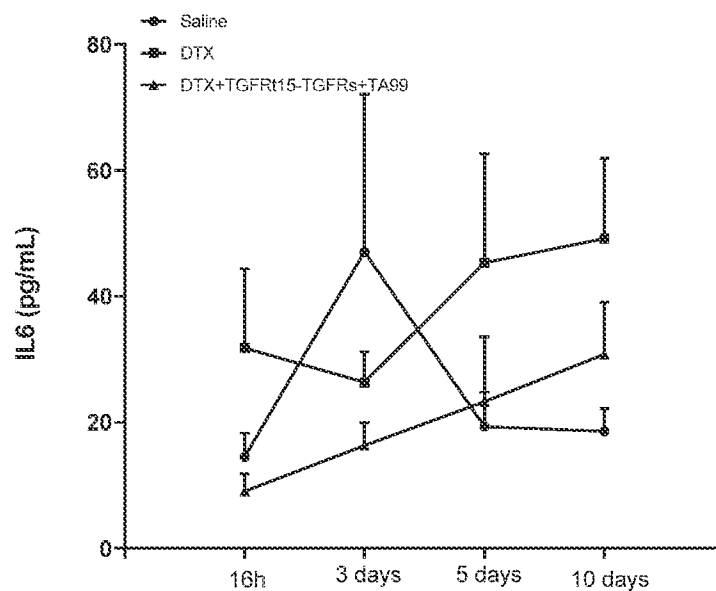


Figure 99C

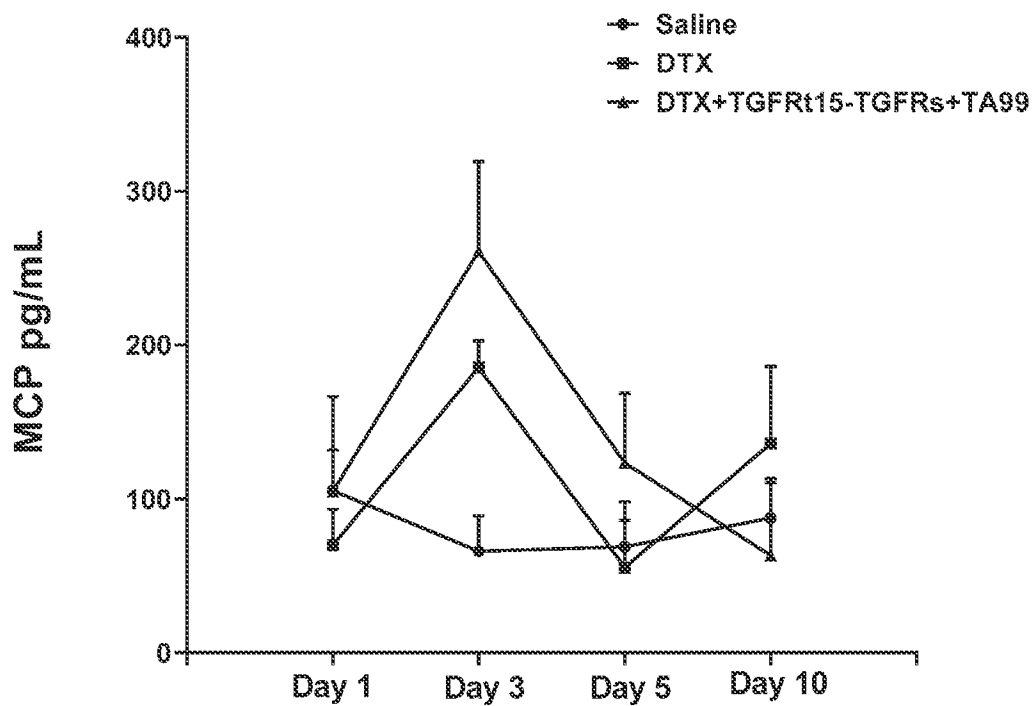


Figure 99D

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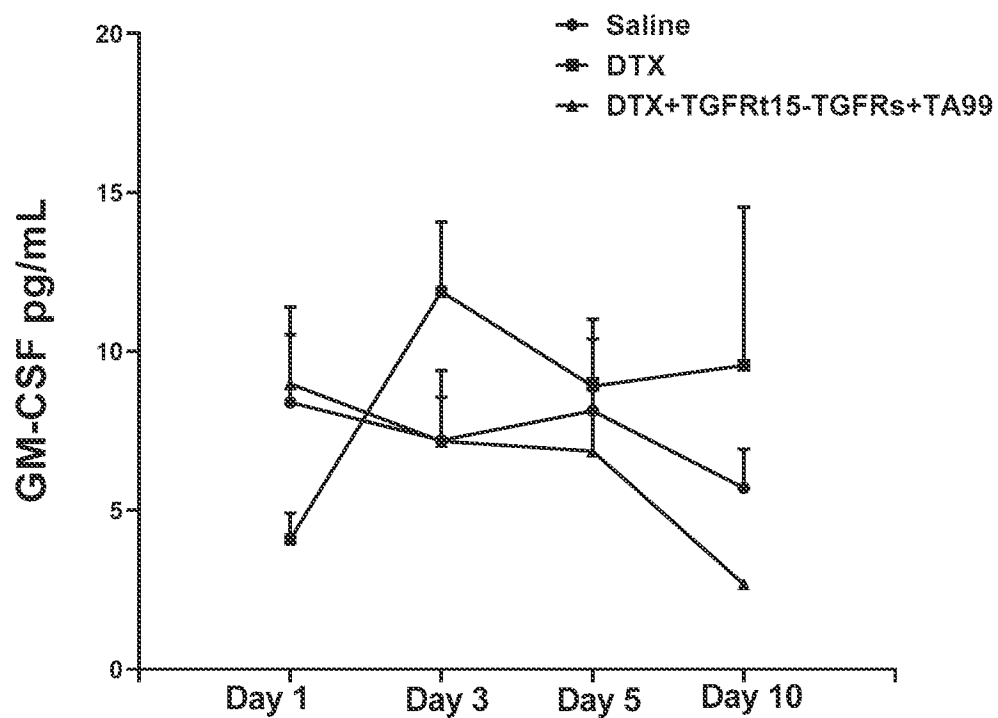


Figure 99E

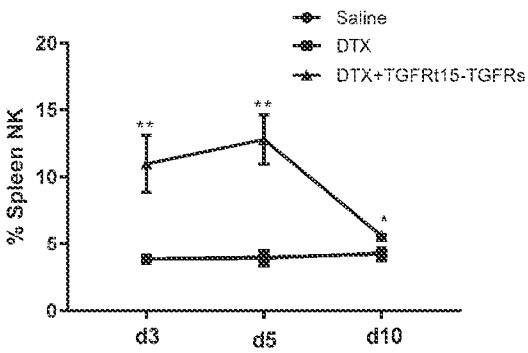


Figure 100A

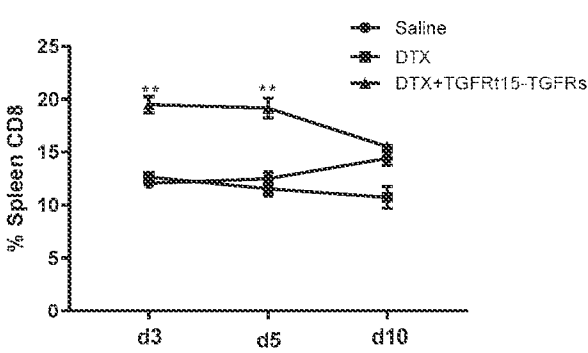


Figure 100B

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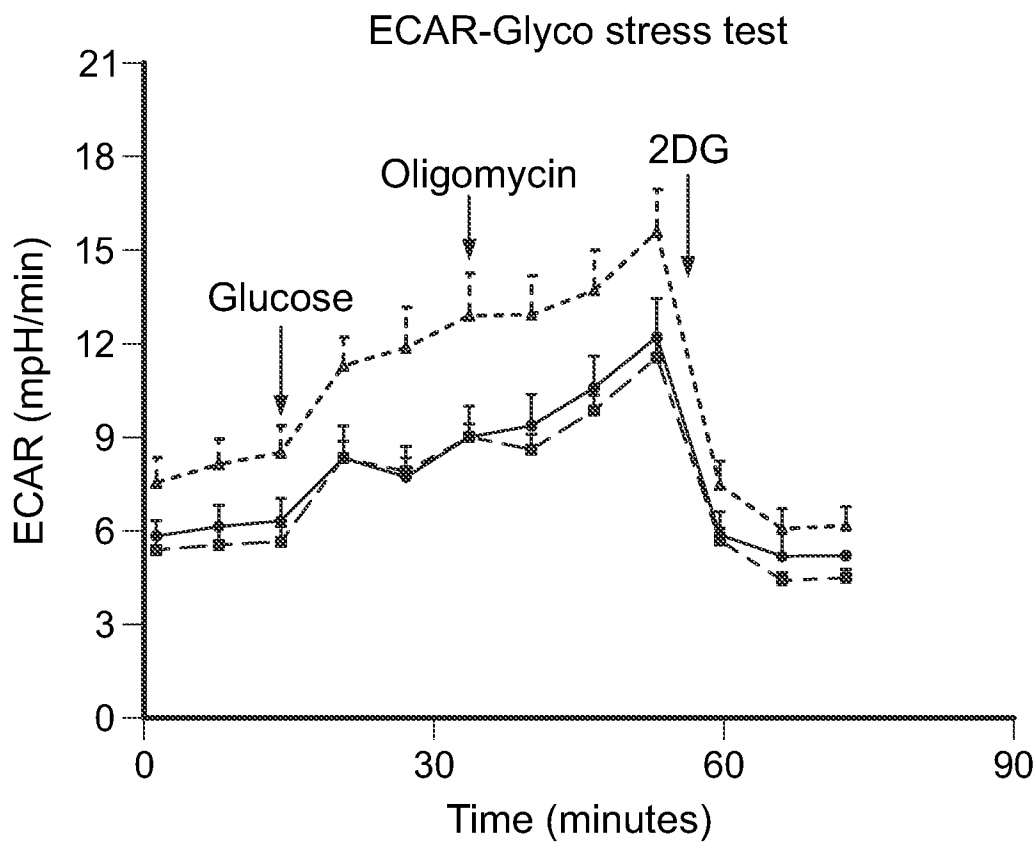


