



(51) International Patent Classification:

A61K 38/18 (2006.01) A61K 39/395 (2006.01)
A61K 38/19 (2006.01) C07K 16/28 (2006.01)
A61K 38/20 (2006.01) C07K 16/46 (2006.01)

(21) International Application Number:

PCT/US2018/040786

(22) International Filing Date:

03 July 2018 (03.07.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/528,411	03 July 2017 (03.07.2017)	US
62/598,433	13 December 2017 (13.12.2017)	US
62/620,418	22 January 2018 (22.01.2018)	US
62/620,107	22 January 2018 (22.01.2018)	US
62/657,455	13 April 2018 (13.04.2018)	US

(71) Applicant: **TORQUE THERAPEUTICS, INC.** [US/US];
450 Kendall Street, Cambridge, Massachusetts 02142 (US).

(72) Inventors: **JONES, Douglas S.**; c/o Torque Therapeutics, Inc., 450 Kendall Street, Cambridge, Massachusetts 02142 (US). **LYONS, Jesse**; c/o Torque Therapeutics, Inc., 450 Kendall Street, Cambridge, Massachusetts 02142 (US). **NIELSEN, Ulrik B.**; c/o Torque Therapeutics, Inc., 450 Kendall Street, Cambridge, Massachusetts 02142 (US).

(74) Agent: **XIE, Fang**; Greenberg Traurig LLP, One International Place, Suite 2000, Boston, Massachusetts 02110 (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, 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, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, 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))

(54) Title: FUSION MOLECULES TARGETING IMMUNE REGULATORY CELLS AND USES THEREOF

Current Treg Manufacturing and ACT



Obstacles for Treg Therapy

- 1: Requires GMP sorting
- 2: Deficiencies in freeze/thaw
- 3: Long and costly ex vivo expansion
- 4: Majority of cells are lost post-transfusion

FIG. 1A

(57) Abstract: Fusion proteins that include an immune (e.g., Treg) targeting moiety and a cytokine or growth factor molecule, pharmaceutical and formulations thereof, and methods of using and making the same, are disclosed.

FUSION MOLECULES TARGETING IMMUNE REGULATORY CELLS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to and the benefit of U.S. Provisional Application Nos. 62/528,411 filed July 3, 2017, 62/598,433 filed December 13, 2017, 62/620,418 filed January 22, 2018, 62/620,107 filed January 22, 2018 and 62/657,455 filed April 13, 2018, the entire disclosure of each of which is incorporated herein by reference.

10 SEQUENCE LISTING

 The ASCII text file submitted herewith via EFS-Web, entitled “174285_011000_sequence.txt” created on July 3, 2018, having a size of 189,027 bytes, is hereby incorporated by reference in its entirety.

15 FIELD

 The present disclosure relates generally to modified immune regulatory cells and engineered fusion molecules. More specifically, the present disclosure relates to modified regulatory T cells and fusion proteins useful for targeted immune therapy and adoptive cell therapy.

20

BACKGROUND

 Adoptive cellular therapies (ACT) are demonstrating clinical benefits across a range of disorders, with potentially fewer risks and greater efficacy than traditional pharmacological strategies. Regulatory T cells (Tregs) are currently undergoing clinical trials in various immune-mediated pathologies, including transplant rejection and autoimmune conditions. In general, cell
25 therapy relies upon *ex vivo* expansion of the cell product. *In vitro* manipulation of cell therapy products, prior to administration to patients, offers the opportunity to enhance the efficacy of the final cell therapy product in other ways. For example, cells can be exposed to reagents that enhance their longevity or functional potency after transfer into the patient. Genetic modification
30 strategies can even permit the design of cells with bespoke functionality. Crucially, *in vitro* manipulation of therapeutic cells in isolation can exert these influences upon the biology of the therapeutic cells, without systemic exposure of the patient to the reagents being used.

 Specifically, current Treg manufacturing and ACT requires sorting of Tregs (*e.g.*, CD4+CD25+CD127- by fluorescence-activated cell sorting, FACS) from a patient sample, *ex*
35 *vivo* expansion of Tregs, often by expanding the Tregs using interleukin 2 (IL-2) or activating

and expanding the T cells by using CD3 and CD28 stimulation and IL-2, and then subsequently administering the Tregs to the patient; see Figure 1A. However, Treg cell therapy presents several key challenges: it requires GMP (good manufacturing practice) level sorting and long and costly *ex vivo* expansion; significant deficiencies in Treg viability and activity after freezing and thaw can exist; and cells can exhibit short-term survival following reinfusion into the patient.

Thus, compositions and methods to improve Treg-based cell therapies hold strong potential for delivering meaningful clinical benefits.

10 SUMMARY

Disclosed herein are methods and compositions for Treg-targeting fusion molecules (“FMs”) to improve engraftment, survival and efficacy of adoptive cell therapies, *e.g.*, immunosuppressive cell therapy. In various embodiments, the Treg-targeting fusions can be combined with the backpack technology as disclosed in, *e.g.*, U.S. Publication No. 2017/0080104, U.S. Patent No. 9,603,944, U.S. Publication No. 2014/0081012, and PCT Application No. PCT/US2017/037249, each of which is incorporated herein by reference in its entirety.

In one aspect, provided herein is a fusion molecule, comprising:

(a) a cytokine or growth factor molecule; and

(b) a regulatory T cell (Treg) targeting moiety comprising an antigen-binding fragment of an antibody having an affinity to an antigen on the surface of a Treg;

wherein the cytokine or growth factor molecule is operatively linked to the antigen-binding fragment.

In some embodiments, the antigen is one or more of CD45, CD4, CD25, CD39, Neuropilin 1 (NRP1), or a variant of any of the foregoing. In some embodiments, the targeting moiety comprises a bispecific molecule having an antigen-binding fragment specific for CD4 and an antigen-binding fragment specific for CD25. In some embodiments, the antigen is one or more of CD4, CD45, CD3, CD2, CD25, CD127, CD197 (CCR7), CXCR3, CXCR4, CXCR5, CD38, CD27, , CCR4, CCR5, CD137, CD39, CCR4, CCR5, CCR6 (CD196), CCR8, CCR10, OX40, GITR, CTLA4, LAG3, CD73, CD103, CD62L, CCR2, CCR9, Neuropilin 1 (NRP1), CD8, CD11a, CD18, or a variant of any of the foregoing. In some embodiments, the cytokine or growth factor molecule is one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, or TGF- β , or a variant of any of the foregoing. In some embodiments, the cytokine or growth factor molecule is one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, TGF- β , IL-7, or IL-21, or a variant of any of the foregoing. In some embodiments, the

cytokine or growth factor molecule is one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, TGF- β , IL-7, IL-21, IL-6, IL-12, or IL-23, or a variant of any of the foregoing. In some embodiments, the Treg is a healthy and/or non-malignant Treg.

In some embodiments, the fusion molecule can further include a linker for operably
5 linking the cytokine or growth factor molecule and the targeting moiety. In some embodiments, the linker is selected from one or more of a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, or a non-helical linker. In some
embodiments, the peptide linker is a human serum albumin or a variant thereof. In some
embodiments, the linker, at either end, is fused to the cytokine or growth factor molecule and the
10 targeting moiety via a peptide comprising Gly and Ser. In some embodiments, the peptide is a
(GGGS)_N (SEQ ID NO: 87) or (GGGGS)_N (SEQ ID NO: 88) linker, where N indicates the
number of repeats of the motif and is an integer selected from 1-10.

In some embodiments, the antigen-binding fragment is a Fab fragment comprising a light
chain and a heavy chain fragment linked by a disulfide bond, and wherein the cytokine or
15 growth factor molecule is operably linked to the Fab fragment at a C-terminus of the light chain,
an N-terminus of the light chain, a C-terminus of the heavy chain fragment, or an N-terminus of
the heavy chain fragment. In some embodiments, the targeting moiety localizes the cytokine or
growth factor molecule to the Treg and enhances its concentration, distribution, duration of
exposure and/or availability on a cell surface of the Treg.

20 A further aspect relates to a fusion molecule, comprising:

- (a) a cytokine or growth factor molecule; and
- (b) a Treg targeting moiety comprising an antibody having an antigen-binding site
specific for an antigen on the surface of a Treg, wherein the antibody comprises a light chain
having a C-terminus and an N-terminus, and a heavy chain having a C-terminus and an N-
25 terminus, wherein the light chain is linked to the heavy chain by a disulfide bond,

wherein the cytokine or growth factor molecule is operably linked to the antibody at the
C-terminus of the light chain, the N-terminus of the light chain, or the N-terminus of the heavy
chain portion.

In some embodiments, the antigen is a CD4 or a CD25 receptor expressed on the cell
30 surface of the Treg. In some embodiments, the cytokine or growth factor molecule comprises an
IL-2, or a variant thereof. In some embodiments, the cytokine or growth factor molecule is
operably linked to the antibody by a linker. In some embodiments, the linker is selected from a
cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical
linker, and a non-helical linker. In some embodiments, the linker is a peptide linker comprising
35 a Gly and a Ser. In some embodiments, the peptide linker is a (GGGS)_N (SEQ ID NO: 87) or

(GGGGS)_N (SEQ ID NO: 88) linker, wherein _N indicates the number of repeats of the motif and is an integer selected from 1-10.

In another aspect, provided herein is a fusion molecule (FM), comprising: (i) a regulatory T cell (Treg) targeting moiety having an affinity with an immune cell surface receptor on the Treg, wherein the targeting moiety is selected from an antibody or antigen-binding fragment thereof, a non-antibody scaffold, or a ligand that binds to the immune cell surface receptor; and (ii) a cytokine or growth factor molecule; wherein the cytokine or growth factor molecule and the targeting moiety are operably linked together as a fusion molecule.

In some embodiments, the immune cell surface receptor is selected from one or more of CD45, CD4, CD25, CD39, Neuropilin 1 (NRP1), or variants thereof. In one embodiment, the targeting moiety comprises a bispecific molecule that binds to both CD4 and CD25. In some embodiments, the immune cell surface receptor is selected from one or more of CD4, CD45, CD3, CD2, CD25, CD127, CD197 (CCR7), CXCR3, CXCR4, CXCR5, CD38, CD27, CCR4, CCR5, CD137, CD39, CCR4, CCR5, CCR6 (CD196), CCR8, CCR10, OX40, GITR, CTLA4, LAG3, CD73, CD103, CD62L, CCR2, CCR9, Neuropilin 1 (NRP1), CD8, CD11a, or CD18, or variants thereof.

In some embodiments, the cytokine or growth factor molecule is selected from one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, or TGF- β , or variants thereof. In some embodiments, the cytokine or growth factor molecule is selected from one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, TGF- β , IL-7, or IL-21, or variants thereof. In some embodiments, the cytokine or growth factor molecule is selected from one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, TGF- β , IL-7, IL-21, IL-6, IL-12, or IL-23, or variants thereof.

In various embodiments, the Treg targeted by the FMs disclosed herein is a healthy and/or non-malignant Treg. In various embodiments, the Treg targeted by the FMs disclosed herein is obtained from patients in need of immunosuppressive therapy that may or may not be marked by Treg dysfunction (e.g., an unhealthy or abnormal Treg). In certain embodiments, the targeting moiety localizes the cytokine or growth factor molecule to the Treg and enhances its concentration, distribution, duration of exposure and/or availability on a cell surface of the Treg.

The FM, in some embodiments, can further include a linker for operably linking the cytokine or growth factor molecule and the targeting moiety. The linker can be selected from one or more of a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, or a non-helical linker. The peptide linker can be human serum albumin or an Fc domain. The linker, at either end, can be fused to the cytokine or growth factor molecule and the targeting moiety via a peptide comprising Gly and Ser. In some embodiments,

the peptide is a (GGGS)_N (SEQ ID NO: 87) or (GGGGS)_N (SEQ ID NO: 88) linker, where N indicates the number of repeats of the motif and is an integer selected from 1-10.

In some embodiments, any of the fusion molecules disclosed herein can further include a nanoparticle, a liposome and/or a biodegradable polymer. In some embodiments, the nanoparticle comprises a protein nanogel, a nucleotide nanogel, a polymer nanoparticle, or a solid nanoparticle. In some embodiments, the nanoparticle comprises a protein nanogel. In some embodiments, the nanoparticle optionally comprises at least one polymer, cationic polymer, or cationic block co-polymer on the nanoparticle surface. In some embodiments, the nanoparticle comprises a nanogel that is cross linked by a reversible linker that is sensitive to redox (disulfide) or pH (hydrolysable groups) or enzymes (proteases).