Figure 101A

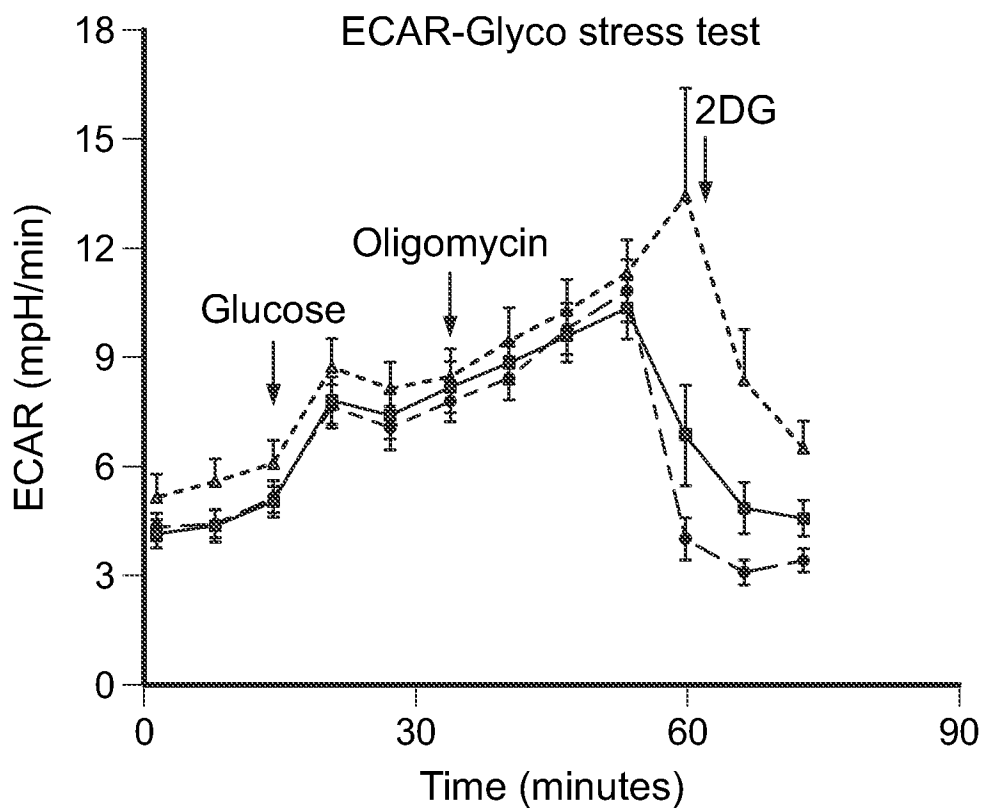


Figure 101B

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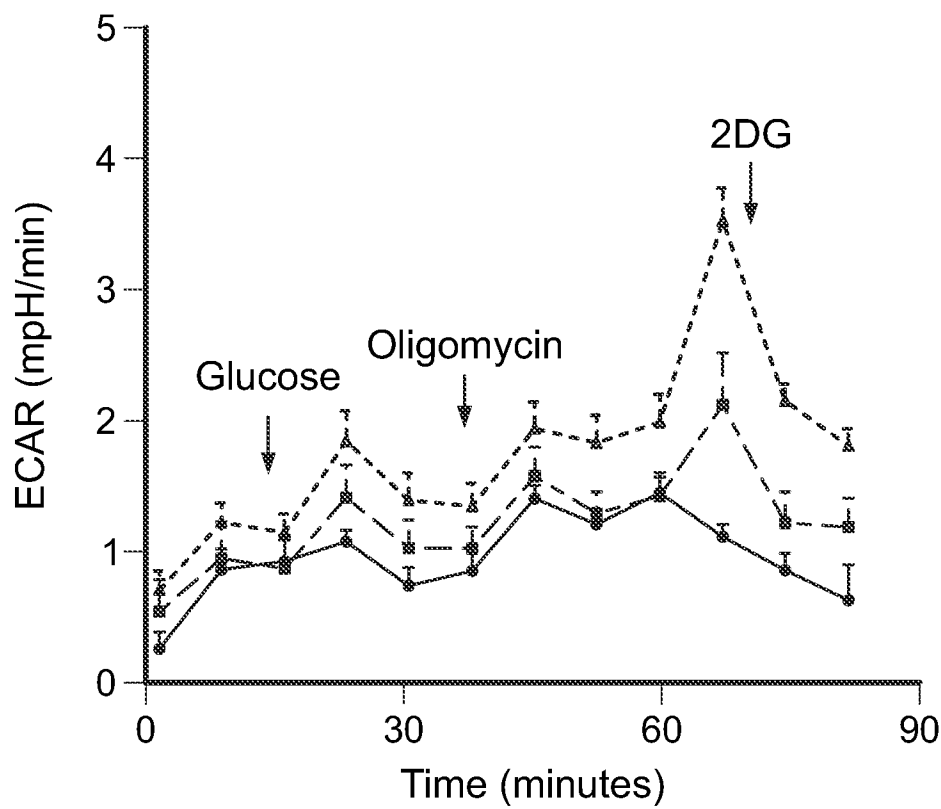


Figure 101C

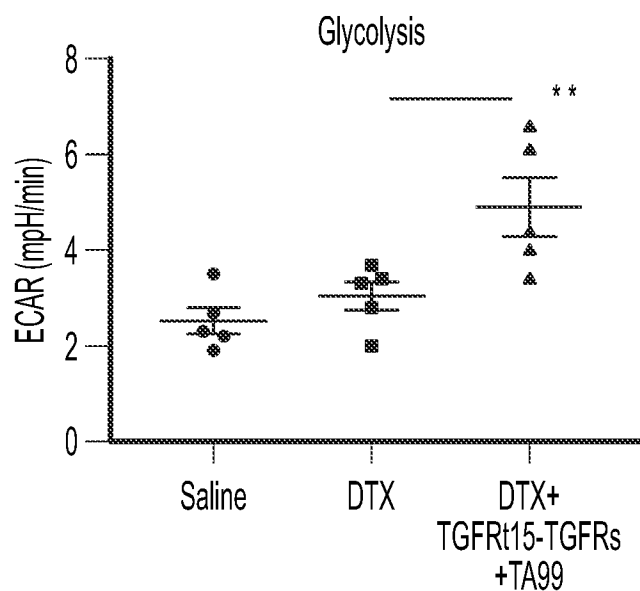


Figure 102A

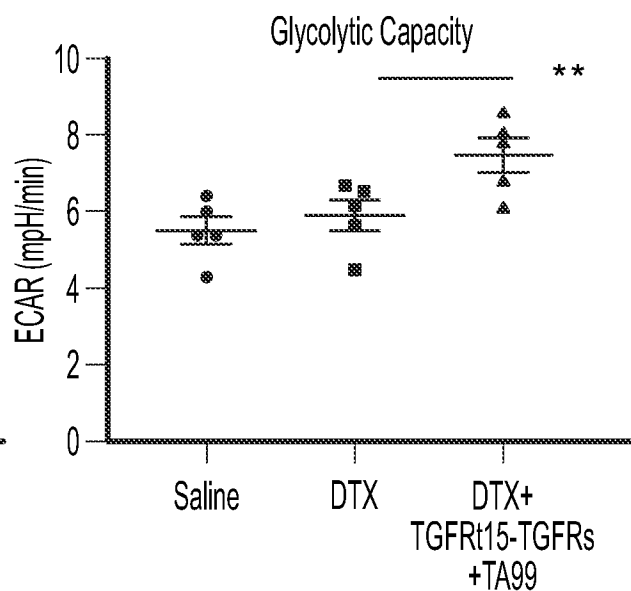


Figure 102B

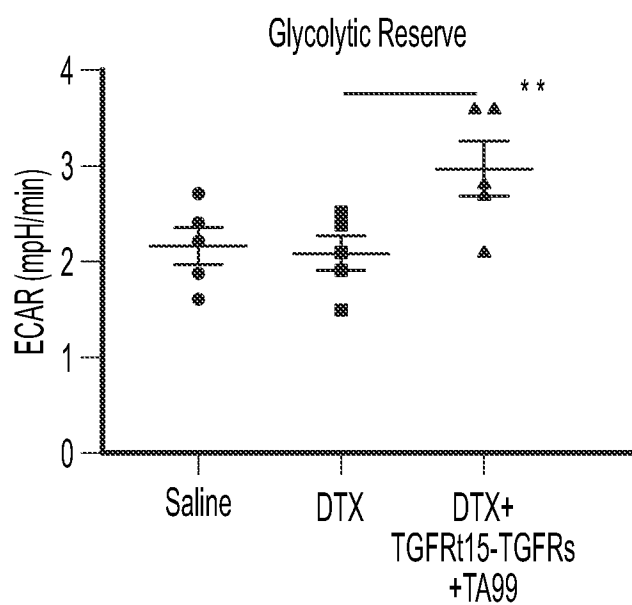


Figure 102C

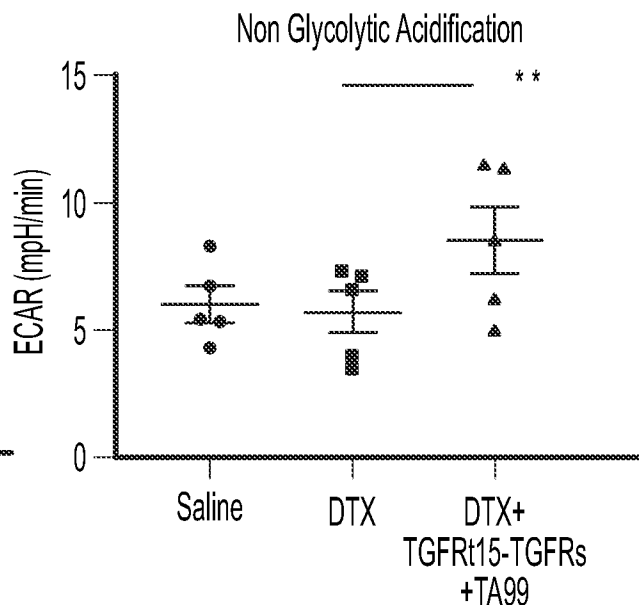


Figure 102D

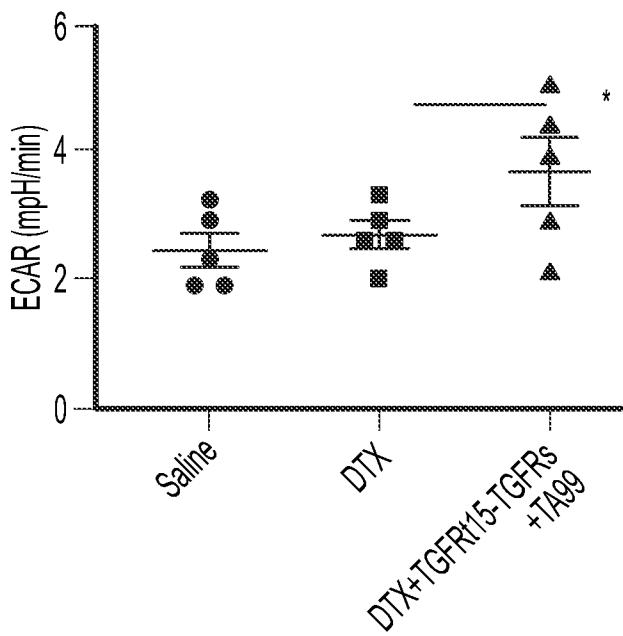


Figure 102E

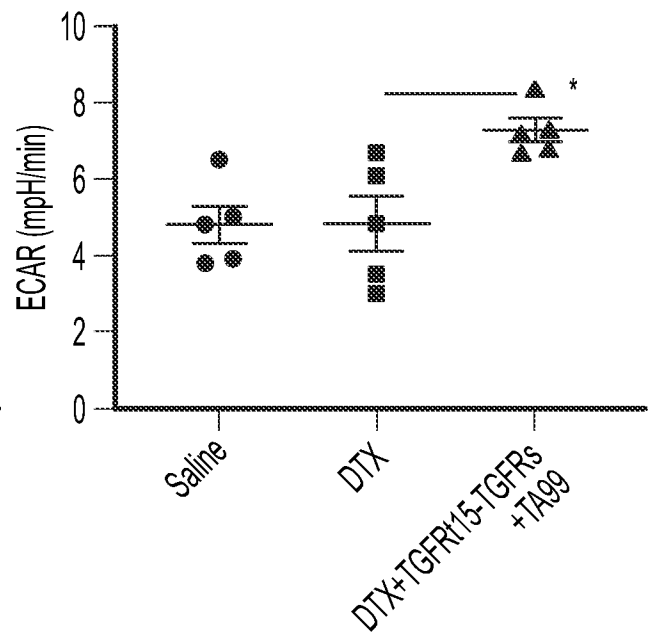


Figure 102F

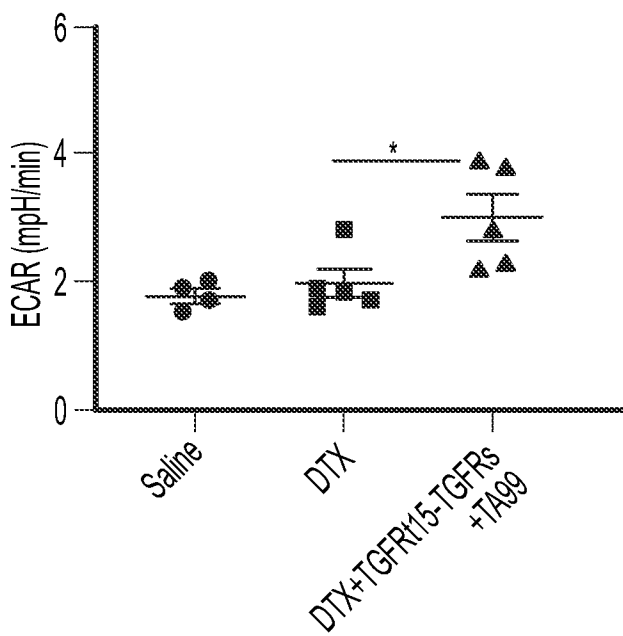


Figure 102G

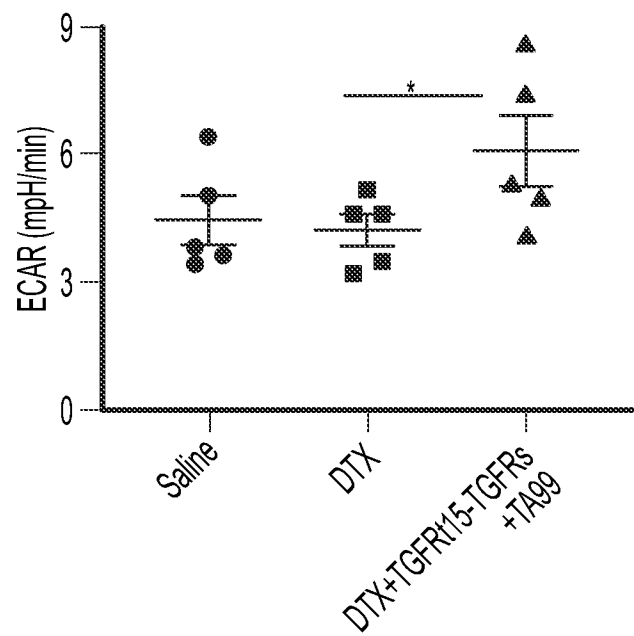


Figure 102H

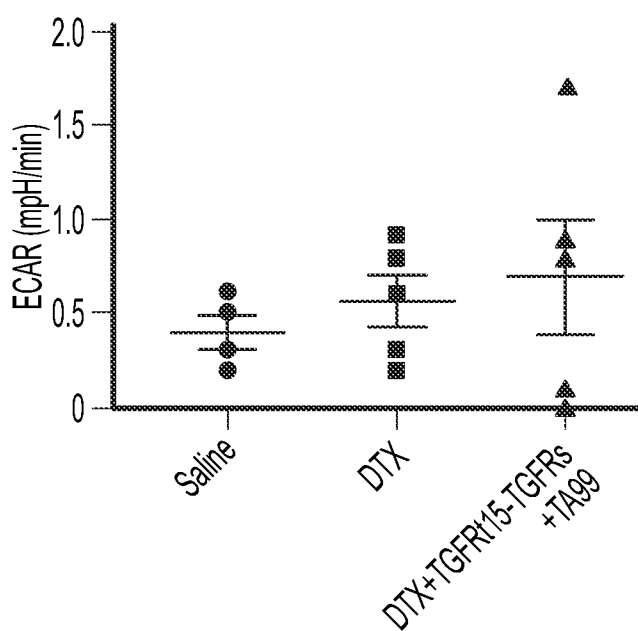


Figure 102I

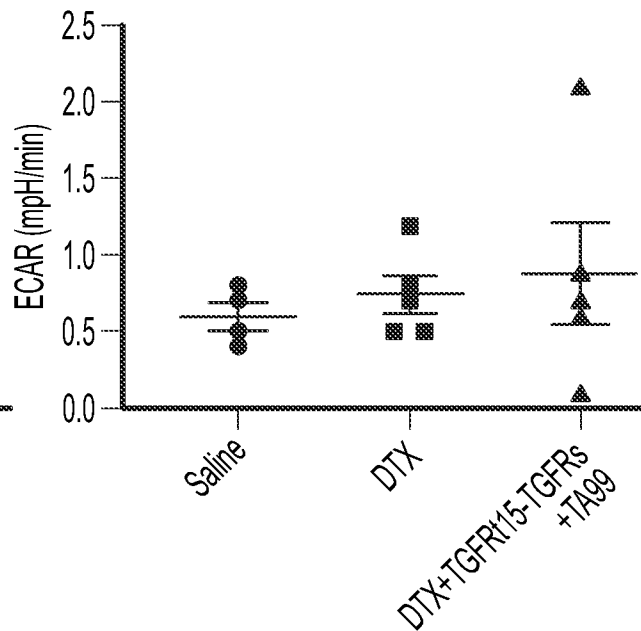


Figure 102J

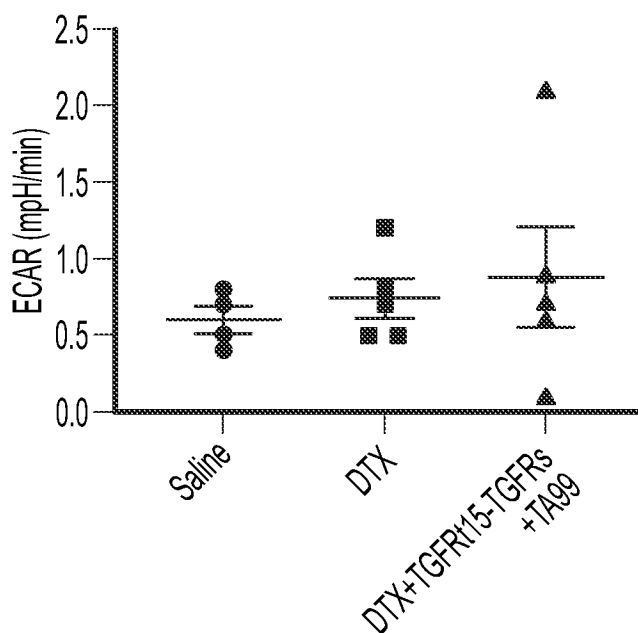


Figure 102K

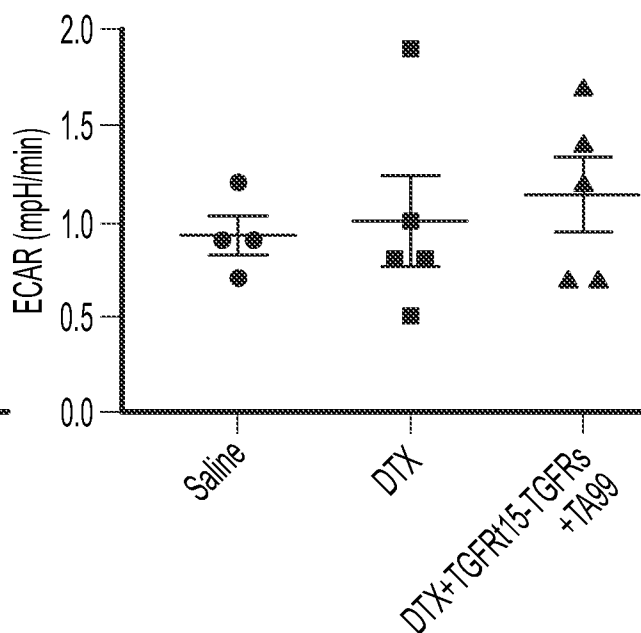