Another aspect relates to a modified Treg comprising:

- (a) a fusion molecule comprising
 - (i) a cytokine or growth factor molecule; and
 - (ii) a Treg targeting moiety having an affinity to a Treg cell surface antigen; and
- (b) a Treg, wherein the fusion molecule is bound to the surface of Treg through interaction with the cell surface antigen.

A further aspect relates to a modified Treg comprising a Treg and any of the fusion molecule disclosed herein bound thereto.

Also provided herein is a method of preparing modified Tregs, comprising:

- (a) providing a population of Tregs; and
- (b) incubating the fusion molecule disclosed herein with the population of Tregs so as to permit targeted binding of the fusion molecule thereto, thereby producing a population of Tregs having fusion molecules bound on the cell surface.

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

- (a) a plurality of fusion molecules, each fusion molecule comprising
 - (i) a cytokine or growth factor molecule; and
 - (ii) a Treg targeting moiety having an affinity to a cell surface antigen of a Treg;
- (b) a population of Tregs, wherein the plurality of fusion molecules are bound to the surface of the Treg through interaction with the cell surface antigen; and
- (c) a pharmaceutically acceptable carrier, excipient, or stabilizer.

A further aspect relates to a method for the suppressing or preventing an immune response in a human subject, the method comprising administering to the human subject a cell

therapeutic composition, the composition comprising:

- (a) a plurality of fusion molecules, each fusion molecule comprising
 - (i) a cytokine or growth factor molecule; and
 - (ii) a Treg targeting moiety having an affinity to a cell surface antigen of a Treg;
- 5 and
- (b) a population of Tregs,

wherein the plurality of fusion molecules are bound to the surface of the Tregs, and wherein the cytokine or growth factor molecule acts *in vivo* upon the population of Tregs in the human subject to suppress or prevent an immune response in the human subject.

10 Also provided herein is a composition comprising:

- (a) a fusion molecule comprising
 - (i) a cytokine or growth factor molecule; and
 - (ii) an immune cell targeting moiety having an affinity to a Treg cell surface antigen;

15 (b) a Treg expressing or otherwise displaying the cell surface antigen, wherein the fusion molecule is bound to the surface of the Treg through interaction with the cell surface antigen; and

- (c) a nanoparticle, nanogel, or liposome.

20 Another aspect relates to an isolated nucleic acid molecule engineered to encode the FM disclosed herein.

A further aspect relates to a vector comprising the nucleic acid molecule disclosed herein.

25 Still another aspect relates to a host cell comprising the nucleic acid molecule disclosed herein or the vector disclosed herein.

Also provided herein is method of making the FM disclosed herein, comprising culturing the host cell disclosed herein under suitable conditions.

In another aspect, a pharmaceutical composition comprising the FM disclosed herein and a pharmaceutically acceptable carrier, excipient, or stabilizer is provided.

30 In a further aspect, a modified Treg, comprising a healthy and/or non-malignant Treg and the FM disclosed herein bound or targeted thereto is provided.

In still another aspect, provided herein is a method for *in vitro* or *ex vivo* expansion of Tregs, comprising providing a population of PBMCs from a subject, and selectively expanding Tregs therein in the presence of a plurality of FMs disclosed herein, thereby producing a
35 plurality of expanded Tregs.

In another aspect, provided herein is a method for providing immunosuppressive therapy, comprising administering to a subject in need thereof a plurality of the fusion molecules disclosed herein, a plurality of the modified Tregs disclosed herein, or the plurality of expanded Tregs disclosed herein.

5 In still another aspect, provided herein is a method for *in vivo* expansion of Tregs, comprising loading a population of sorted Tregs or PBMCs obtained from a subject with CD3/CD28/IL-2 backpacks to provide loaded cells, and administering the loaded cells to the subject without *in vitro* culturing.

10 In a further aspect, provided herein is a method for providing immunosuppressive therapy, comprising administering to a subject in need thereof a plurality of FMs disclosed herein, a plurality of the modified Tregs disclosed herein, the plurality of expanded Tregs disclosed herein, or the loaded cells disclosed herein. The immunosuppressive therapy can be used to treat diseases such as allo-immune diseases, auto-immune diseases, allergy, and inflammatory diseases. The allo-immune diseases can include, for example, organ transplant
15 rejection, graft versus host disease or rejection (GVHD) (*e.g.*, post-allogeneic hematopoietic stem cell transplant (HSCT) and other post-allogeneic stem cell transplantation (SCT), and rejection of autologous stem cells and/or genetically modified autologous stem cells (*e.g.*, where CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) or the like is used to modify cells *ex vivo* to fix some deficiency which are then transplanted back into the patient,
20 resulting in rejection due to any new or modified proteins being recognized as antigenic). The auto-immune diseases can include, for example, Type 1 diabetes, Multiple Sclerosis and Alopecia. The inflammatory diseases can include, for example, Inflammatory Bowel Disease, Rheumatoid Arthritis and Lupus.

In another aspect, the disclosure provides a particle, *e.g.*, a nanoparticle, that comprises
25 an FM as described herein, *e.g.*, nanoparticle that comprises a protein (*e.g.*, a protein nanogel as described herein). In one embodiment, the particle comprises the same FM. In other embodiments, the particle comprises one or more different types of FM. Nanoparticles and methods of making are disclosed in PCT International Application No. PCT/US2017/037249 filed June 13, 2017, *e.g.*, on pages 57-79, which is incorporated herein by reference in its
30 entirety.

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

In some embodiments, the FM is a bifunctional or bispecific molecule, *e.g.*, it has at least
35 two different kinds of members, *e.g.*, with different functions and/or binding specificities. For

example, the FM can comprise, or consist of, a modulatory moiety, *e.g.*, a cytokine molecule, and a targeting moiety, wherein the modulatory moiety and the targeting moiety bind to two different cell surface targets or receptors in the same or different immune regulatory cells. In some embodiments, the modulatory moiety and the targeting moiety bind to two different targets on the same regulatory T cell. A bifunctional or bispecific molecule can further comprise additional moieties, *e.g.*, further binding and/or functional moieties. For example, the FM can be a multifunctional or multispecific molecule, *e.g.*, it is a trifunctional or trispecific, or a tetrafunctional or tetraspecific, fusion molecule.

In certain embodiments, the FM can be represented with the following formula in an N to C terminal orientation: R1-(optionally L1)-R2 or R2-(optionally L1)-R1; wherein R1 comprises a targeting moiety, L1 comprises a linker (*e.g.*, a peptide linker described herein), and R2 comprises a modulatory moiety, *e.g.*, a cytokine or growth factor molecule.

In some embodiments, the modulatory moiety, *e.g.*, the cytokine molecule, is functionally linked, *e.g.*, covalently linked (*e.g.*, by chemical coupling, genetic or protein fusion, noncovalent association or otherwise) to the targeting moiety. For example, the modulatory moiety can be covalently coupled indirectly, *e.g.*, via a linker to the targeting moiety. In some embodiments, the linker is chosen from: a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, or a non-helical linker. In some embodiments, the linker is a peptide linker. The peptide linker can be 1-30, 1-5, 5-20, 8-18, 10-15, or about 8, 9, 10, 11, 12, 13, 14, 15-20, 20-25, or 25-30 amino acids long. In some embodiments, the peptide linker can be 30 amino acids or longer; *e.g.*, 30-35, 35-40, 40-50 50-60 amino acids long. In some embodiments, the peptide linker comprises Gly and Ser, *e.g.*, a linker comprising the amino acid sequence (Gly₃-Ser)_n or (Gly₄-Ser)_n, wherein n indicates the number of repeats of the motif, *e.g.*, n=1, 2, 3, 4 or 5 (*e.g.*, a (Gly₃-Ser)₂ or (Gly₄-Ser)₂, or a (Gly₃-Ser)₃ or a (Gly₄-Ser)₃ linker). In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 36, 37, 38, or 39, or an amino acid sequence substantially identical thereto (*e.g.*, having 1, 2, 3, 4, or 5 amino acid substitutions). In one embodiment, the linker comprises an amino acid sequence GGGSGGGS (SEQ ID NO: 37). In another embodiment, the linker comprises amino acids derived from an antibody hinge region. In certain embodiments, the linker comprises amino acids derived from the hinge regions of IgG1, IgG2, IgG3, IgG4, IgGM, or IgGA antibodies. In embodiments, the linker comprises amino acids derived from an IgG hinge region, *e.g.*, an IgG1, IgG2 or IgG4 hinge region. For example, the linker comprises a variant amino acid sequence from an IgG hinge, *e.g.*, a variant having one or more cysteines replaced, *e.g.*, with serines. In some embodiments, the linker comprises DKTHTCPPSCAPE (SEQ ID NO: 91), having one or both cysteines replaced with another amino acid, *e.g.*, a serine.

In some embodiments, the linker comprises amino acids DKTHTSPPSPAP (SEQ ID NO: 38), EPKSSDKTHTSPPSPAPE (SEQ ID NO: 92), or a derivative thereof. In embodiments, the linker comprises amino acids derived from an IgG2 hinge region, *e.g.*, amino acids SVESPPSP (SEQ ID NO: 93), ERKSSVESPPSP (SEQ ID NO: 94), or a derivative thereof. In embodiments, the linker comprises amino acids derived from an IgG4 hinge region, *e.g.*, amino acids PPSPSSP (SEQ ID NO: 95), ESKYGPPSPSSP (SEQ ID NO: 96), or a derivative thereof.

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

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

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

In other embodiments, the linker is a reversible linker, in which the association between targeting moiety and the modulatory moiety can be reversed. In some embodiments the linker can be degraded by hydrolysis, reducing environments, or enzymes. In other embodiments, the linker can be degraded by hydrolysis in a pH-dependent manner. In other embodiments the linker can be reversed by redox state (*e.g.* linker containing disulfide bonds). In other embodiments the linker can be reversed by enzymatic cleavage. In some embodiments the linker can be cleaved by a matrix metalloproteinase (MMP; *e.g.* MMP-1, MMP-2, MMP-3, or MMP-9), a disintegrin and metalloprotease (ADAM; *e.g.* ADAM-17), a plasminogen activator (*e.g.* urokinase-type plasminogen activator, uPA, or tissue plasminogen activator, tPA), or a granzyme (*e.g.* granzyme A or granzyme B).

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

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this present

disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A depicts the current Treg manufacturing and ACT, and obstacles associated therewith.

FIG. 1B depicts exemplary fusion proteins of the present disclosure combining a cytokine and an immunoglobulin moiety for cell-surface targeting and stimulation.

FIG. 1C depicts an exemplary process for *ex vivo* expansion of Tregs.

FIG. 1D depicts an exemplary process for *in vivo* expansion of Tregs, without culturing *ex vivo*.

FIG. 1E depicts exemplary process for IL-2 loading-driven survival, expansion and engraftment of Tregs.

FIG. 1F depicts exemplary applications of Treg cell therapy in connection with various cytokines or growth factors.

FIG. 1G depicts generation of Treg specific scFvs via phase display and Treg-targeting of exemplary tethered fusions.

FIG. 1H depicts exemplary diseases that can benefit from Treg cell therapies disclosed herein.

FIGS. 2A-2C depict FMs comprising various antibody formats. **FIG. 2A** depicts a schematic of IL-15 FMs comprising various antibody formats. **FIGS 2B** and **2C** depict evaluation of FMs on CD8 T cell expansion in pulse assay using CellTiter Blue (**FIG. 2B**) or flow cytometry counting beads (**FIG. 2C**).

FIGS. 3A-3D depict a schematic for various fusion strategies between the IL-15/sushi complex and an anti-CD45 antibody. **FIGS 3A** and **3B** depict schematics for fusion of IL-15 to the N- or C-terminus of chBC8 IgG (**FIG. 3A**) or chBC8 Fab (**FIG. 3B**) light-chain. Antibody fusions comprise a complex with IL-15R α -sushi through a high-affinity interaction with IL-15. **FIGS 3C** and **3D** depict schematics for fusion of IL-15R α -sushi to the N- or C-terminus of chBC8 IgG (**FIG. 3C**) or chBC8Fab (**FIG. 3D**) light-chain. Antibody fusions comprise a complex with IL-15^{N72D} through a high-affinity interaction with IL-15R α -sushi.