Figure 102L

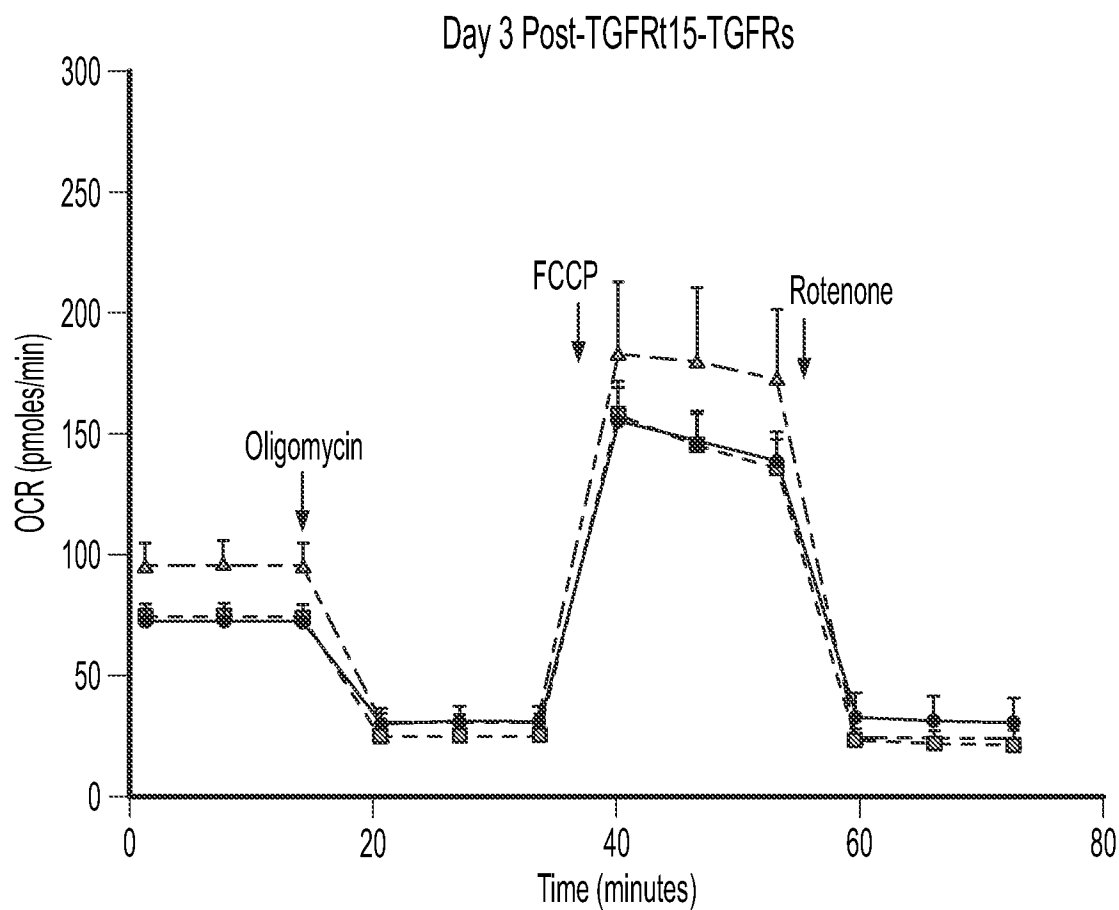


Figure 103A

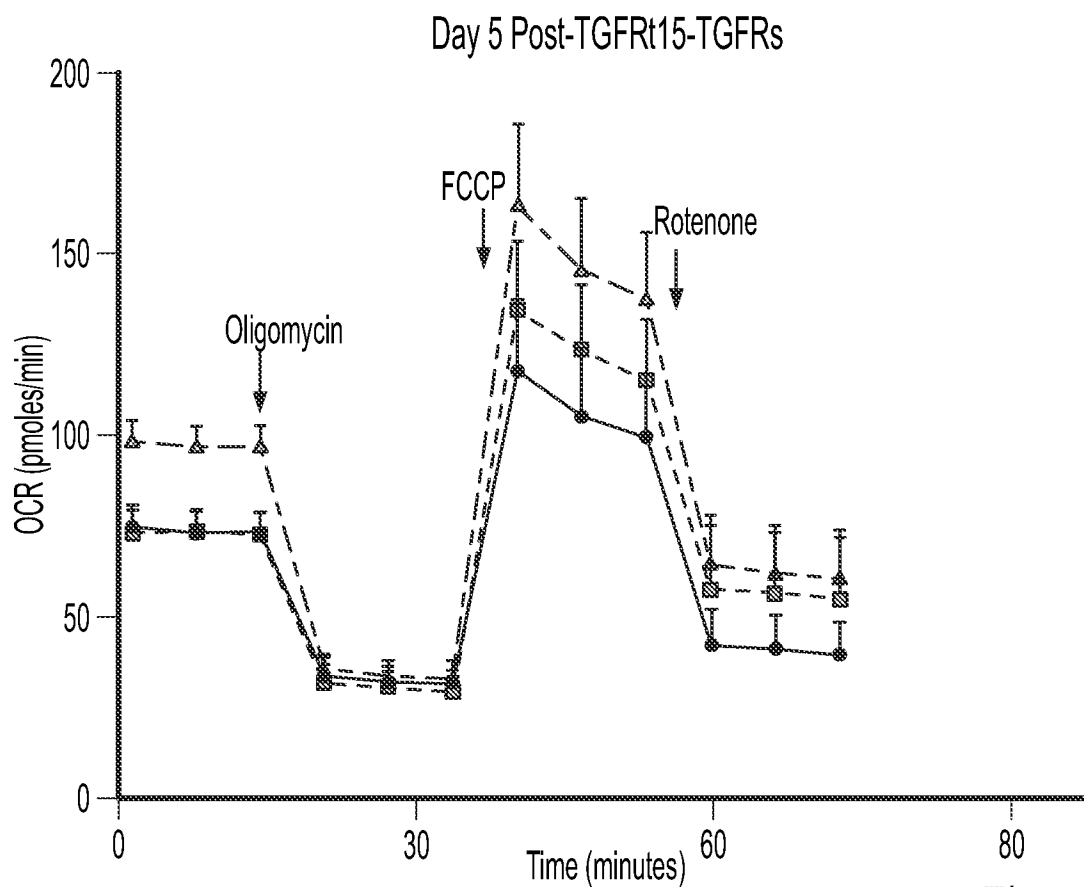


Figure 103B

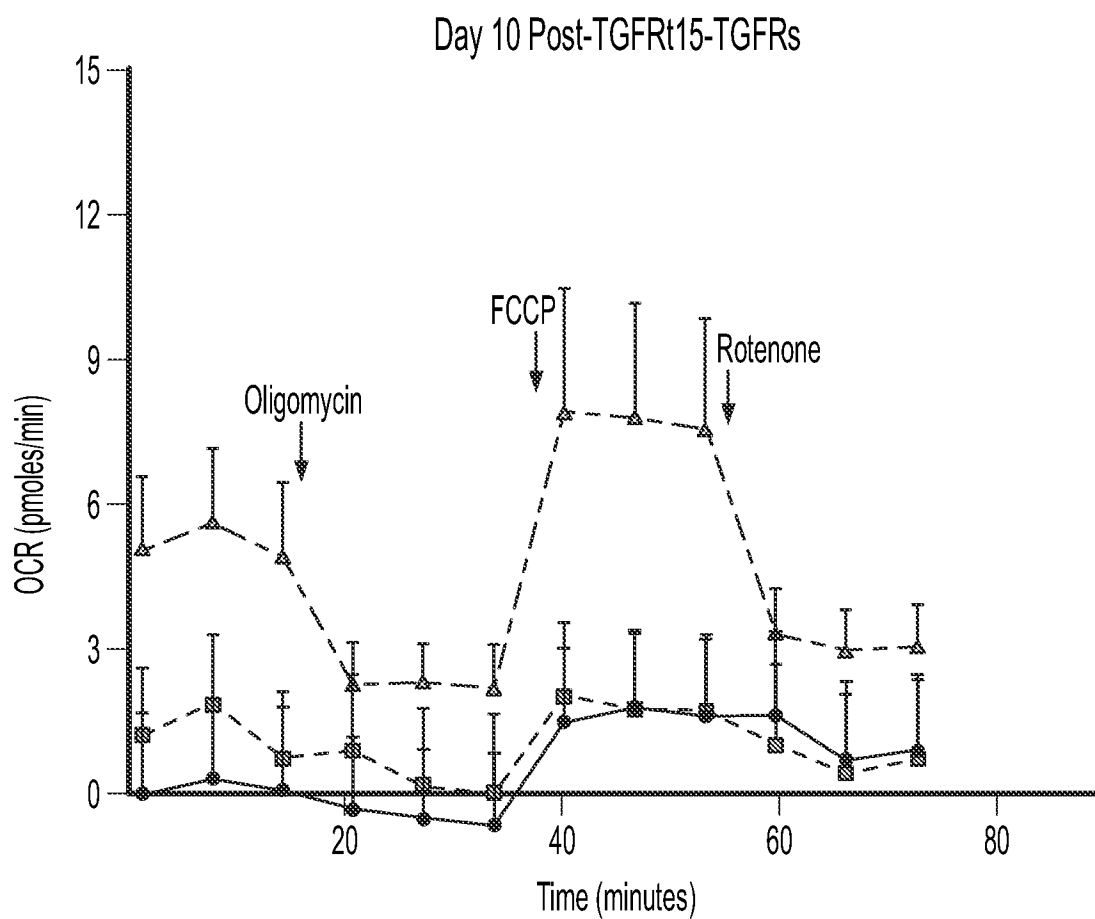


Figure 103C

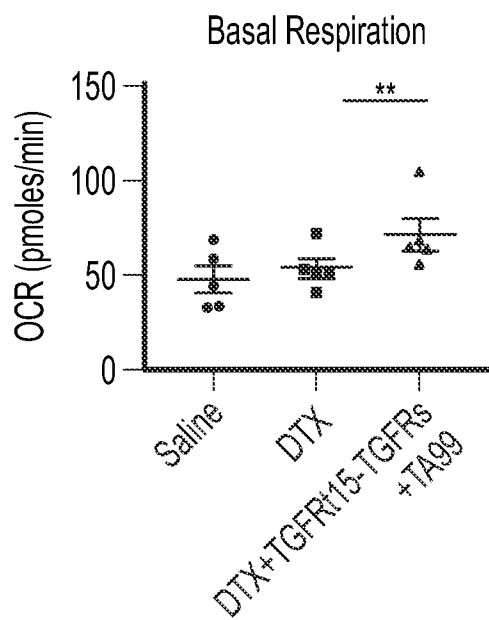


Figure 104A

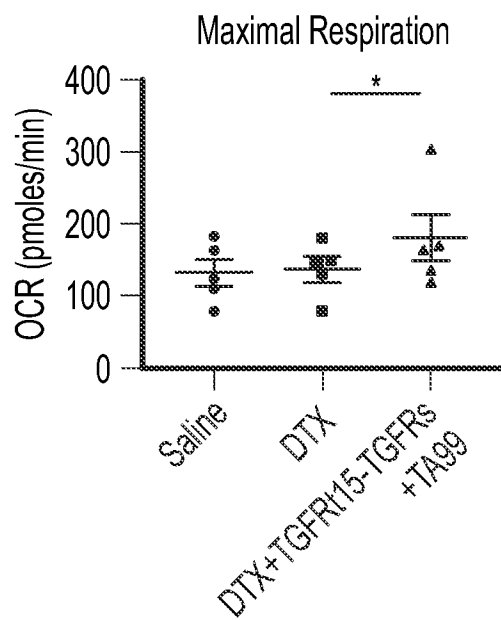


Figure 104B

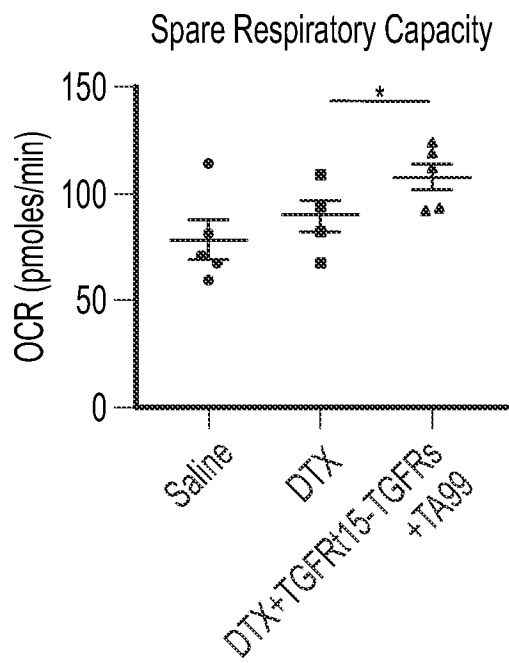


Figure 104C

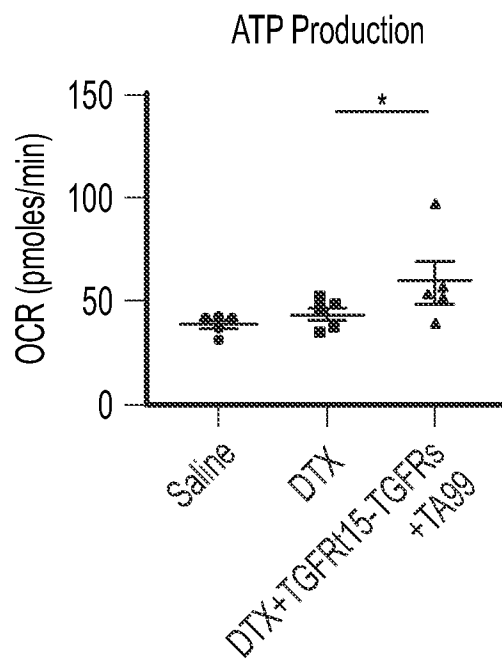


Figure 104D

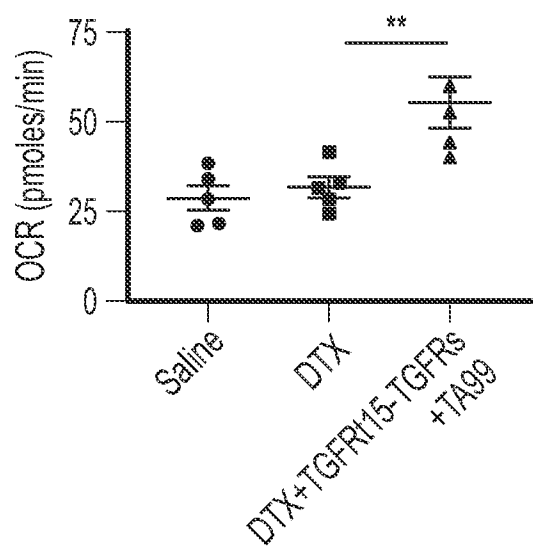


Figure 104E

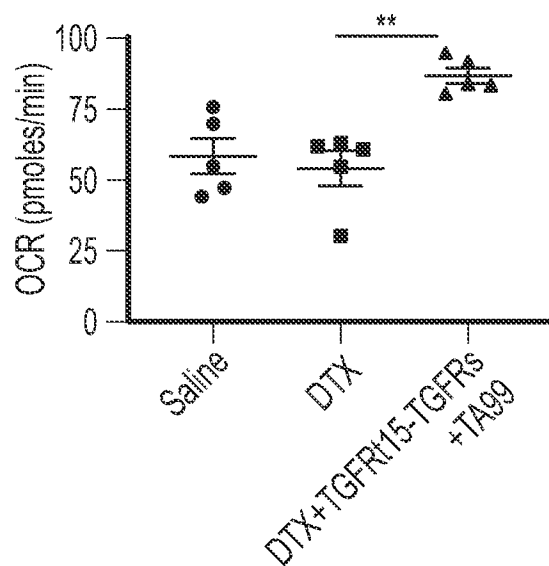


Figure 104F

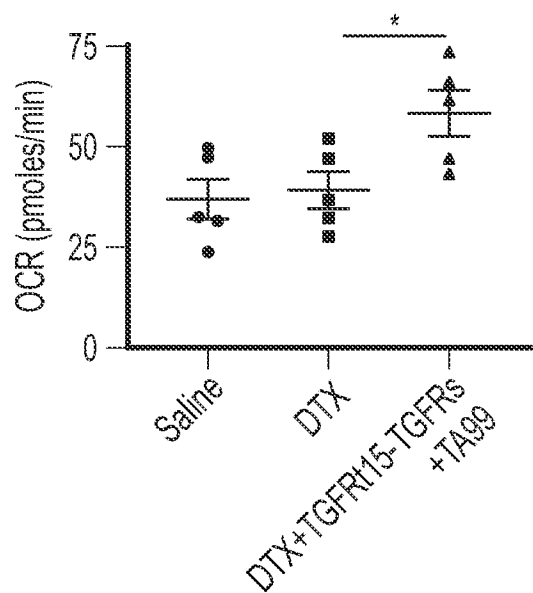


Figure 104G

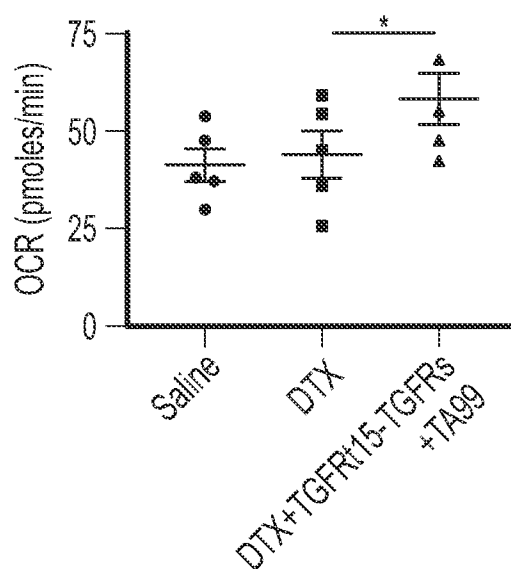


Figure 104H

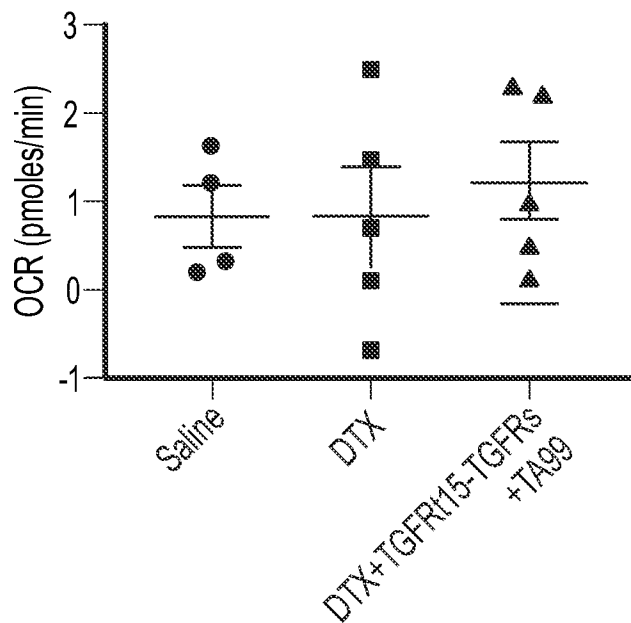