FIG. 3E depicts a schematic design for a bivalent (bispecific) fusion, comprising 2 anti-

CD45 antibodies (h9.4 Fab and BC8 scFv) fused to the IL-15/sushi complex.

FIGS. 4A-4B depict the effect of FMs comprising various fusion strategies on CD8 T cell expansion. (**FIGS 4A** and **4B**) FMs comprising IL-15^{N72D} and chBC8 Fab (**FIG. 4A**) or chBC8 IgG (**FIG. 4B**) were evaluated for T cell expansion in pulse bioassay. CD8 T cell proliferation was analyzed using CellTiter Blue; IL15^{N72D}/sushi^{L771}-Fc and unstimulated T-cells (neg ctrl) were included for comparison.

FIGS. 5A-5D depict biological activity of FMs comprising wild-type or mutated IL-15 and various fusion strategies of IL-15/sushi complex to anti-CD45 antibody. **FIGS. 5A** and **5B** depict FMs comprising wild-type IL-15 or IL-15^{N72D} and chBC8 Fab (**FIG. 5A**) or chBC8 IgG (**FIG. 5B**) that were evaluated for CD8 T cell expansion in pulse assay using CellTiter Blue three days after pulse incubation with FMs. **FIGS. 5C** and **5D** depict the evaluation of FMs comprising chBC8 Fab (**FIG. 5C**) or chBC8 IgG (**FIG. 5D**) on CD8 T cell expansion in a constant exposure, *e.g.*, static, assay format using CellTiter Blue following incubation with FMs for three days.

FIGS. 6A-6B depict FMs comprising C-terminal fusion to chBC8 IgG light- or heavy-chain. **FIG. 6A** depicts schematics of FMs comprising IL-15 fused to the C-terminus of chBC8 IgG light- or heavy-chain and a noncovalent complex between IL-15 and IL-15R α -sushi. **FIG. 6B** depicts effects of FMs on CD8 T cell expansion in a pulse bioassay.

FIGS. 7A-7C depict FMs require functional binding of the antibody fragment for improved activity. **FIGS. 7A** and **7B** depict IL-15 FMs comprising chBC8 Fab (**FIG. 7A**) or chBC8 IgG (**FIG. 7B**) in the presence or absence of soluble BC8 IgG competitor that were evaluated for expansion of CD8 T cells in pulse assay using CellTiter Blue. **FIG. 7C** depicts evaluation of IL-15 FM comprising a humanized BC8 Fab fragment or a humanized BC8 Fab fragment containing a mutated CDR-H3 that ablates binding to CD45 for CD8 T cell expansion in pulse bioassay.

FIGS. 8A-8C depict cell surface loading and persistence of FMs. **FIG. 8A** depicts flow cytometry histograms showing MFI after pulse of FMs as detected by staining with fluorescently labeled anti-IL15 or anti-IgG antibodies. **FIG. 8B** depicts persistence of cell surface staining of IL-15 over time. **FIG. 8C** depicts cell density over time, measured using flow cytometry counting beads.

FIG. 9A-9B depict evaluation of FMs comprising varied linker composition between IL-15 and chBC8. **FIG. 9A** depicts amino acid sequences of the three different linkers for fusion of IL-15 to the C-terminus of chBC8 Fab light-chain. **FIG. 9B** depicts evaluation of FMs comprising different linker compositions for CD8 T cell expansion in pulse bioassay using CellTiter Blue.

FIGS. 10A-10B depict evaluation of FMs comprising alternative CD45 antibody clones. **FIG. 10A** depicts binding affinity of various CD45 antibody clones to CD45 expressed on T cells. **FIG. 10B** depicts evaluation of IL-15 FMs comprising different anti-CD45 antibody clones in pulse bioassay using CellTiter Blue.

FIGS. 11A-11B depict FMs comprising antibodies targeting CD8, CD11a, or CD18. **FIG. 11A** depicts a schematic of IL-15 FMs comprising fusion of IL-15 to the light-chain C-terminus of a Fab antibody fragment targeting alternative cell surface receptors; FMs comprise a noncovalent association between IL-15 and IL-15R α -sushi. **FIG. 11B** depicts evaluation of IL-15 FMs for CD8 T cell expansion in pulse bioassay using CellTiter Blue.

FIGS. 12A-12B depict evaluation of FM potency comprising anti-CD8 on CD4 or CD8 T cells. **FIG. 12A** depicts a schematic of IL-15 FM comprising fusion of IL-15 to the C-terminus of an anti-CD8 Fab light-chain, and a noncovalent association with IL-15R α -sushi. **FIG. 12B** depicts evaluation of potency of anti-CD8 FM on purified CD4 or CD8 T cells in pulse bioassay using CellTiter Blue. CD4 or CD8 T cells were purified from total T cells (which naturally comprise mixtures of CD4 and CD8 T cells) using magnetic bead sorting.

FIGS. 13A-13C depict graphs showing that nanoparticles comprising IL-15 FMs support T cell expansion. **FIGS. 13A** and **13B** depict crosslinking of FM into protein nanogels analyzed by size-exclusion chromatography (**FIG. 13A**); protein nanogels comprising IL15^{N72D}/sushiL77I-Fc are shown for comparison (**FIG. 13B**). Backpacks comprising IL-15 FM or IL15^{N72D}/sushiL77I-Fc were evaluated for CD8 T cell expansion in pulse bioassay (**FIG. 13C**). Cell density was analyzed using flow cytometry counting beads; data are presented as fold expansion.

FIG. 14 shows example immunotargeted surface receptors and cytokines, illustrating versatility of the platform.

FIGS. 15A-15G show tethered fusion platform enables selective cell targeting and improved expansion of CD8 T cells in cultures of activated total CD3 human T cells by pulse incubation with CD8-targeted FMs.

FIGS. 16A-16E shows cellular activities of various mutated forms of IL-15 and surface persistence of FMs comprising IL-15 mutants.

FIGS. 17A-17C shows selective cell targeting and CD8 T cell expansion by CD8-targeted FMs comprising wild-type or mutated IL-15.

FIG. 17D shows surface persistence of CD45-targeted FM comprising wild-type or mutated IL-15.

FIG. 18 shows enrichment of Tregs following bead-based magnetic sorting for CD4⁺/CD25⁺/CD127^{dim} cells. These Treg enriched CD4s were used to test *in vitro* and *in vivo* expansion driven by IL-2 tethered fusions.

FIG. 19 depicts a strategy for limiting inflammatory and auto-immune diseases via specific targeting of IL-2 to Treg cells.

FIG. 20 shows titration of Fab-HSA-IL2 on Treg-enriched CD4s. Fab-HSA-IL2 shows titratable surface binding immediately post-loading and for more than 3 days. In addition, Fab-HSA-IL2 – loaded cells show dose-dependent proliferation, and even very low doses maintains viability.

FIG. 21 relates surface staining of pulsed IL-2 tethered fusions (left panel) with proliferation and survival of Treg-enriched cells (right panels). All three tested tethered fusions exhibited strong staining and drove proliferation and survival of the cells. Proliferation and survival were comparable to constant stimulation with recombinant IL-2.

FIG. 22 illustrates the effects of pulsed IL-2 tethered fusions over time. Cells pulsed with any of the TF constructs showed equal or better expansion compared to constant stimulation with rhIL2 through 6 days post-pulse, and comparable expansion and survival out to 9 days. IL-2 tethered fusions promote proliferation and long-term survival of Treg-enriched cell populations *in vitro*.

FIG. 23 shows increased expansion of IL-2 tethered fusion pulsed Treg-enriched CD4 cells in the blood of NSG mice. There were increased numbers of CD4 cells in the IL-2 tethered fusion-pulsed group throughout the course of the experiment. This indicates that IL-2 tethered fusions can increase expansion and engraftment *in vivo*.

FIG. 24 shows that the Fab-HSA-IL2 preferentially supports proliferation and survival of loaded cells. Despite having some pro-proliferative effect on non-pulsed cells, there is still stronger effects on the pulsed cells, and the CD4:CD8 ratio is higher in the Fab-HSA-IL2 group than the control group.

FIG. 25 provides a schematic describing how dual-targeting, and cytokine affinity modulation will increase on-target effects while reducing off-target effects of IL-2 antibody tethered fusion molecules.

DETAILED DESCRIPTION

The present disclosure provides, *inter alia*, compositions and methods for preparation and use of fusion molecules or FMs. An “FM” as described herein includes a modulatory moiety, *e.g.*, a cytokine or growth factor molecule (*e.g.*, a biologically active cytokine), and a targeting moiety, *e.g.*, an antibody molecule (*e.g.*, an antibody or antibody fragment) capable of

binding to Tregs. In embodiments, the modulatory moiety and the targeting moiety are operably linked or tethered together (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise). As such, the fusion molecules are also referred to as “tethered fusions” herein. In some embodiments, the targeting moiety is capable of binding to Treg cell surface target, thereby targeting the modulatory moiety, *e.g.*, cytokine molecule, to the Treg.

In embodiments, the Treg-targeting tethered fusions disclosed herein can be used to improve engraftment, survival and efficacy of adoptive cell therapies, *e.g.*, immunosuppressive cell therapy. In various embodiments, the Treg-targeting tethered fusions, or the cytokine molecules alone (*e.g.*, without Treg-targeting moiety) can be combined with nanoparticle, nanogel and liposome technology as disclosed in, *e.g.*, U.S. Publication No. 2017/0080104, U.S. Patent No. 9,603,944, U.S. Publication No. 2014/0081012, and PCT Application No. PCT/US2017/037249, each of which is incorporated herein by reference in its entirety.

The Treg-targeting tethered fusions and related immunosuppressive cell therapy disclosed herein are advantageous compared to conventional Treg manufacturing and ACT. Conventional Treg manufacturing requires GMP sorting of pure Tregs. In contrast, as shown in FIG. 1C, using Treg-targeting tethered fusions such as Treg-targeted IL-2, selective expansion (*ex vivo*) of Tregs from a mixed population of peripheral blood mononuclear cells (PBMC) can be achieved, thereby avoiding expensive and time-consuming GMP sorting of Tregs. Furthermore, by further subjecting the cells to loading (*e.g.*, with IL-2 backpacks), survival and activity of the cells post-thaw can be improved.

Conventional Treg manufacturing additionally requires long and costly *ex vivo* expansion. In contrast, as shown in FIG. 1D, sorted Tregs or PBMC without sorting can be subject to loading (*e.g.*, CD3/CD28/IL-2) followed by direct infusion to patient, whereby the Tregs are expanded *in vivo*, without *in vitro* culturing. Furthermore, loaded Tregs (*e.g.*, with IL-2 backpacks), prepared either from *ex vivo* expansion or without culturing, can receive pro-survival signal *in vivo*, increasing expansion and engraftment (FIG. 1E).

Without wishing to be bound by theory, binding of the tethered fusions to Tregs, *e.g.*, via Treg cell surface target is believed to increase the concentration, *e.g.*, the concentration over time, of the cytokine or growth factor molecule, on the surface of the Treg. This can result in an effect (*e.g.*, stimulatory) on the Treg itself bound by the tethered fusions (autocrine signaling), or on another (*e.g.*, neighboring) immune cell (paracrine signaling). In embodiments, the targeting moiety results in an increase in one or more of: binding, availability, activation and/or signaling of the cytokine or growth factor on the Treg, *e.g.*, over a specified amount of time. For example, CD4/CD25 targeting moieties can result in binding specifically to Tregs. CD45 targeting moieties can be used to target Tregs, although not specifically. In embodiments, the FM does

not substantially interfere with the signaling function of the cytokine or growth factor. Such targeting effect results in localized and prolonged stimulation of proliferation and activation of the Tregs, thus inducing controlled Treg expansion and/or activation, and Treg-mediated suppression of an immune response.

5 In some embodiments, the targeting moiety can be an antibody molecule or a ligand molecule that binds to a Treg cell surface receptor, *e.g.*, CD45, CD4, CD25, CD39, or Neuropilin 1 (NRP1). In some embodiments, the Treg cell surface receptor is selected from one or more of CD4, CD45, CD3, CD2, CD25, CD127, CD197 (CCR7), CXCR3, CXCR4, CXCR5, CD38, CD27, CCR4, CCR5, CD137, CD39, CCR4, CCR5, CCR6 (CD196), CCR8, CCR10,
10 OX40, GITR, CTLA4, LAG3, CD73, CD103, CD62L, CCR2, CCR9, NRP1, CD8, CD11a, CD18, or variants thereof.