Figure 104I

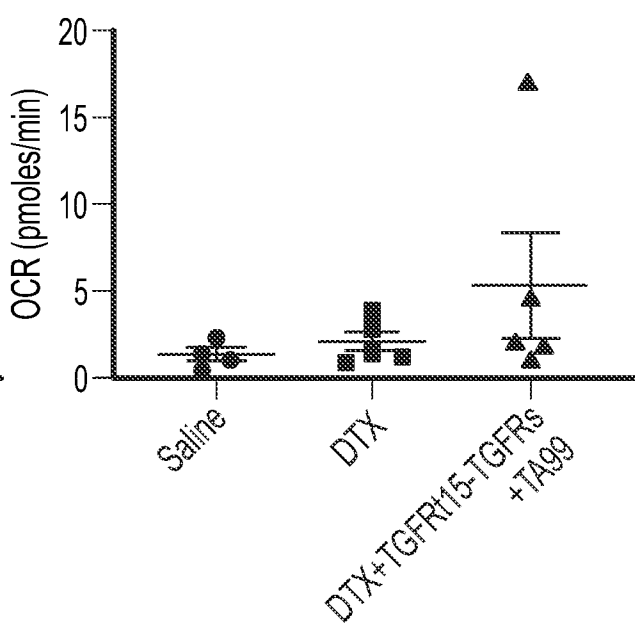


Figure 104J

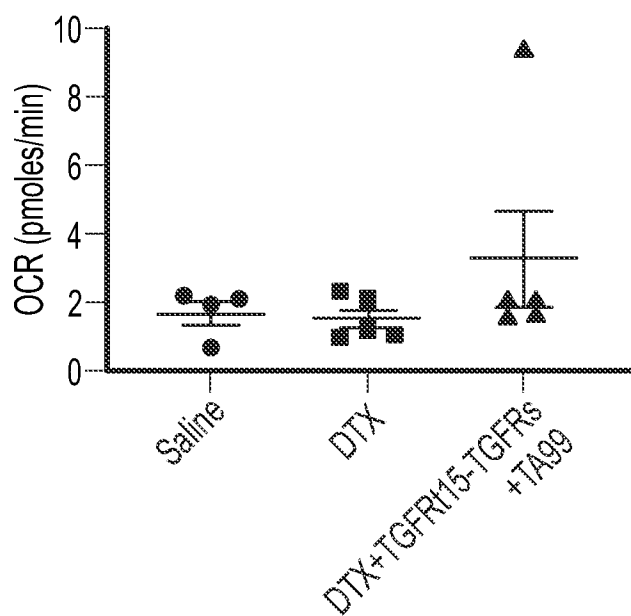


Figure 104K

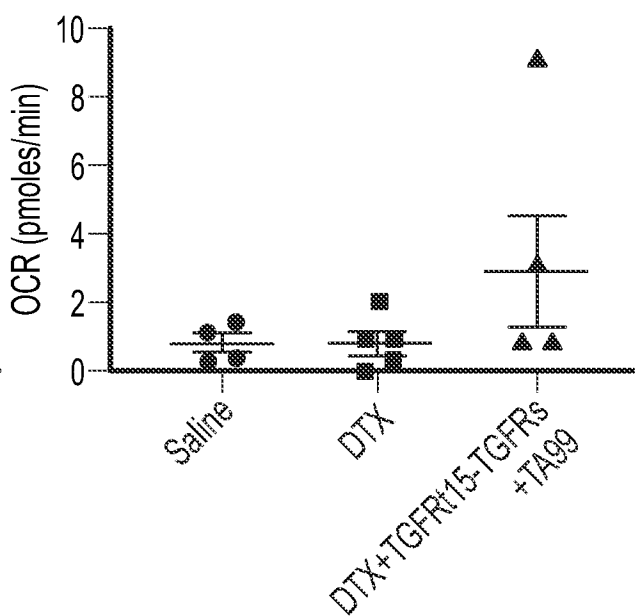


Figure 104L

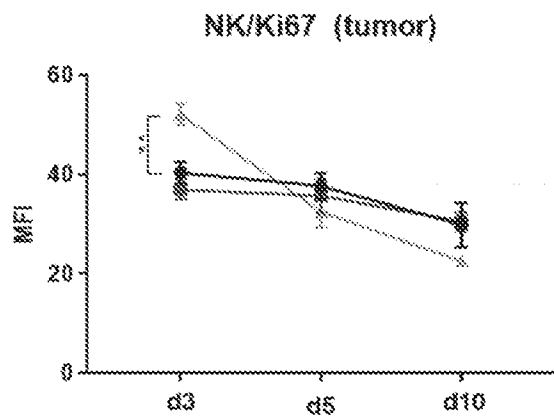


Figure 105A

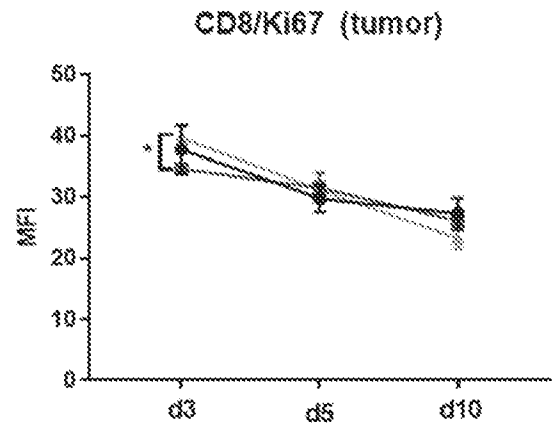


Figure 105B

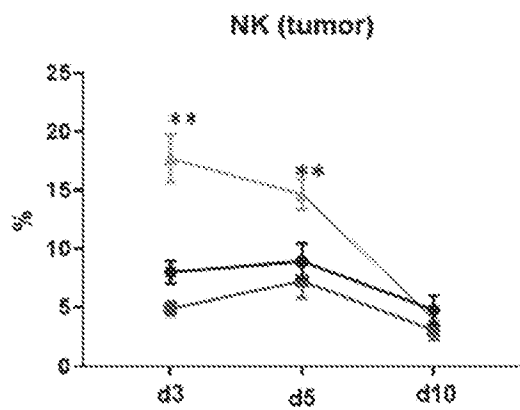


Figure 105C

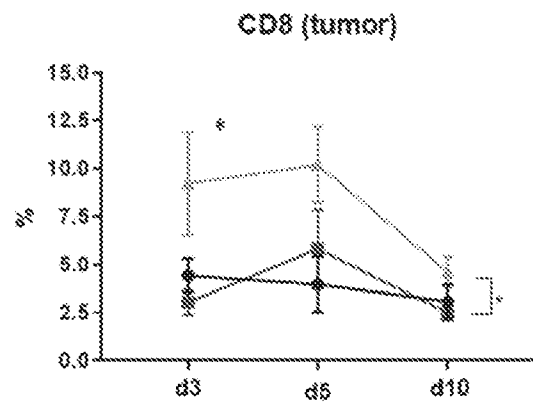


Figure 105D

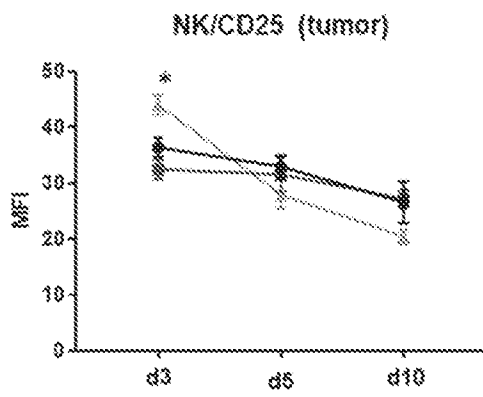


Figure 105E

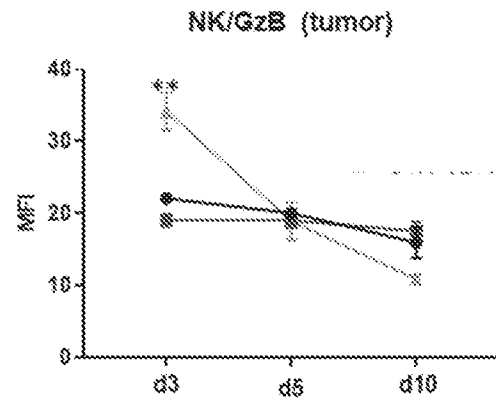


Figure 105F

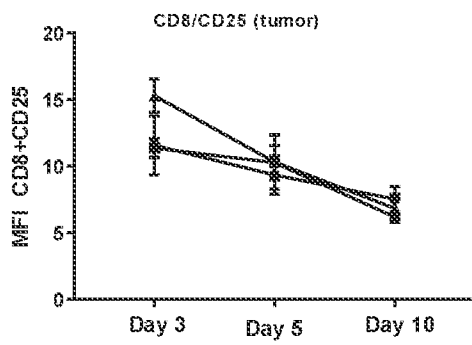


Figure 105G

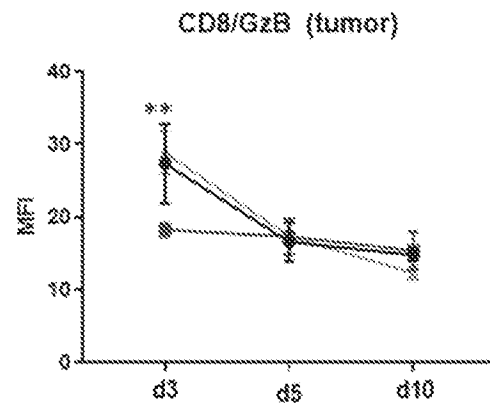


Figure 105H

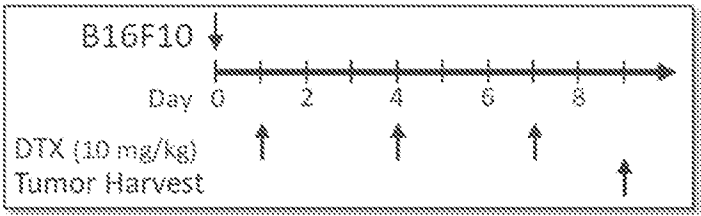


Figure 106A

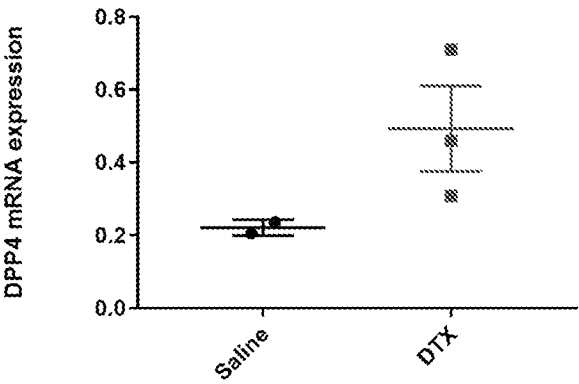


Figure 106B

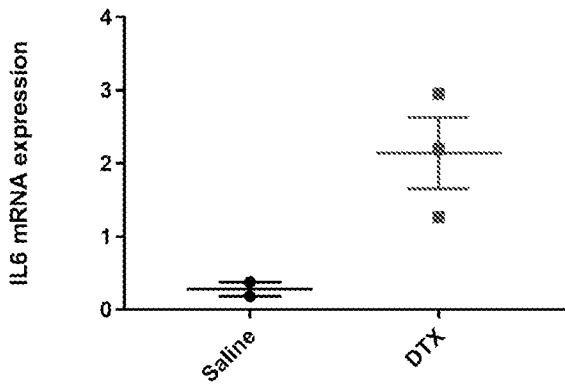


Figure 106C

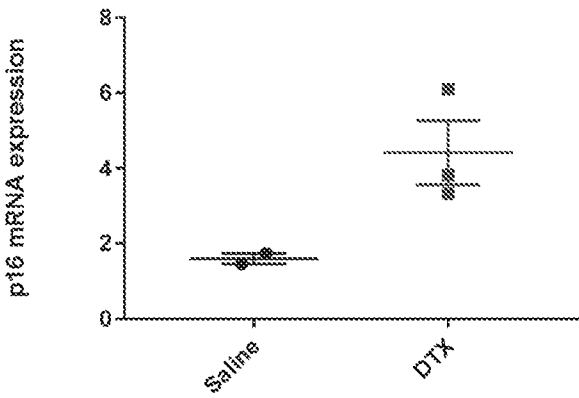


Figure 106D

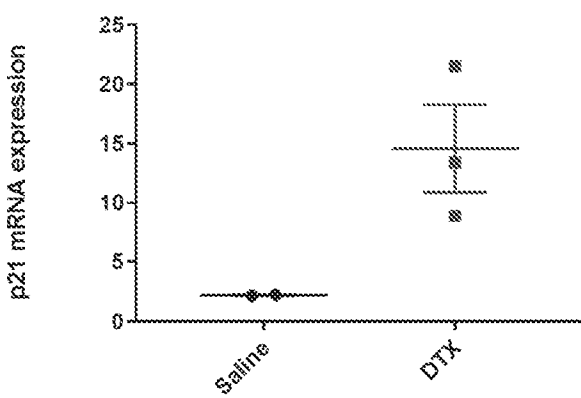


Figure 106E

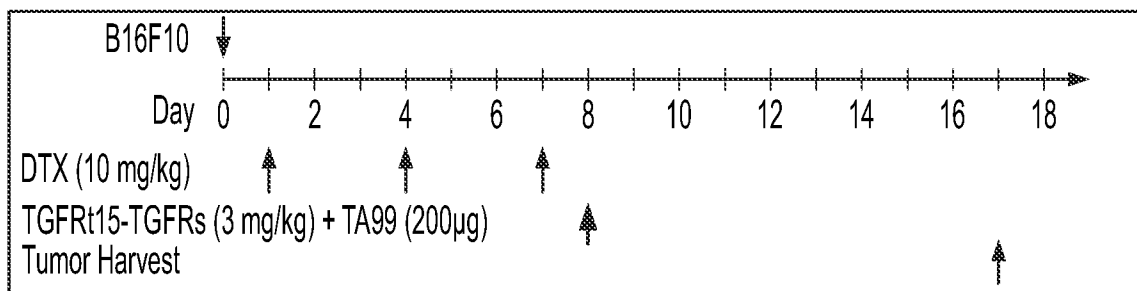


Figure 107A

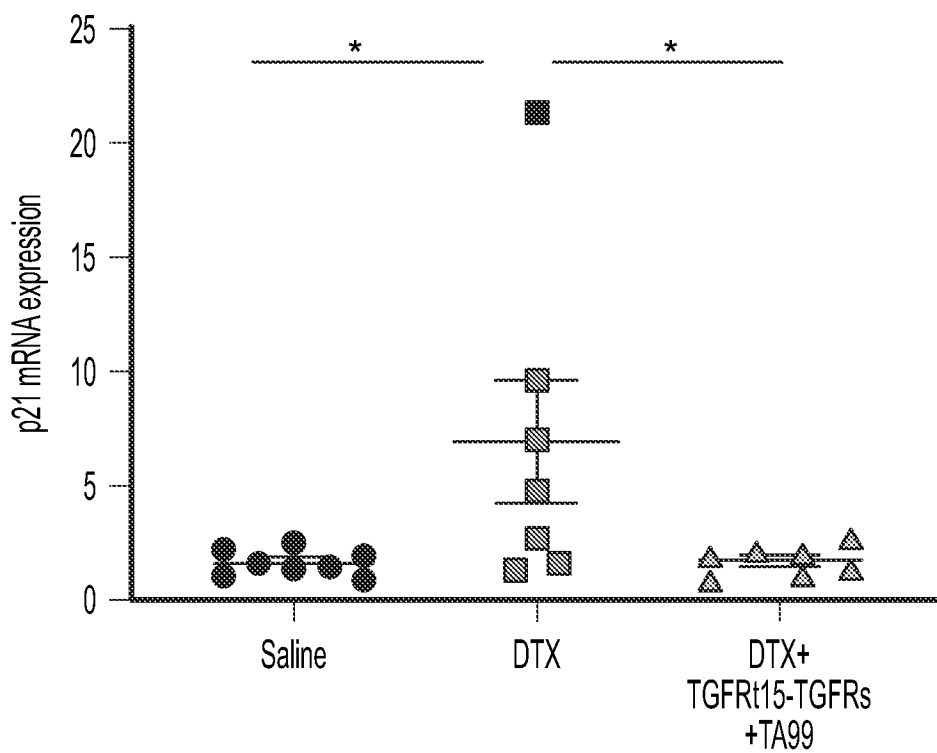


Figure 107B

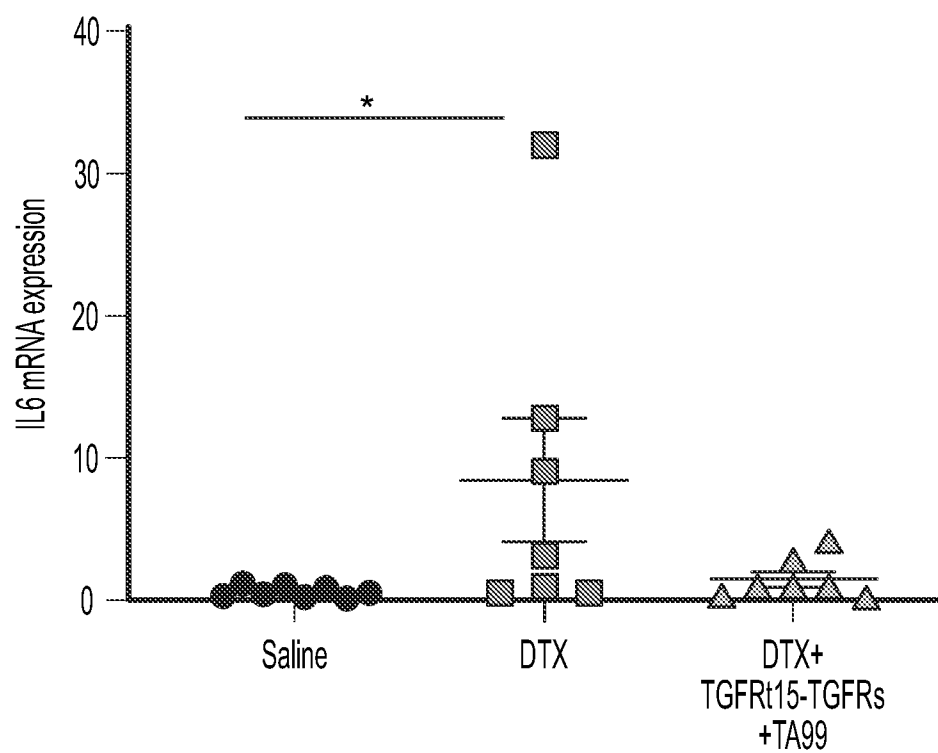


Figure 107C

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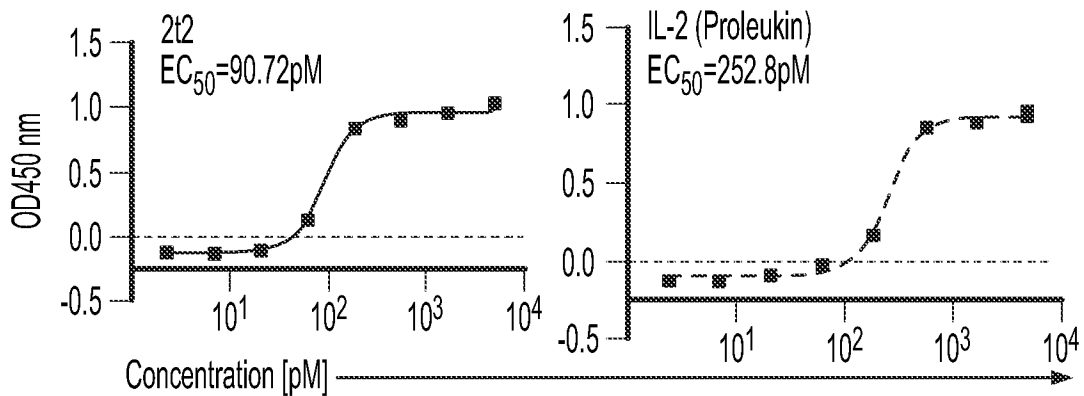
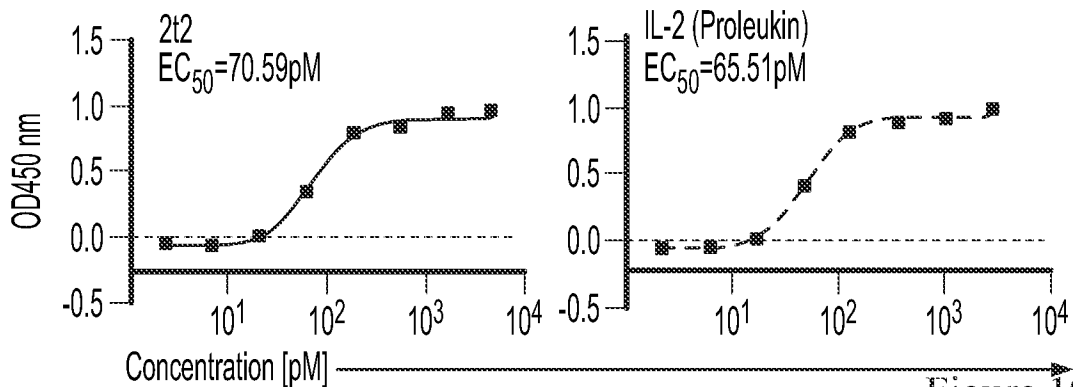
Activation of cells with IL-2R $_{\alpha\beta\gamma}$ (CTLL-2)Activation of cells with IL-2R $_{\alpha\beta\gamma}$ (32D $_{\beta}$)