In some embodiments, the cytokine or growth factor molecule is selected from one or more IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, or TGF- β , or variants thereof. In some embodiments, the cytokine or growth factor molecule is selected from one or
15 more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin (Areg), IL-33, TGF- β , IL-7, or IL-21, or variants thereof. In some embodiments, the cytokine or growth factor molecule is selected from one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, TGF- β , IL-7, IL-21, IL-6, IL-12, or IL-23, or variants thereof. For example, as shown in FIG. 1F, TGF- β , IL10 and IL-33 can promote Treg differentiation and prevent conversion to
20 inflammatory cells. IL-35 and IL-10 can promote iTreg in the periphery. Areg drives tissue regeneration during inflammation.

In one exemplary embodiment, the targeting moiety is derived from an anti-CD45 antibody molecule and the cytokine molecule is IL-2 or variants thereof.

Exemplary applications of immunosuppressive cell therapy include allo-immune
25 diseases, auto-immune diseases, allergy, and inflammatory diseases (FIG. 1H). Allo-immune diseases can include, for example, organ transplant rejection, graft versus host disease or rejection (GVHD) (*e.g.*, post-allogeneic hematopoietic stem cell transplant (HSCT) and other post-allogeneic stem cell transplantation (SCT), and rejection of autologous stem cells and/or genetically modified autologous stem cells (*e.g.*, where CRISPR (Clustered Regularly
30 Interspaced Short Palindromic Repeats) or the like is used to modify cells *ex vivo* to fix some deficiency which are then transplanted back into the patient, resulting in rejection due to any new or modified proteins being recognized as antigenic). Treg number has been shown to correlate with GVHD control and organ transplant tolerance. For allergy, expansion of antigen-specific Tregs has been shown to associate with loss of milk allergy, and Treg depletion
35 enhances allergy. Auto-immune diseases include Type 1 diabetes, Multiple Sclerosis and

alopecia. These diseases have functional defects in Tregs, phenotypic instability and decreased number of Tregs. Inflammatory diseases include Crohn's Disease, Inflammatory Bowel Disease, Rheumatoid Arthritis and Lupus. These diseases can have increased Treg number, but are marked by decreased Treg function. Tregs have been shown to control/prevent these diseases in mouse models.

Definitions

Certain terms are defined herein below. Additional definitions are provided throughout the application.

As used herein, the articles "a" and "an" refer to one or more than one, *e.g.*, to at least one, of the grammatical object of the article. The use of the words "a" or "an" when used in conjunction with the term "comprising" herein may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

As used herein, "about" and "approximately" generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Exemplary degrees of error are within 20 percent (%), typically, within 10%, and more typically, within 5% of a given range of values. The term "substantially" means more than 50%, preferably more than 80%, and most preferably more than 90% or 95%.

As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are present in a given embodiment, yet open to the inclusion of unspecified elements.

As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the disclosure.

The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

The term "autologous" refers to any material derived from the same individual to whom it is later to be re-introduced into the individual.

The term "allogeneic" refers to any material derived from a different animal of the same species as the individual to whom the material is introduced.

"Antibody" or "antibody molecule" as used herein refers to a protein, *e.g.*, an immunoglobulin chain or fragment thereof, comprising at least one immunoglobulin variable domain sequence. An antibody molecule encompasses antibodies (*e.g.*, full-length antibodies) and antibody fragments. In an embodiment, an antibody molecule comprises an antigen binding

or functional fragment of a full length antibody, or a full length immunoglobulin chain. For example, a full-length antibody is an immunoglobulin (Ig) molecule (e.g., IgG) that is naturally occurring or formed by normal immunoglobulin gene fragment recombinatorial processes). In embodiments, an antibody molecule refers to an immunologically active, antigen-binding

5 portion of an immunoglobulin molecule, such as an antibody fragment. An antibody fragment, e.g., functional fragment, is a portion of an antibody, e.g., Fab, Fab', F(ab')₂, F(ab)₂, variable fragment (Fv), domain antibody (dAb), or single chain variable fragment (scFv). A functional antibody fragment binds to the same antigen as that recognized by the intact (e.g., full-length) antibody. The terms "antibody fragment" or "functional fragment" also include isolated

10 fragments consisting of the variable regions, such as the "Fv" fragments consisting of the variable regions of the heavy and light chains or recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("scFv proteins"). In some embodiments, an antibody fragment does not include portions of antibodies without antigen binding activity, such as Fc fragments or single amino acid residues. Exemplary

15 antibody molecules include full length antibodies and antibody fragments, e.g., dAb (domain antibody), single chain, Fab, Fab', and F(ab')₂ fragments, and single chain variable fragments (scFvs). The terms "Fab" and "Fab fragment" are used interchangeably and refer to a region that includes one constant and one variable domain from each heavy and light chain of the antibody, i.e., V_L, C_L, V_H, and C_H1.

20 As used herein, an "immunoglobulin variable domain sequence" refers to an amino acid sequence which can form the structure of an immunoglobulin variable domain. For example, the sequence may include all or part of the amino acid sequence of a naturally-occurring variable domain. For example, the sequence may or may not include one, two, or more N- or C-terminal amino acids, or may include other alterations that are compatible with formation of the protein

25 structure.

In embodiments, an antibody molecule is monospecific, *e.g.*, it comprises binding specificity for a single epitope. In some embodiments, an antibody molecule is multispecific, *e.g.*, it comprises more than one immunoglobulin variable domain sequence, where a first immunoglobulin variable domain sequence has binding specificity for a first epitope and a

30 second immunoglobulin variable domain sequence has binding specificity for a second epitope. In some embodiments, an antibody molecule is a bispecific antibody molecule. "Bispecific antibody molecule" as used herein refers to an antibody molecule that has specificity for at least two (*e.g.*, two, three, four, or more) epitopes and/or antigens.

"Antigen" (Ag) as used herein refers to a macromolecule, including all proteins or

35 peptides. In some embodiments, an antigen is a molecule that can provoke activation of certain

immune cells (including immune regulatory cells) and/or antibody generation. Any macromolecule, including almost all proteins or peptides, can be an antigen. Antigens can also be derived from genomic recombinant or DNA. For example, any DNA comprising a nucleotide sequence or a partial nucleotide sequence that encodes a protein capable of eliciting an immune response encodes an “antigen.” In embodiments, an antigen does not need to be encoded solely by a full-length nucleotide sequence of a gene, nor does an antigen need to be encoded by a gene at all. In embodiments, an antigen can be synthesized or can be derived from a biological sample, *e.g.*, a tissue sample, a tumor sample, a cell, or a fluid with other biological components.

The “antigen-binding site” or “antigen-binding fragment” or “antigen-binding portion” (used interchangeably herein) of an antibody molecule refers to the part of an antibody molecule, *e.g.*, an immunoglobulin (Ig) molecule such as IgG, that participates in antigen binding. In some embodiments, the antigen-binding site is formed by amino acid residues of the variable (V) regions of the heavy (H) and light (L) chains. Three highly divergent stretches within the variable regions of the heavy and light chains, referred to as hypervariable regions, are disposed between more conserved flanking stretches called “framework regions” (FRs). FRs are amino acid sequences that are naturally found between, and adjacent to, hypervariable regions in immunoglobulins. In embodiments, in an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface, which is complementary to the three-dimensional surface of a bound antigen. The three hypervariable regions of each of the heavy and light chains are referred to as “complementarity-determining regions,” or “CDRs.” The framework region and CDRs have been defined and described, *e.g.*, in Kabat, E.A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al. (1987) J. Mol. Biol. 196:901-917. Each variable chain (*e.g.*, variable heavy chain and variable light chain) is typically made up of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the amino acid order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Variable light chain (VL) CDRs are generally defined to include residues at positions 27-32 (CDR1), 50-56 (CDR2), and 91-97 (CDR3). Variable heavy chain (VH) CDRs are generally defined to include residues at positions 27-33 (CDR1), 52-56 (CDR2), and 95-102 (CDR3). One of ordinary skill in the art would understand that the loops can be of different length across antibodies and the numbering systems such as the Kabat or Chotia control so that the frameworks have consistent numbering across antibodies.

In some embodiments, the antigen-binding fragment of an antibody (*e.g.*, when included

as part of the fusion molecule of the present disclosure) can lack or be free of a full Fc domain. In certain embodiments, an antibody-binding fragment does not include a full IgG or a full Fc but may include one or more constant regions (or fragments thereof) from the light and/or heavy chains. In some embodiments, the antigen-binding fragment can be completely free of any Fc domain. In some embodiments, the antigen-binding fragment can be substantially free of a full Fc domain. In some embodiments, the antigen-binding fragment can include a portion of a full Fc domain (e.g., CH2 or CH3 domain or a portion thereof). In some embodiments, the antigen-binding fragment can include a full Fc domain. In some embodiments, the Fc domain is an IgG domain, e.g., an IgG1, IgG2, IgG3, or IgG4 Fc domain. In some embodiments, the Fc domain comprises a CH2 domain and a CH3 domain.

As used herein, “backpacks” refer to protein clusters that can be prepared by reacting, e.g., cross-linking various therapeutic protein monomers. Optionally, a surface modification such as polycation can be introduced on the protein cluster, which can be loaded onto cell surface as cell membranes are negatively charged. Exemplary backpacking technology is disclosed in, e.g., U.S. Publication No. 2017/0080104, U.S. Patent No. 9,603,944, U.S. Publication No. 2014/0081012, U.S. Provisional App. No. 62/554,058 filed September 5, 2017 and PCT Application No. PCT/US2017/037249, each of which is incorporated herein by reference in its entirety.

As used herein, a “cytokine molecule” refers to a full length, a fragment, or a variant of a naturally-occurring, wild type cytokine (including fragments and functional variants thereof having at least 10% of the activity of the naturally-occurring cytokine molecule). In embodiments, the cytokine molecule has at least 30, 50, or 80% of the activity, e.g., the immunomodulatory activity, of the naturally-occurring molecule. In embodiments, the cytokine molecule further comprises a receptor domain, e.g., a cytokine receptor domain, optionally, coupled to an immunoglobulin Fc region. In other embodiments, the cytokine molecule is coupled to an immunoglobulin Fc region. In other embodiments, the cytokine molecule is coupled to an antibody molecule (e.g., an immunoglobulin Fab or scFv fragment, a Fab fragment, a FAB₂ fragment, or an affibody fragment or derivative, e.g. a sdAb (nanobody) fragment, a heavy chain antibody fragment, single-domain antibody, a bispecific or multispecific antibody), or non-antibody scaffolds and antibody mimetics (e.g., lipocalins (e.g. anticalins), affibodies, fibronectin (e.g., monobodies or Adnectins), knottins, ankyrin repeats (e.g. DARPins), and A domains (e.g. avimers)).

A “cytokine agonist,” as used herein can include an agonist of a cytokine receptor, e.g., an antibody molecule (e.g., an agonistic antibody) to a cytokine receptor that elicits at least one activity of a naturally-occurring cytokine.

As used herein, an “immune cell” refers to any of various cells that function in the immune system, *e.g.*, to protect against agents of infection and foreign matter. In embodiments, this term includes leukocytes, *e.g.*, neutrophils, eosinophils, basophils, lymphocytes, and monocytes. The term “immune cell” includes immune regulatory cells (*e.g.*, Tregs) and immune effector cells described herein. “Immune cell” also refers to modified versions of cells involved in an immune response, *e.g.* modified NK cells, including NK cell line NK-92 (ATCC cat. No. CRL-2407), haNK (an NK-92 variant that expresses the high-affinity Fc receptor FcγRIIIa (158V)) and taNK (targeted NK-92 cells transfected with a gene that expresses a CAR for a given tumor antigen), *e.g.*, as described in Klingemann *et al. supra*.