Figure 108A

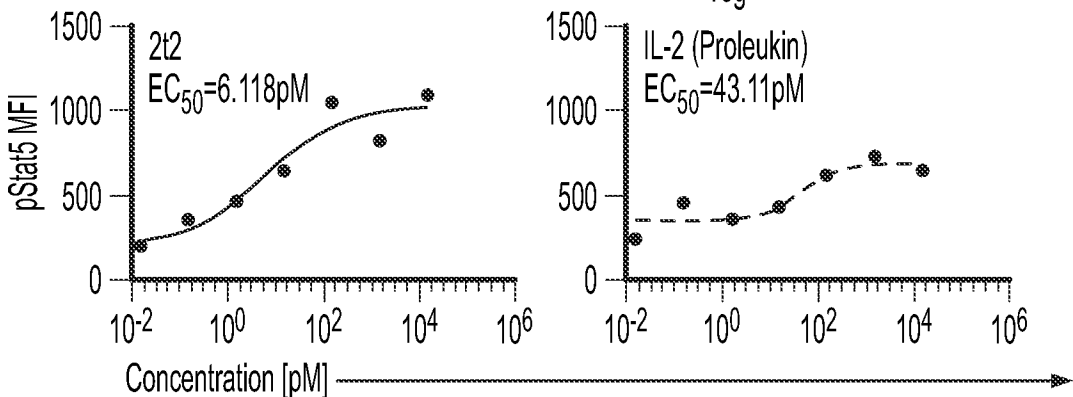
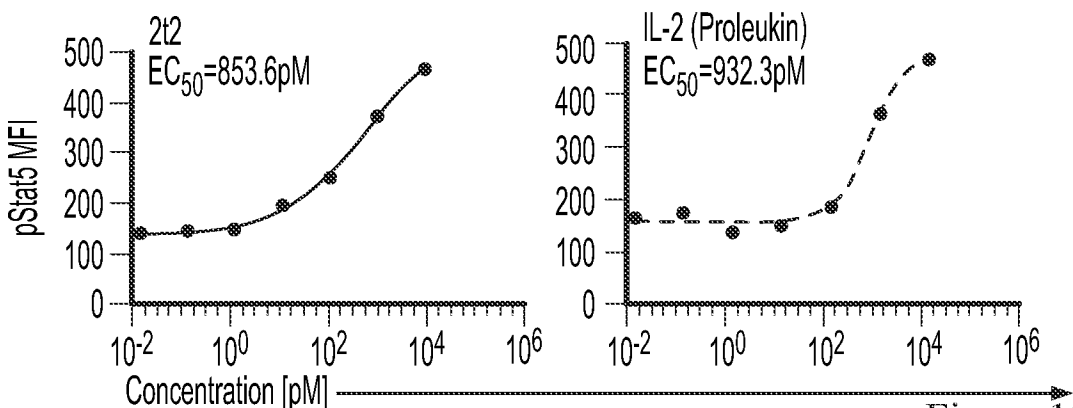
Activation of human CD4⁺CD25⁺T_{reg} pSTAT5Activation of human CD8⁺T_{con} pSTAT5

Figure 108B

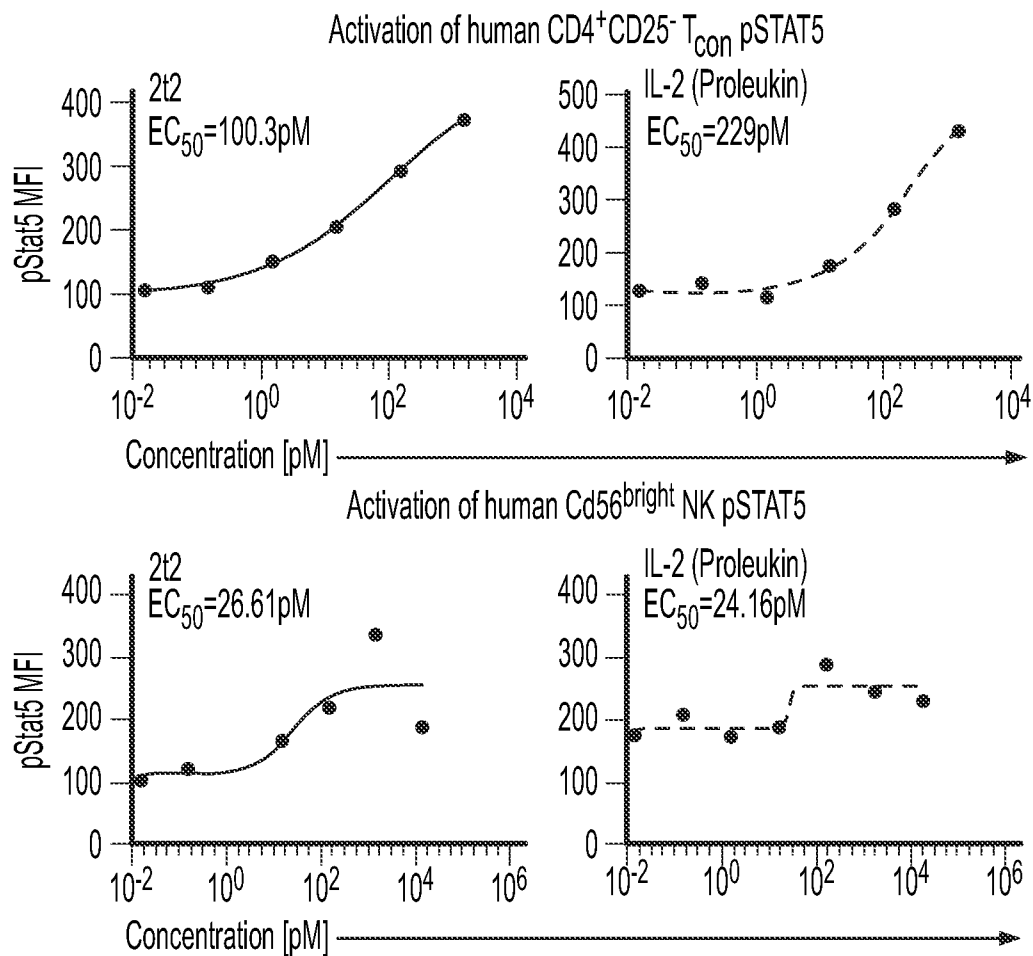


Figure 108C

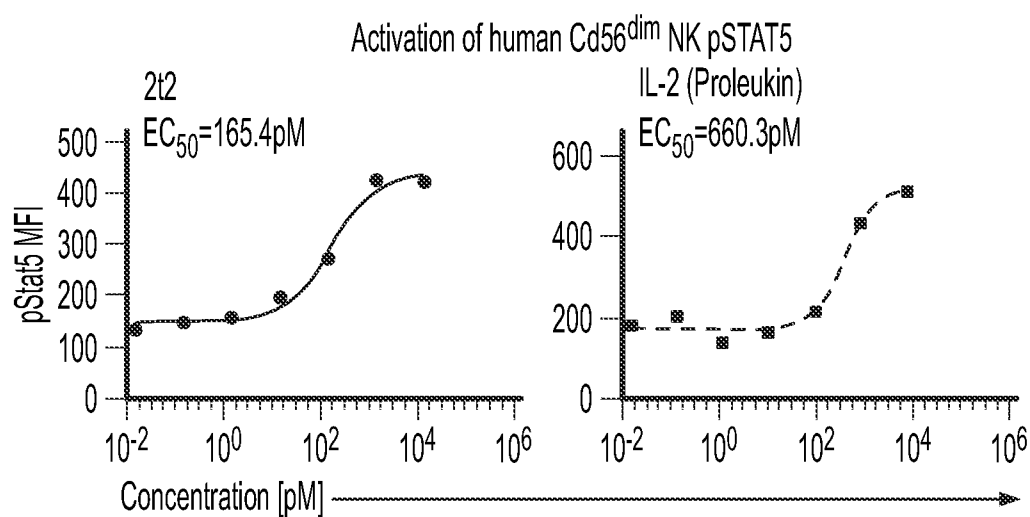


Figure 108D

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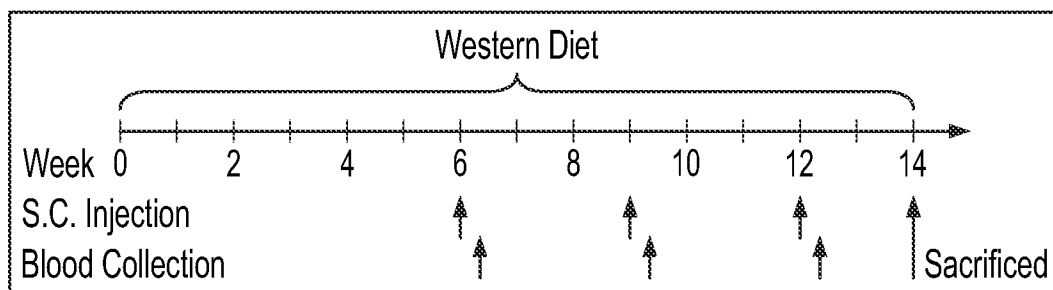