“Immune effector cell,” as that term is used herein, refers to a cell that is involved in an immune response, *e.g.*, in the promotion of an immune effector response. Examples of immune effector cells include, but are not limited to, T cells, *e.g.*, CD4⁺T cells, CD8⁺ T cells, alpha T cells, beta T cells, gamma T cells, and delta T cells; B cells; natural killer (NK) cells; natural killer T (NKT) cells; dendritic cells; and mast cells. In some embodiments, the immune cell is an immune cell (*e.g.*, T cell or NK cell) that comprises, *e.g.*, expresses, a Chimeric Antigen Receptor (CAR), *e.g.*, a CAR that binds to a cancer antigen. In other embodiments, the immune cell expresses an exogenous high affinity Fc receptor. In some embodiments, the immune cell comprises, *e.g.*, expresses, an engineered T-cell receptor. In some embodiments, the immune cell is a tumor infiltrating lymphocyte. In some embodiments, the immune cells comprise a population of immune cells and comprise T cells that have been enriched for specificity for a tumor-associated antigen (TAA), *e.g.* enriched by sorting for T cells with specificity towards MHCs displaying a TAA of interest, *e.g.* MART-1. In some embodiments, immune cells comprise a population of immune cells and comprise T cells that have been “trained” to possess specificity against a TAA by an antigen presenting cell (APC), *e.g.* a dendritic cell, displaying TAA peptides of interest. In some embodiments, the T cells are trained against a TAA chosen from one or more of MART-1, MAGE-A4, NY-ESO-1, SSX2, Survivin, or others. In some embodiments the immune cells comprise a population of T cells that have been “trained” to possess specificity against a multiple TAAs by an APC, *e.g.* a dendritic cell, displaying multiple TAA peptides of interest. In some embodiments, the immune cell is a cytotoxic T cell (*e.g.*, a CD8⁺ T cell). In some embodiments, the immune cell is a helper T cell, *e.g.*, a CD4⁺ T cell.

The term “effector function” or “effector response” refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines.

“Immune regulatory cell”, as used herein, refers to an immune cell that participates in immunomodulation or suppression of immune responses, *e.g.*, a regulatory T cell or regulatory

B cell.

“Tregs” or “Treg cells” refer to Regulatory T cells. Regulatory T cells are a class of T cells that suppress the activity of other immune cells, and are defined using flow cytometry by the cell marker phenotype CD4+CD25+FOXP3+. Because FOXP3 is an intracellular protein and requires cell fixation and permeabilization for staining, the cell surface phenotype CD4+CD25+CD127⁻ can be used for defining live Tregs. Tregs also include various Treg subclasses, such as tTregs (thymus-derived) and pTregs (peripherally-derived, differentiated from naïve T cells in the periphery). All Tregs express the IL2R $\alpha\beta\gamma$ receptor, do not produce their own IL-2 and are dependent on IL-2 for growth, and someone skilled in the art will recognize that both classes will be selectively activated by an IL2R $\alpha\beta\gamma$ selective agonist.

“Cytotoxic T lymphocytes” (CTLs) as used herein refer to T cells that have the ability to kill a target cell. CTL activation can occur when two steps occur: 1) an interaction between an antigen-bound MHC molecule on the target cell and a T cell receptor on the CTL is made; and 2) a costimulatory signal is made by engagement of costimulatory molecules on the T cell and the target cell. CTLs then recognize specific antigens on target cells and induce the destruction of these target cells, *e.g.*, by cell lysis. In some embodiments, the CTL expresses a CAR. In some embodiments, the CTL expresses an engineered T-cell receptor.

“Modulation” or “modulatory” as used herein refers to any alteration of an existing or potential immune responses against an autoimmune or allergy provoking epitope, including, *e.g.*, nucleic acids, lipids, phospholipids, carbohydrates, self-polypeptides, protein complexes, or ribonucleoprotein complexes, that occurs as a result of administration of the compositions disclosed herein. Such modulation includes any alteration in presence, capacity, or function of an immune regulatory cell, such as Treg, involved in, or capable of being involved in, an immune response. “Modulation” includes any change imparted on an existing immune response, a developing immune response, a potential immune response, or the capacity to induce, regulate, influence, or respond to an immune response. Modulation includes any alteration in the expression and/or function of genes, proteins and/or other molecules in immune cells as part of an immune response.

“Modulation of an immune response” includes, for example, the following: elimination, deletion, or sequestration of immune cells; induction or generation of immune cells that can modulate the functional capacity of other cells such as autoreactive lymphocytes, antigen presenting cells, or inflammatory cells; induction of an unresponsive state in immune cells (*i.e.*, anergy); increasing, decreasing, or changing the activity or function of immune cells or the capacity to do so, including, but not limited to, altering the pattern of proteins expressed by these cells. Examples include altered production and/or secretion of certain classes of molecules such

as cytokines, chemokines, growth factors, transcription factors, kinases, costimulatory molecules, or other cell surface receptors; or any combination of these modulatory events. In some embodiments, modulation includes suppression or immunosuppression of certain immune responses, e.g., in conditions or diseases where the immune system displays an excessive or overactive response such as autoimmune diseases, allergies, bone marrow or organ transplant, and other inflammatory diseases. Suppression or immunosuppression as used herein includes reduction, inhibition and lessening of an immune response.

"Sample" or "tissue sample" refers to a biological sample obtained from a tissue or bodily fluid of a subject or patient. The source of the tissue sample can be solid tissue as from a fresh, frozen and/or preserved organ, tissue sample, biopsy, or aspirate; blood or any blood constituents (e.g., serum, plasma); bone marrow or any bone marrow constituents; bodily fluids such as urine, cerebral spinal fluid, whole blood, plasma and serum. The sample can include a non-cellular fraction (e.g., urine, plasma, serum, or other non-cellular body fluid). In other embodiments, the body fluid from which the sample is obtained from an individual comprises blood (e.g., whole blood).

The term "subject" includes living organisms in which an immune response can be elicited (e.g., mammals, human). In one embodiment, the subject is a patient, e.g., a patient in need of immune cell therapy. In another embodiment, the subject is a donor, e.g. an allogenic donor of immune cells, e.g., intended for allogenic transplantation.

The compositions and methods of the present disclosure encompass polypeptides and nucleic acids having the sequences specified, or sequences substantially identical or similar thereto, e.g., sequences at least 85%, 90%, 95% identical or higher to the sequence specified. In the context of an amino acid sequence, the term "substantially identical" is used herein to refer to a first amino acid that contains a sufficient or minimum number of amino acid residues that are i) identical to, or ii) conservative substitutions of aligned amino acid residues in a second amino acid sequence such that the first and second amino acid sequences can have a common structural domain and/or common functional activity. For example, amino acid sequences that contain a common structural domain having at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to a reference sequence, e.g., a sequence provided herein.

In the context of nucleotide sequence, the term "substantially identical" is used herein to refer to a first nucleic acid sequence that contains a sufficient or minimum number of nucleotides that are identical to aligned nucleotides in a second nucleic acid sequence such that the first and second nucleotide sequences encode a polypeptide having common functional activity, or encode a common structural polypeptide domain or a common functional polypeptide activity.

For example, nucleotide sequences having at least about 85%, 90%, 91%, 92%, 93%, 94%,

95%, 96%, 97%, 98% or 99% identity to a reference sequence, *e.g.*, a sequence provided herein. Calculations of homology or sequence identity between sequences (the terms are used interchangeably herein) are performed as follows.

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology").

The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch ((1970) *J. Mol. Biol.* 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that should be used unless otherwise specified) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5. The percent identity between two amino acid or nucleotide sequences can be determined using the algorithm of E. Meyers and W. Miller ((1989) *CABIOS*, 4:11-17) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences described herein can be used as a "query

sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12
5 to obtain nucleotide sequences homologous to a nucleic acid (*e.g.*, SEQ ID NO: 1) molecules of the disclosure. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules of the disclosure. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25:3389-3402. When utilizing
10 BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. It is understood that the molecules of the present disclosure may have additional conservative or non-essential amino acid substitutions, which do not have a substantial effect on their functions.

The term "amino acid" is intended to embrace all molecules, whether natural or
15 synthetic, which include both an amino functionality and an acid functionality and capable of being included in a polymer of naturally-occurring amino acids. Exemplary amino acids include naturally-occurring amino acids; analogs, derivatives and congeners thereof; amino acid analogs having variant side chains; and all stereoisomers of any of any of the foregoing. As used herein the term "amino acid" includes both the D- or L- optical isomers and peptidomimetics.

A "conservative amino acid substitution" is one in which the amino acid residue is
20 replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine,
25 threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine).

The term "functional variant" or "variant" or "variant form" in the context of a polypeptide refers to a polypeptide that is capable of having at least 10% of one or more
30 activities of the naturally-occurring sequence. In some embodiments, the functional variant has substantial amino acid sequence identity to the naturally-occurring sequence, or is encoded by a substantially identical nucleotide sequence, such that the functional variant has one or more activities of the naturally-occurring sequence.

The term "molecule" as used herein can refer to a polypeptide or a nucleic acid encoding
35 a polypeptide, as indicated by the context. This term includes full length, a fragment or a variant

of a naturally-occurring, wild type polypeptide or nucleic acid encoding the same, *e.g.*, a functional variant, thereof. In some embodiments, the variant is a derivative, *e.g.*, a mutant, of a wild type polypeptide or nucleic acid encoding the same.

The terms "polypeptide", "peptide" and "protein" (if single chain) are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. The polypeptide can be isolated from natural sources, can be produced by recombinant techniques from a eukaryotic or prokaryotic host, or can be a product of synthetic procedures.

The terms "nucleic acid," "nucleic acid sequence," "nucleotide sequence," or "polynucleotide sequence," and "polynucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. The polynucleotide may be either single-stranded or double-stranded, and if single-stranded may be the coding strand or non-coding (antisense) strand. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. The nucleic acid may be a recombinant polynucleotide, or a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in a non-natural arrangement.

The term "parent polypeptide" refers to a wild-type polypeptide and the amino acid sequence or nucleotide sequence of the wild-type polypeptide is part of a publicly accessible protein database (*e.g.*, EMBL Nucleotide Sequence Database, NCBI Entrez, ExPasy, Protein Data Bank and the like).

The term "mutant polypeptide" or "polypeptide variant" or "mutin" refers to a form of a polypeptide, wherein its amino acid sequence differs from the amino acid sequence of its corresponding wild-type (parent) form, naturally existing form or any other parent form. A mutant polypeptide can contain one or more mutations, *e.g.*, replacement, insertion, deletion, etc. which result in the mutant polypeptide.

The term "corresponding to a parent polypeptide" (or grammatical variations of this term) is used to describe a polypeptide of the present disclosure, wherein the amino acid sequence of the polypeptide differs from the amino acid sequence of the corresponding parent polypeptide only by the presence of at least amino acid variation. Typically, the amino acid

sequences of the variant polypeptide and the parent polypeptide exhibit a high percentage of identity. In one example, "corresponding to a parent polypeptide" means that the amino acid sequence of the variant polypeptide has at least about 50% identity, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or at least about 98% identity to the amino acid sequence of the parent polypeptide. In another example, the nucleic acid sequence that encodes the variant polypeptide has at least about 50% identity, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or at least about 98% identity to the nucleic acid sequence encoding the parent polypeptide.

The term "introducing (or adding etc.) a variation into a parent polypeptide" (or grammatical variations thereof), or "modifying a parent polypeptide" to include a variation (or grammatical variations thereof) do not necessarily mean that the parent polypeptide is a physical starting material for such conversion, but rather that the parent polypeptide provides the guiding amino acid sequence for the making of a variant polypeptide. In one example, "introducing a variant into a parent polypeptide" means that the gene for the parent polypeptide is modified through appropriate mutations to create a nucleotide sequence that encodes a variant polypeptide. In another example, "introducing a variant into a parent polypeptide" means that the resulting polypeptide is theoretically designed using the parent polypeptide sequence as a guide. The designed polypeptide may then be generated by chemical or other means.

The term "isolated," as used herein, refers to material that is removed from its original or native environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated by human intervention from some or all of the co-existing materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of the environment in which it is found in nature.

Various aspects of the disclosure are described in further detail below. Additional definitions are set out throughout the specification.

Modulatory Moiety

An modulatory moiety can be any agent or molecule capable of modulating, *e.g.*, stimulating or suppressing, a function or activity of another agent, molecule or cell. In some embodiments, the modulatory moiety reduces, inhibits or suppresses a function or activity. In some embodiments, the modulatory moiety enhances, activates or stimulates a function or activity. In certain embodiments, the modulatory moiety activates or enhances proliferation of

immune regulatory cells. In one embodiment, the modulatory moiety activates or enhances proliferation of Tregs, which in turn, regulates, reduces or suppresses certain immune response, thereby providing an immunosuppressive activity.