Figure 109A

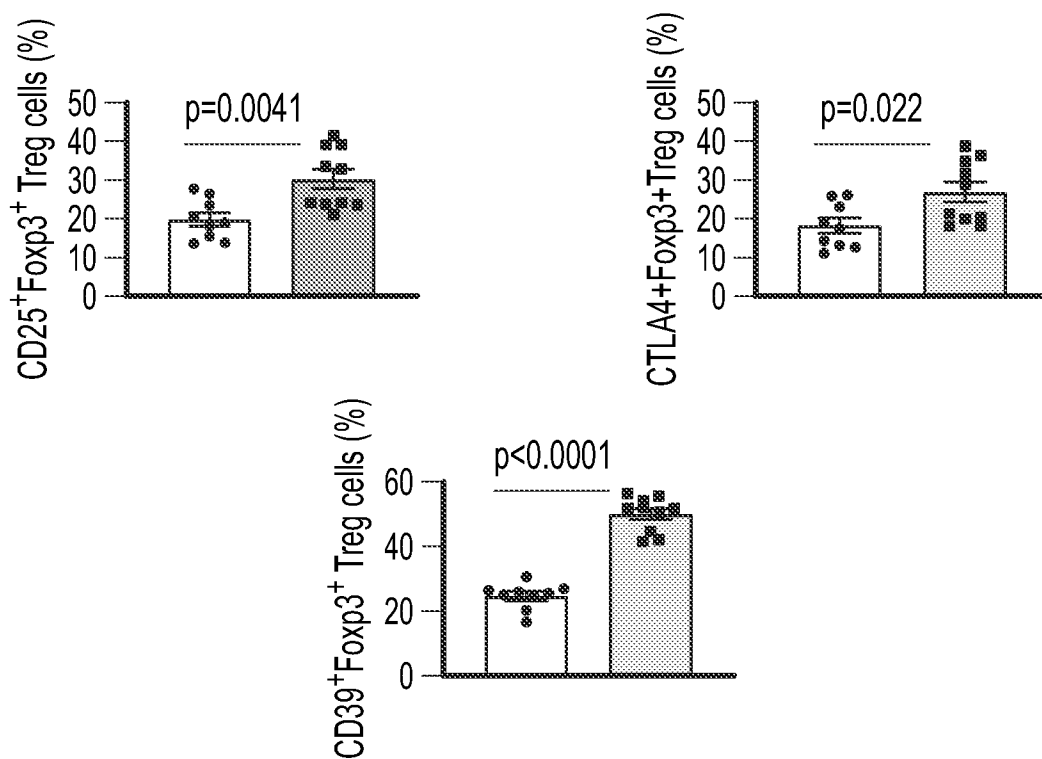


Figure 109B

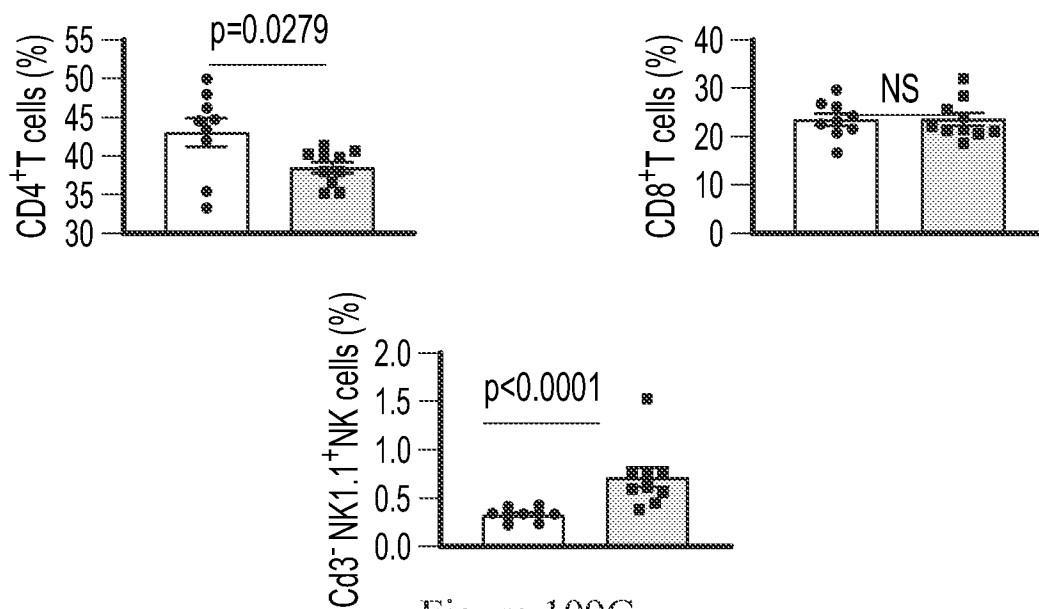


Figure 109C

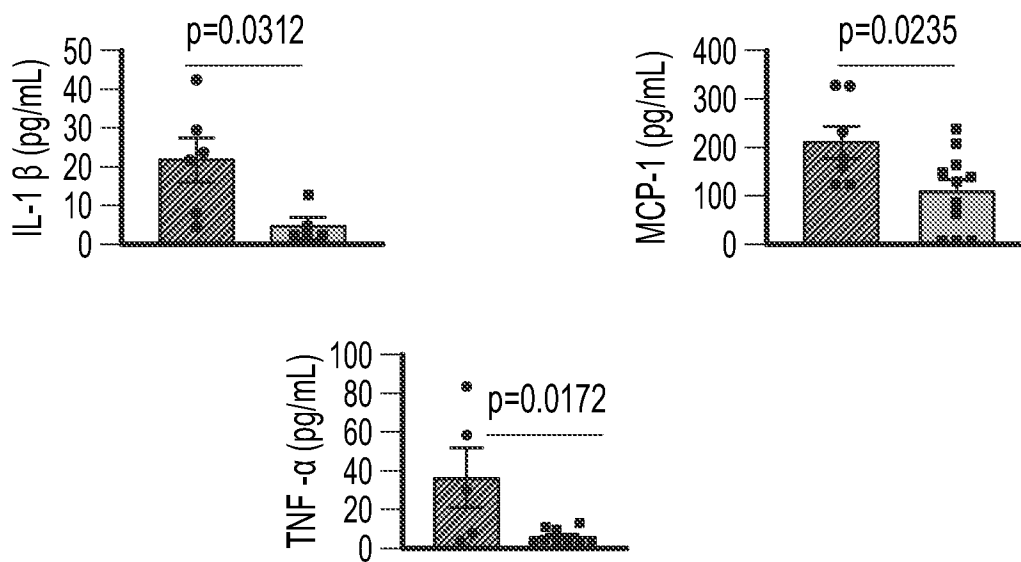


Figure 109D

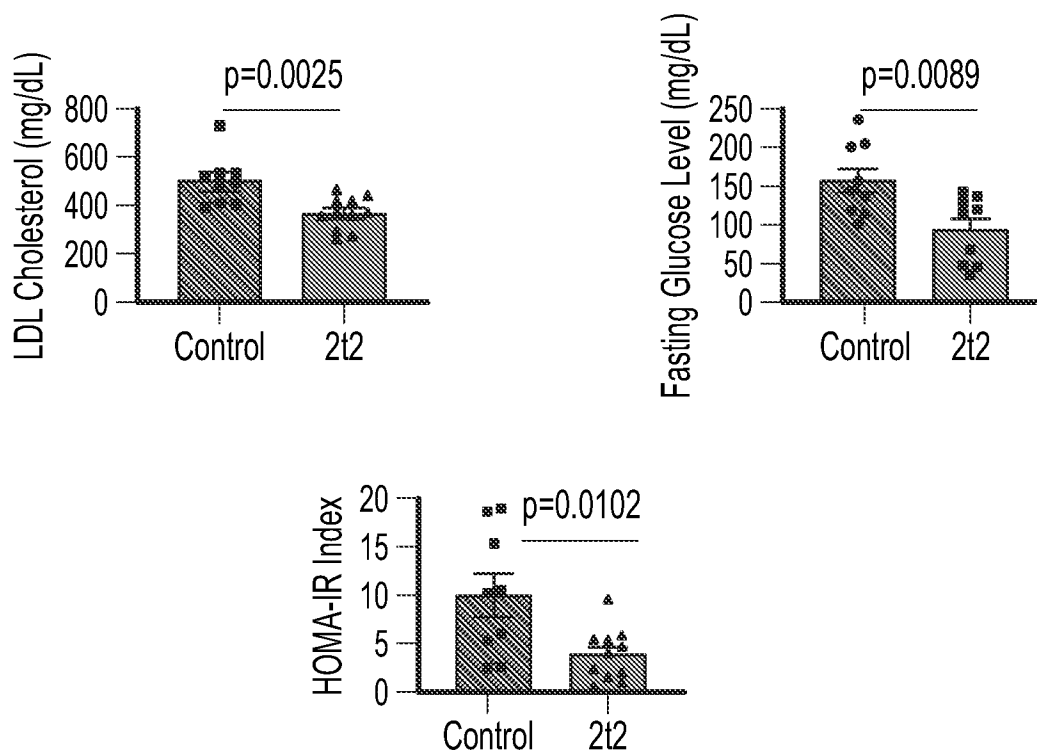


Figure 109E

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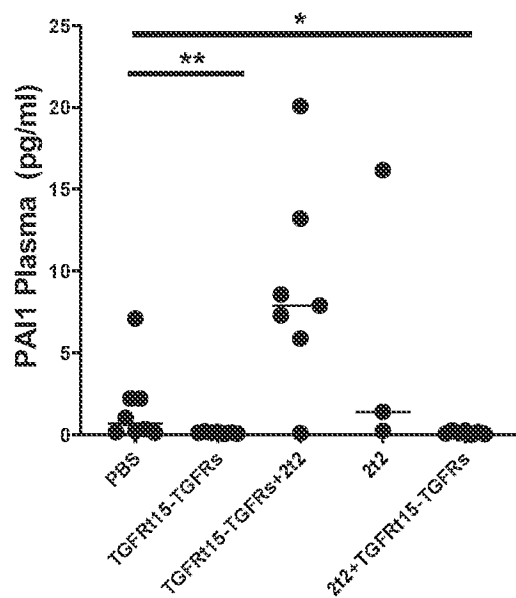


Figure 110A

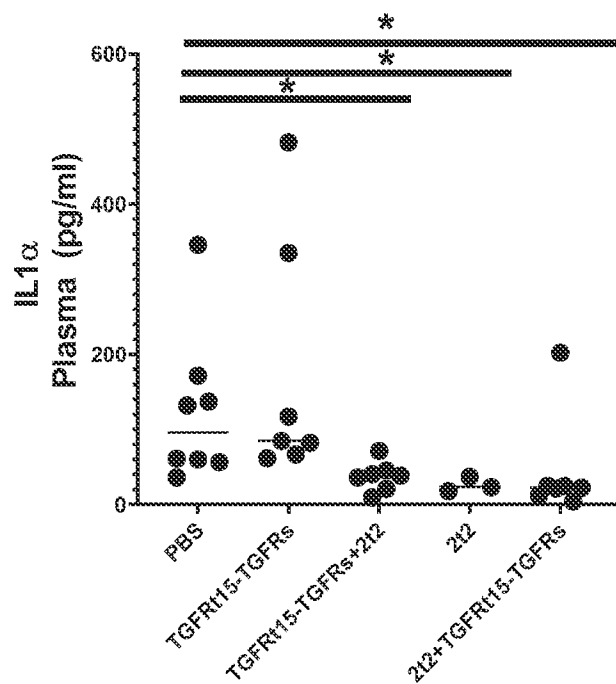


Figure 110B

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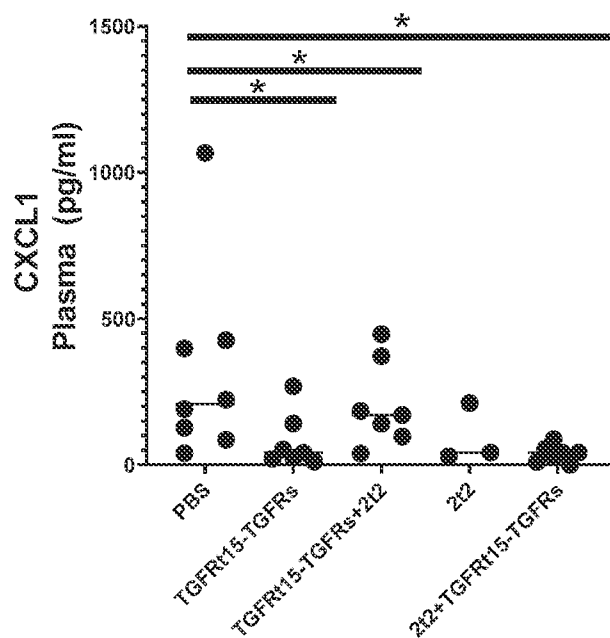


Figure 110C

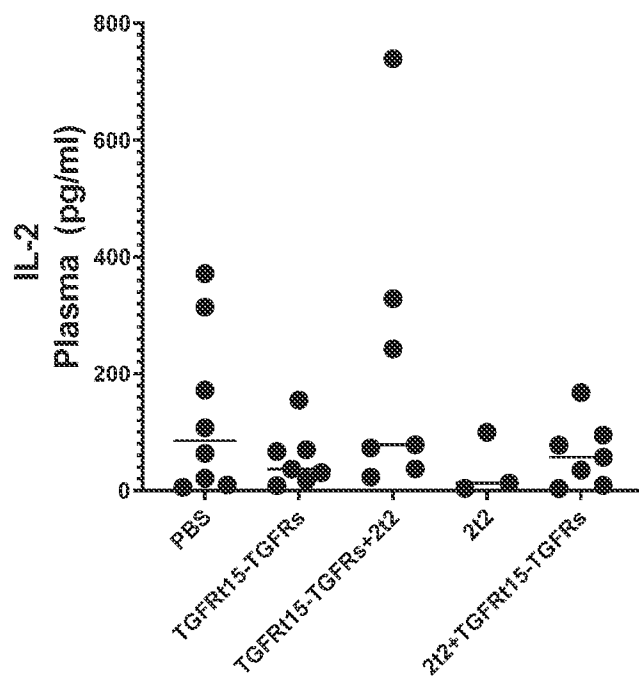


Figure 110D