Various modulatory moieties can be used. In some embodiments, the modulatory moiety
5 can be a cytokine or growth factor.

Cytokines are proteinaceous signaling compounds that are mediators of the immune response. They control many different cellular functions including proliferation, differentiation and cell survival/apoptosis; cytokines are also involved in several pathophysiological processes including viral infections and autoimmune diseases. Cytokines are synthesized under various
10 stimuli by a variety of cells, including those of both the innate (monocytes, macrophages, dendritic cells) and adaptive (T- and B-cells) immune systems. Cytokines can be classified into two groups: pro- and anti-inflammatory. Pro-inflammatory cytokines, including IFN γ , IL-1 (IL-1 α , IL-1 β), IL-6 and TNF- α , are predominantly derived from the innate immune cells and Th1 cells. Anti-inflammatory cytokines, including IL-10, IL-4, IL-13 and IL-5, are synthesized from
15 Th2 immune cells.

Growth factors are capable of stimulating cellular growth, proliferation, healing, and cellular differentiation. Usually it is a protein or a steroid hormone. Growth factor is sometimes used interchangeably with the term cytokine.

The present disclosure provides, *inter alia*, FMs (*e.g.*, FM), that include or are
20 engineered to contain one or more cytokine/growth factor molecules, *e.g.*, cytokines, growth factors and/or functional variants thereof. Accordingly, in some embodiments, the cytokine molecule is an interleukin (IL) or a variant, *e.g.*, a functional variant thereof. In some embodiments, the cytokine is a proinflammatory interleukin. In other embodiments, the cytokine is anti-inflammatory.

In embodiments, the cytokine molecule is full length, a fragment or a variant of a cytokine, *e.g.*, a cytokine comprising one or more mutations. In some embodiments, the cytokine molecule of the FM includes an immunomodulatory cytokine, *e.g.*, a pro-inflammatory cytokine or an anti-inflammatory cytokine. In some embodiments, the cytokine is a member of the common γ -chain (γ c) family of cytokines. In some embodiments, the cytokine molecule
30 comprises a cytokine chosen from one or more of IL-15, IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-13), IL-18, IL-21, IL-27, IL-35), IFN γ , TNF β , IFN α , IFN β , Areg, GM-CSF, or GCSF, including variant forms thereof (*e.g.*, a cytokine derivative, a complex comprising the cytokine molecule with a polypeptide, *e.g.*, a cytokine receptor complex, and other agonist forms thereof). In some embodiments, the cytokine molecule is a pro-inflammatory cytokine molecule chosen from an
35 IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, or TGF- β molecule. In certain

embodiments, the cytokine is IL-7, IL-21, IL-6, IL-12, or IL-23. In some embodiments, the cytokine molecule is an anti-inflammatory cytokine molecule chosen from an IL-4, IL-10, IL-13, IL-35 cytokine molecule. In some embodiments, the cytokine molecule is a superagonist (SA). For example, the superagonist can have increased cytokine activity, *e.g.*, by at least 10%,
 5 20%, or 30%, compared to the naturally-occurring cytokine. In some embodiments, the cytokine molecule is a monomer or a dimer. In embodiments, the cytokine molecule further comprises a receptor or a fragment thereof, *e.g.*, a cytokine receptor domain.

In one embodiment, the cytokine molecule comprises an amino acid sequence that is identical or substantially identical to the wild-type cytokine sequence, *e.g.*, a human cytokine
 10 sequence. In some embodiments, the cytokine molecule comprises an amino acid sequence at least 95% to 100% identical, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to a wild-type cytokine sequence, *e.g.*, a human cytokine sequence. In embodiments, the cytokine molecule comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or
 15 insertions, *e.g.*, conservative substitutions) relative to the wild-type cytokine sequence, *e.g.*, the human cytokine sequence.

In some embodiments, the cytokine molecule comprises a complex of a cytokine and an anti-cytokine antibody molecule. For example, the cytokine can be IL-2 and the anti-cytokine antibody can be a non-neutralizing anti-IL-2 antibody molecule (see *e.g.*, Spangler, J.B., et al.
 20 (2015) [dx.doi.org/10.1016/j.immuni.2015.04.015](https://doi.org/10.1016/j.immuni.2015.04.015); Letourneau, S. et al. (2010) PNAS Vol 107(5): 2171-2176; Boyman, O. et al. (2006) Science 311, 1924); and Spangler, J.B. (2015) Annu Rev Immunol 33:139-167, the contents of which are entirely incorporated by reference).

In some embodiments, the cytokine molecule is an IL-15 molecule, *e.g.*, a full length, a fragment or a variant of IL-15, *e.g.*, human IL-15. In embodiments, the IL-15 molecule is a
 25 wild-type, human IL-5, *e.g.*, having the amino acid sequence of SEQ ID NO: 10. In other embodiments, the IL-15 molecule is a variant of human IL-5, *e.g.*, having one or more amino acid modifications.

In some embodiments, the IL-15 variant comprises, or consists of, a mutation at position 45, 51, 52, or 72, *e.g.*, as described in US 2016/0184399. In some embodiments, the IL-15
 30 variant comprises, or consists of, an N, S or L to one of D, E, A Y or P substitution. In some embodiments, the mutation is chosen from L45D, L45E, S51D, L52D, N72D, N72E, N72A, N72S, N72Y, or N72P (in reference to the sequence of human IL-15, SEQ ID NO: 11). As those of skill will realize, any combination of the positions can be mutated. In some embodiments, the IL-15 variant comprises two or more mutations. In some embodiments, the IL-15 variant
 35 comprises three or more mutations. In some embodiments, the IL-15 variant comprises four,

five, or six or more mutations.

In some embodiments, the IL-15 molecule comprises a mutation, e.g., an N72D point mutation as shown in SEQ ID NO: 11 herein. In some embodiments, the IL-15 molecule comprises a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to
5 SEQ ID NO: 11, wherein the sequence comprises an N72D mutation relative to wild-type human IL-15, and having IL-15R α binding activity.

In some embodiments, the IL-15 variant comprises, or consists of, one or more mutations at amino acid position 8, 10, 61, 64, 65, 72, 101, or 108 (in reference to the sequence of human IL-15, SEQ ID NO: 11). In some embodiments the IL-15 variant possesses increased activity as
10 compared with wild-type IL-15. In some embodiments the IL-15 variant possesses decreased activity as compared with wild-type IL-15. In some embodiments the IL-15 variant possesses approximately two-fold, four-fold, ten-fold, 20-fold, 40-fold, 60-fold, 100-fold, or more than 100-fold decreased activity as compared with wild-type IL-15. In some embodiments, the mutation is chosen from D8N, K10Q, D61N, D61H, E64H, N65H, N72A, N72H, Q101N,
15 Q108N, or Q108H (in reference to the sequence of human IL-15, SEQ ID NO: 11). As those of ordinary skill in the art would realize, any combination of the positions can be mutated. In some embodiments, the IL-15 variant comprises two or more mutations. In some embodiments, the IL-15 variant comprises three or more mutations. In some embodiments, the IL-15 variant comprises four, five, or six or more mutations. In some embodiments the IL-15 variant
20 comprises mutations at positions 61 and 64. In some embodiments the mutations at positions 61 and 64 are D61N or D61H and E64Q or E64H. In some embodiments the IL-15 variants comprises mutations at positions 61 and 108. In some embodiments the mutations at positions 61 and 108 are D61N or D61H and Q108N or Q108H.

In embodiments, the cytokine molecule further comprises a receptor domain, e.g., a
25 cytokine receptor domain. In one embodiment, the cytokine molecule comprises an IL-15 receptor, or a fragment thereof (e.g., an IL-15 binding domain of an IL-15 receptor alpha) as described herein. In some embodiments, the cytokine molecule is an IL-15 molecule, e.g., IL-15 or an IL-15 superagonist as described herein. As used herein, a “superagonist” form of a cytokine molecule shows increased activity, e.g., by at least 10%, 20%, 30%, compared to the
30 naturally-occurring cytokine. An exemplary superagonist is an IL-15 SA. In some embodiments, the IL-15 SA comprises a complex of IL-15 and an IL-15 binding fragment of an IL-15 receptor, e.g., IL-15 receptor alpha or an IL-15 binding fragment thereof, e.g., as described herein. In other embodiments, the cytokine molecule further comprises a receptor domain, e.g., an extracellular domain of an IL-15R alpha, optionally, coupled to an
35 immunoglobulin Fc or an antibody molecule. In embodiments, the cytokine molecule is an IL-

15 superagonist (IL-15SA) as described in WO 2010/059253. In some embodiments, the cytokine molecule comprises IL-15 and a soluble IL-15 receptor alpha domain fused to an Fc (e.g., a sIL-15Ra-Fc fusion protein), e.g., as described in Rubinstein et al *PNAS* 103:24 p. 9166-9171 (2006).

5 The IL-15 molecule can further comprise a polypeptide, e.g., a cytokine receptor, e.g., a cytokine receptor domain, and a second, heterologous domain. In one embodiment, the heterologous domain is an immunoglobulin Fc region. In other embodiments, the heterologous domain is an antibody molecule, e.g., a Fab fragment, a FAB₂ fragment, a scFv fragment, or an affibody fragment or derivative, e.g. a sdAb (nanobody) fragment, a heavy chain antibody
10 fragment. In some embodiments, the polypeptide also comprises a third heterologous domain. In some embodiments, the cytokine receptor domain is N-terminal of the second domain, and in other embodiments, the cytokine receptor domain is C-terminal of the second domain.

The wild-type IL-15 Receptor alpha sequence and fragment and variants of this sequence are set out below.

15 Wild-type IL-15 Receptor alpha sequence (Genbank Acc. No. AAI21141.1): SEQ ID NO: 41.

Wild-type IL-15 Receptor alpha extracellular domain (portion of accession number Q13261): SEQ ID NO: 63.

Isoform CRA_d IL-15 Receptor alpha extracellular domain (portion of accession number
20 EAW86418): SEQ ID NO: 64.

The wild-type IL-15 Receptor alpha sequence is provided above as SEQ ID NO: 41. IL-15 receptor alpha contains an extracellular domain, a 23 amino acid transmembrane segment, and a 39 amino acid cytoplasmic tail. The extracellular domain of IL-15 Receptor alpha is provided as SEQ ID NO: 63.

25 In other embodiments, an IL-15 agonist can be used. For example, an agonist of an IL-15 receptor, e.g., an antibody molecule (e.g., an agonistic antibody) to an IL-15 receptor, that elicits at least one activity of a naturally-occurring cytokine. In embodiments, the IL-15 receptor or fragment thereof is from human or a non-human animal, e.g., mammal, e.g., non-human primate.

30 The wild-type IL-15 Receptor alpha sequence is provided above as SEQ ID NO: 64. IL-15 receptor alpha contains an extracellular domain, a 23 amino acid transmembrane segment, and a 39 amino acid cytoplasmic tail. The sushi domain has been described in the literature including, e.g., Bergamaschi *et al.* (2008), *JBC* VOL. 283, NO. 7, pp. 4189–4199; Wei *et al.* (2001), *Journal of Immunology* 167:277-282; Schluns *et al.* (2004) *PNAS* Vol 110 (15) 5616–
35 5621; US 2016/0184399 (the contents of each of which is incorporated by reference herein).

The extracellular domain of IL-15 Receptor alpha is provided as SEQ ID NO: 63. The extracellular domain of IL-15 Receptor alpha comprises a domain referred to as the sushi domain, which binds IL-15. The general sushi domain, also referred to as complement control protein (CCP) modules or short consensus repeats (SCR), is a protein domain found in several proteins, including multiple members of the complement system. The sushi domain adopts a beta-sandwich fold, which is bounded by the first and fourth cysteine of four highly conserved cysteine residues, comprising a sequence stretch of approximately 60 amino acids (Norman, Barlow, et al. J Mol Biol. 1991 Jun 20;219(4):717-25). The amino acid residues bounded by the first and fourth cysteines of the sushi domain in IL-15Ralpha comprise a 62 amino acid polypeptide that we refer to as the minimal domain (SEQ ID NO: 52). Including additional amino acids of IL-15Ralpha at the N- and C-terminus of the minimal sushi domain, such as inclusion of N-terminal Ile and Thr and C-terminal Ile and Arg residues result in a 65 sushi amino acid domain (SEQ ID NO: 9).

A sushi domain as described herein may comprise one or more mutations relative to a wild-type sushi domain. For instance, residue 77 of IL-15Ra is leucine in the wild-type gene (and is underlined in SEQ ID NO: 41), but can be mutated to isoleucine (L77I). Accordingly, a minimal sushi domain comprising L77I (with the numbering referring to the wild-type IL-15Ra of SEQ ID NO: 41) is provided as SEQ ID NO: 65. An extended sushi domain comprising L77I (with the numbering referring to the wild-type IL-15Ra of SEQ ID NO: 41) is provided as SEQ ID NO: 66.

Minimal sushi domain, wild-type:

CPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSL
KC (SEQ ID NO: 52)

Extended sushi domain, wild-type:

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTTP
SLKCIR (SEQ ID NO: 9)

Minimal sushi domain, L77I:

CPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVINKATNVAHWTTPSL
KCI (SEQ ID NO: 65)

Extended sushi domain, L77I:

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVINKATNVAHWTTPS

LKCIR (SEQ ID NO: 66)

In some embodiments, a sushi domain consists of 62-171 amino acids of SEQ ID NO: 63 or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto, and having IL-15 binding activity. In some embodiments, a sushi domain consists of 65-171 amino acids of SEQ ID NO: 63 or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto, and having IL-15 binding activity. In some embodiments, a sushi domain consists of up to 171 amino acids of SEQ ID NO: 63 or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto, and having IL-15 binding activity. In some embodiments, a sushi domain consists of 62-171, 62-160, 62-150, 62-140, 62-130, 62-120, 62-110, 62-100, 62-90, 62-80, 62-70, 65-171, 65-160, 65-150, 65-140, 65-130, 65-120, 65-110, 65-100, 65-90, 65-80, or 65-70 amino acids of SEQ ID NO: 63 or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto, and having IL-15 binding activity. In some embodiments, a sushi domain consists of 62-171, 62-160, 62-150, 62-140, 62-130, 62-120, 62-110, 62-100, 62-90, 62-80, 62-70, 65-171, 65-160, 65-150, 65-140, 65-130, 65-120, 65-110, 65-100, 65-90, 65-80, or 65-70 amino acids of SEQ ID NO: 63. In some embodiments, the sushi domain comprises, or consists of, an amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 52.

In some embodiments, a sushi domain consists of 62-171 amino acids of SEQ ID NO: 63 or a sequence having up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, or 40 modifications (e.g., substitutions) relative thereto, and having IL-15 binding activity. In some embodiments, a sushi domain consists of up to 171 amino acids of SEQ ID NO: 5 or a sequence having up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, or 40 modifications (e.g., substitutions) relative thereto, and having IL-15 binding activity. In some embodiments, a sushi domain consists of 62-171, 62-160, 62-150, 62-140, 62-130, 62-120, 62-110, 62-100, 62-90, 62-80, 62-70, 65-171, 65-160, 65-150, 65-140, 65-130, 65-120, 65-110, 65-100, 65-90, 65-80, or 65-70 amino acids of SEQ ID NO: 63 or a sequence having up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, or 40 modifications (e.g., substitutions) relative thereto, and having IL-15 binding activity.

In some embodiments, a sushi domain comprises at least 62 amino acids of SEQ ID NO: 63 or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto, wherein the sequence comprises an L77I mutation relative to wild-type IL-15Ra, and having IL-15 binding activity. In some embodiments, a sushi domain comprises at least 65 amino acids of SEQ ID NO: 63 or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto, wherein the sequence comprises an L77I mutation relative to wild-type IL-15Ra, and having IL-15 binding activity. In some embodiments, a sushi domain comprises a portion of SEQ ID NO: 63 or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%,

or 99% identity thereto, wherein the sequence comprises an L77I mutation relative to wild-type IL-15Ra, and having IL-15 binding activity. In some embodiments, the sushi domain comprises an amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 52.

In some embodiments, a sushi domain comprises at least 62 amino acids of SEQ ID NO: 63 or a sequence having up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, or 40 modifications (e.g., substitutions) relative thereto, wherein the sequence comprises an L77I mutation relative to wild-type IL-15Ra, and having IL-15 binding activity. In some embodiments, a sushi domain comprises a portion of SEQ ID NO: 66 or a sequence having up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, or 40 modifications (e.g., substitutions) relative thereto, wherein the sequence comprises an L77I mutation relative to wild-type IL-15Ra, and having IL-15 binding activity.

In embodiments, the sushi domain comprises at least 10, 20, 30, 40, 50, 60, 62, 65, 70, 80, 90, 100, 110, 120, 130, 140, 150, or 160 consecutive amino acids of SEQ ID NO: 63, or a sequence having an L77I mutation relative thereto. In embodiments, the sushi domain consists of 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-110, 110-120, 120-130, 130-140, 140-150, 150-160, or 160-170 consecutive amino acids of SEQ ID NO: 63, or a sequence having an L77I mutation relative thereto.

In embodiments, the sushi domain is a sushi domain from human or a non-human animal, e.g., mammal, e.g., non-human primate.

In some embodiments, the polypeptide can have a second, heterologous domain, e.g., an Fc domain or a Fab domain.

In some embodiments, the polypeptide comprising the IL-15 receptor or fragment thereof comprises an Fc domain. In embodiments, the Fc domain is an effector-attenuated Fc domain, e.g., a human IgG2 Fc domain, e.g., a human IgG2 Fc domain of SEQ ID NO: 54 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

In embodiments, the effector-attenuated Fc domain has reduced effector activity, e.g., compared to a wild-type IgG1 Fc domain, e.g., compared to a wild-type IgG1 Fc domain of SEQ ID NO: 67. In some embodiments, effector activity comprises antibody-dependent cellular toxicity (ADCC). In embodiments, the effector activity is reduced by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% in an ADCC assay, e.g., compared to a wild-type IgG1 Fc domain of SEQ ID NO: 67. In some embodiments, effector activity comprises complement dependent cytotoxicity (CDC). In embodiments, the effector activity is reduced by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% in a CDC assay such as a CDC assay described in Armour et al., "Recombinant human IgG molecules lacking Fc gamma receptor I binding and monocyte triggering activities." Eur J Immunol (1999) 29:2613-24" e.g.,

compared to a wild-type IgG1 Fc domain of SEQ ID NO: 67.

In some embodiments, the Fc domain comprises an IgG1 Fc domain of SEQ ID NO: 67 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

5 In some embodiments, the Fc domain comprises an IgG2 constant region of SEQ ID NO: 68 or fragment thereof, or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

In some embodiments, the Fc domain comprises an IgG2Da Fc domain of SEQ ID NO: 55 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto. In embodiments, the Fc domain comprises one or both of A330S and P331S mutations using Kabat numbering system. In embodiments, the Fc domain is one described in Armour et al. "Recombinant human IgG molecules lacking Fc gamma receptor I binding and monocyte triggering activities." Eur J Immunol (1999) 29:2613-24.

In some embodiments, the Fc domain has dimerization activity.

15 In some embodiments, the Fc domain is an IgG domain, e.g., an IgG1, IgG2, IgG3, or IgG4 Fc domain. In some embodiments, the Fc domain comprises a CH2 domain and a CH3 domain.

In some embodiments, the nanoparticle comprises a protein having a sequence of SEQ ID NO: 56 (sushi-IgG2Da-Fc) or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

In some embodiments, the FM that comprises a sushi domain described herein (e.g., in SEQ ID NO: 9) and an Fc domain described herein, e.g., an IgG2 Fc domain (e.g., SEQ ID NO: 54 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto). In some embodiments, the FM comprises a sushi domain of SEQ ID NO: 9 and an Fc domain described herein, e.g., an IgG1 Fc domain, e.g., an Fc domain of SEQ ID NO: 67 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto. In some embodiments, the FM comprises a sushi domain of SEQ ID NO: 9 and an IgG2 Fc domain, e.g., an Fc domain of SEQ ID NO: 54 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto. In some embodiments, the FM comprises a sushi domain of SEQ ID NO: 9 and an IgG1 Fc domain, e.g., an Fc domain of SEQ ID NO: 67 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

In some embodiments, the FM comprises a sushi domain of SEQ ID NO: 9 and an IgG2Da Fc domain of SEQ ID NO: 56 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto. In some embodiments, the FM comprises

a sushi domain of SEQ ID NO: 9 and an IgG2Da Fc domain of SEQ ID NO: 56 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto. In some embodiments, the FM comprises a sushi-IgG2Da-Fc protein having a sequence of SEQ ID NO: 56 or an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto.

In embodiments, the IL-15 molecule is a molecule described in PCT International Application Publication No. WO2017/027843, which is herein incorporated by reference in its entirety.

In some embodiments, the cytokine molecule is interleukin 2 (IL-2). IL-2 is chiefly secreted by T cells in response to antigenic stimuli, and is the main cytokine for T cell proliferation. IL-2 receptor is expressed by T lymphocytes, NK cells, B cells, macrophages, and monocytes; however, only T lymphocytes are capable of producing this cytokine.

The high affinity IL-2 receptor (IL-2R) is a heterotrimeric cell surface receptor composed of α , β , and γ_c -polypeptide chains ($K_D 10^{-11}$ M). The 55 kDa α -chain, also known as IL-2R α , CD25, p55, and Tac (T cell activation) antigen, is unique to the IL-2R. The β (CD122; P75) and γ_c (CD132) chains are part of a cytokine receptor superfamily (hematopoietin receptors) and are functional components of other cytokine receptors, such as IL-15R. The intermediate affinity receptor is a dimer composed of a β - and a γ_c -chain ($K_D 10^{-9}$ M) while the low affinity receptor consists of a monomeric α -subunit that has no signal transduction capacity ($K_D 10^{-8}$ M).

Treg cells constitutively express high levels of IL-2 α -chain, having thus a higher affinity to IL-2, and compete for this growth factor with proliferating cells. By depriving proliferating effector cells from IL-2, Treg cells do not only prevent them from continuing the proliferative process but also leave them without a vital cytokine, causing metabolic interruption and cell death.

In one embodiment, the IL-2 molecule (*e.g.*, IL-2 polypeptide molecule) comprises a wild-type IL-2 amino acid sequence, *e.g.*, a human IL-2 amino acid sequence, *e.g.*, including the amino acid sequence of SEQ ID NO: 80. In some embodiments, the cytokine molecule is an IL-2 molecule, *e.g.*, a full length, a fragment or a variant of IL-2, *e.g.*, human IL-2 that retains at least some IL-2 activity such as binding to IL-2R. In other embodiments, the IL-2 molecule is a variant of human IL-2, *e.g.*, having one or more amino acid alterations, *e.g.*, substitutions, to the human IL-2 amino acid sequence. In some embodiments, the IL-2 variant comprises, or consists of, one or more mutations while maintaining the IL-2R binding activity.

In some embodiments, the IL-2 of the present disclosure can have the following

sequence (or a functional variant thereof):

APTSSSTKKTQLQLEHLLLDLQMLNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCL
EEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNR
WITFCQSIISTLT (SEQ ID NO: 80)

5 In certain embodiments, variant forms of IL-2 can be made by introducing one or more mutations into the wild-type form, so as to fine tune its binding affinity to IL-2R. For example, a library of IL-2 mutants or variants can be made to test their affinity to IL-2R. These variants can also be conjugated or coupled to various targeting moieties, and the resulting FMs can be tested for targeting. In some embodiments, the IL-2 variant comprises, or consists of, one or
10 more mutations.

In some embodiments, the IL-2 variants can be IL-2 $\alpha\beta\gamma$ selective agonists. Functionally they selectively activate the IL2R $\alpha\beta\gamma$ receptor complex relative to the IL2R $\beta\gamma$ receptor complex. It is derived from a wild type IL-2 protein structurally defined as having at least 95% sequence identity to the wild type IL-2 (e.g., SEQ ID NO: 80) and functionally defined by the ability to
15 preferentially activate Treg cells. The variants can also be functionally defined by its ability to selectively activate IL-2 receptor signaling in Tregs, as measured by the levels of phosphorylated STAT5 protein in Treg cells compared to CD4⁺ CD25⁻/low T cells or NK cells, or by the selective activation of Phytohemagglutinin-stimulated T cells versus NK cells.

In some embodiments, modifications to the cytokine or growth factor amino acid
20 sequence can be made to improve persistence and/or selectivity for an immune regulatory cell such as a regulatory T cell. One of the obstacles for prolonged surface persistence and signaling of cytokine fusions is cytokine/receptor internalization and degradation through the endosomal/lysosomal pathway. Extending the persistence of cytokine fusions can be achieved, in some embodiments, by altering the affinity of cytokines in the altered-pH environment of the
25 endosome. By decreasing the affinity of cytokine for its receptor in the endosome, there is decreased lysosomal degradation and increased recycling to the cell surface. To promote endosomal dissociation of IL-2 from its receptor, histidine mutants can be engineered in receptor-binding motifs of IL-2. Histidine changes protonation state in the altered pH of the endosome, and this reduces the affinity of IL-2/receptor interactions. A number of different
30 substitutions can be tested to find ones that increase recycling and persistence without decreasing surface affinity for the receptor. Alternatively, reduced surface affinity for the receptor can be desirable for enhancing Treg-selective IL-2 signaling. In embodiments, histidine substitutions can be focused in motifs that are involved in IL2R β interaction in order to enhance IL2R α binding and Treg selectivity.

35 In certain embodiments, altered receptor affinity for increased Treg selectivity can also

be engineered. One of the hallmarks of Tregs is increased requirement and sensitivity to IL-2 signaling. This is mediated in part by significant induction of the IL-2 receptor alpha subunit (IL2R α , CD25). This increased CD25 expression can be used in order to promote selectivity of IL-2 signaling on Treg cells. IL-2 signaling is mediated by hetero-trimers of IL2R α / β / γ or IL2R β / β / γ . Due to increased CD25 (IL2R α) expression, IL-2 signaling on Tregs is driven primarily by the α / β / γ trimer. Antibodies can be used that disrupt IL-2 binding to IL2R α or IL2R β bias IL-2 signaling and expansion towards T-effector cells (Teffs) or Tregs respectively. Instead of antibody-based approaches, disruption of IL-2 binding to IL2R β can be achieved via amino acid substitution. This can be tested using the histidine substitution approach detailed above, and through standard binding interface modulation substitutions (sterics, charge, etc.).

In some embodiments, the cytokine is IL-15 and its variants such as those disclosed in PCT International Application No. PCT/US2017/037249 filed June 13, 2017, *e.g.*, on pages 46-57, which is incorporated herein by reference in its entirety.

Amphiregulin (Areg) is a polypeptide growth factor that belongs to the epidermal growth factor (EGF) family. Areg is synthesized as a type I transmembrane protein precursor (proAR) and expressed on the cell surface. EGFR may be activated by Areg in several ways: autocrine or paracrine activation by the soluble form of Areg, a juxtacrine mode enabling the un-cleaved transmembrane form to activate EGFR, or by a newly described mode of signaling entailing Areg containing exosomes, that better enhance invasion of recipient cells in comparison to exosomes containing high affinity ligands.

TGF- β is a multifunctional cytokine belonging to the transforming growth factor superfamily that includes four different isoforms (TGF- β 1 to 4) and many other signaling proteins produced by all white blood cell lineages. Activated TGF- β complexes with other factors to form a serine/threonine kinase complex that binds to TGF- β receptors, which is composed of both type 1 and type 2 receptor subunits. After the binding of TGF- β , the type 2 receptor kinase phosphorylates and activates the type 1 receptor kinase that activates a signaling cascade. This leads to the activation of different downstream substrates and regulatory proteins, inducing transcription of different target genes that function in differentiation, chemotaxis, proliferation, and activation of many immune cells.

Targeting Moieties

Fusion molecules according to the present disclosure disclosed herein include a targeting moiety to target a regulatory immune cell, such as a regulatory T cell or regulatory B cell. In some embodiments, the targeting moiety may be an antibody molecule (*e.g.*, an antigen binding

domain as described herein), a receptor or a receptor fragment, or a ligand or a ligand fragment, or a combination thereof. In certain embodiments, the targeting moiety associates with or binds to a molecule, *e.g.*, surface marker or receptor, present on the surface of a cell (a “targeting moiety”). According to some embodiments, the FM may be selectively targeted to a regulatory T cell by the binding of the targeting moiety to the cell surface. For example, the FM can be targeted to general Tregs by binding to general Treg surface markers such as CD4 and/or CD25 (FIG. 1G). The FM can also be specifically targeted to subsets of functional Tregs by binding to subset-specific surface markers such as CD29 and/or NRP1 (FIG. 1G). In some embodiments, combinatorial targeting of two or more FMs can be achieved

In some embodiments, the targeting moiety is chosen from an antibody molecule (*e.g.*, a full antibody (*e.g.*, an antibody that includes at least one, and preferably two, complete heavy chains, and at least one, and preferably two, complete light chains), or an antigen-binding fragment (*e.g.*, a Fab, F(ab')₂, Fv, a single chain Fv, a single domain antibody, a diabody (dAb), a bivalent antibody, or bispecific antibody or fragment thereof, a single domain variant thereof, or a camelid antibody)), non-antibody scaffold, or ligand that binds to the CD45 receptor.

In some embodiments, the targeting moiety targets the FM to markers, cell surface receptors, or other molecules present on the surface of a regulatory T cell, including, for example, CD45 (via, *e.g.*, BC8 (ACCT: HB-10507), 9.4 (ATTC: HB- 10508)), CD4, CD3, CD2, CD25, CD127, CD197 (CCR7), CXCR3, CXCR4, CXCR5, CD38, CD27, CD8, CCR4, CCR5, CD137, CCR6 (CD196), CCR8, CCR10, OX40, GITR, NRP1, CTLA4, LAG3, CD73, CD103, CD62L, CCR2, CCR9, CD11a (via, for example, MHM24 monoclonal antibody), CD18 (via, for example, chimeric1B4 monoclonal antibody). In other embodiments, the targeting moiety targets CD19 on a regulatory B cell. In some embodiments, a targeting moiety is chosen from an antibody molecule, *e.g.*, an antigen binding domain, non-antibody scaffold, or ligand that binds to CD45, CD4, CD3, CD2, CD25, CD127, CD197 (CCR7), CXCR3, CXCR4, CXCR5, CD38, CD27, CD8, CCR4, CCR5, CD137, CCR6 (CD196), CCR8, CCR10, OX40, GITR, NRP1, CTLA4, LAG3, CD73, CD103, CD62L, CCR2, CCR9, CD11a, or CD18.

According to some embodiments, the targeting moiety selectively targets the FM to regulatory T cells over other immune cells or other cell types. In some embodiments, the targeting moiety directs the FM to regulatory T cells by targeting cell surface receptors or other molecules that are expressed on the surface of a regulatory cell but may also be present on other immune cells or other cell types. In certain embodiments, the targeting moiety targets the FM to cell surface receptors or other molecules that are present in higher abundance on the surface of regulatory T cells as compared with other immune cells or other cell types that may be co-located with the regulatory T cells of interest.

In some embodiments, the targeting moiety can be a bispecific moiety that binds to more than one target. Exemplary bispecific Treg targeting moieties include CD4:CD45, CD8:CD45, CD4:CD39, CD4:NRP1, and CD4:CD25. In some embodiments, while CD127 is a negative surface marker for Tregs, the pairing of CD127 with a positive Treg surface marker (e.g., CD4:CD127, CD25:CD127) can be used to negatively enrich and/or sort Tregs.

“CD45,” also known as leukocyte common antigen, refers to human CD45 protein and species, isoforms, and other sequence variants thereof. Thus, CD45 can be the native, full-length protein or can be a truncated fragment or a sequence variant (e.g., a naturally occurring isoform, or recombinant variant) that retains at least one biological activity of the native protein. CD45 is a receptor-linked protein tyrosine phosphatase that is expressed on leukocytes, and which plays an important role in the function of these cells (reviewed in Altin, JG (1997) Immunol Cell Biol. 75(5):430-45, incorporated herein by reference). For example, the extracellular domain of CD45 is expressed in several different isoforms on T cells, and the particular isoform(s) expressed depends on the particular subpopulation of cell, their state of maturation, and antigen exposure. Expression of CD45 is important for the activation of T cells via the TCR, and that different CD45 isoforms display a different ability to support T cell activation.

“CD4” is a co-receptor for MHC Class II (with TCR, T-cell receptor); found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells. CD4+T cells are crucial in achieving a regulated effective immune response to pathogens. Naive CD4+T cells are activated after interaction with antigen-MHC complex and differentiate into specific subtypes depending mainly on the cytokine milieu of the microenvironment. Besides the classical T-helper 1 and T-helper 2, other subsets have been identified, including T-helper 17, regulatory T cell, follicular helper T cell, and T-helper 9, each with a characteristic cytokine profile. CD4+T cells carry out multiple functions, ranging from activation of the cells of the innate immune system, B-lymphocytes, cytotoxic T cells, as well as nonimmune cells, and also play critical role in the suppression of immune reaction. See e.g., Rishi Vishal et al. “CD4+T Cells: Differentiation and Functions,” Clinical and Developmental Immunology, vol. 2012, Article ID 925135, 12 pages, 2012. doi:10.1155/2012/925135.

“CD25” (also called interleukin-2 receptor alpha chain) is a type I transmembrane protein present on Tregs as well as activated T cells, activated B cells, some thymocytes, myeloid precursors, and oligodendrocytes.

“CD127” is part of the heterodimeric IL-7 receptor that is composed of CD127 and the common γ chain, which is shared by other cytokine receptors (IL-2R, IL-4R, IL-9R, IL-15R, and IL-21R). CD127 is expressed on thymocytes, T- and B-cell progenitors, mature T cells, monocytes, and some other lymphoid and myeloid cells. CD127 expression is down-modulated

on the Treg cells, inversely correlating with the expression of Treg marker FoxP3. Studies have shown that IL-7R plays an important role in the proliferation and differentiation of mature T cells, and *in vitro* experiments show that the expression of CD127 is down-regulated following T cell activation. It is believed that FoxP3 interacts with the CD127 promoter and might
5 contribute to reduced expression of CD127 in Tregs.

“CD8” is a transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR). Like the TCR, CD8 binds to a major histocompatibility complex (MHC) molecule, but is specific for the class I MHC protein. There are two isoforms of the protein, alpha and beta, each encoded by a different gene. In humans, both genes are located on
10 chromosome 2 in position 2p12. The CD8 co-receptor is predominantly expressed on the surface of cytotoxic T cells, but can also be found on natural killer cells, cortical thymocytes, and dendritic cells. It is expressed in T cell lymphoblastic lymphoma and hypo-pigmented mycosis fungoides. To function, CD8 forms a dimer, consisting of a pair of CD8 chains. The most common form of CD8 is composed of a CD8- α and CD8- β chain, both members of the
15 immunoglobulin superfamily with an immunoglobulin variable (IgV)-like extracellular domain connected to the membrane by a thin stalk, and an intracellular tail. Less-common homodimers of the CD8- α chain are also expressed on some cells. The extracellular IgV-like domain of CD8- α interacts with the $\alpha 3$ portion of the Class I MHC molecule. This affinity keeps the T cell receptor of the cytotoxic T cell and the target cell bound closely together during antigen-specific
20 activation. Cytotoxic T cells with CD8 surface protein are called CD8+ T cells. See e.g., Leahy DJ et al., (March 1992) "Crystal structure of a soluble form of the human T cell coreceptor CD8 at 2.6 Å resolution" Cell 68 (6): 1145–62; Gao G et al., (2000) "Molecular interactions of coreceptor CD8 and MHC class I: the molecular basis for functional coordination with the T-cell receptor" Immunol Today, 21 (12): 630–6; and Devine L et al. (1999) "Orientation of the Ig
25 domains of CD8 alpha beta relative to MHC class I" J Immunol., 162 (2): 846–51.

“CD39” is an integral membrane protein with two transmembrane domains and a large extracellular region with nucleoside triphosphate diphosphohydrolase activity. In humans, CD39 is mainly expressed by regulatory T cells but also other leukocytes. It is also named as ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1). CD39 is an ectoenzyme that
30 hydrolases ATP/UTP and ADP/UDP to the respective nucleosides such as AMP. A recent study demonstrates that CD39 is a cell surface marker of Foxp3 Treg cells which may regulate immune T cell suppression by the downstream production of adenosine and thus, might represent a target for the development of novel therapeutic methods useful for treating diseases associated with Treg activity (Deaglio et al., J Exp Med. 2007 Jun. 11; 204(6):1257-65).

“NRP1” is a multi-functional receptor that contributes to the development of the nervous

and vascular systems. NRP1 was described as a receptor that binds the semaphorin 3A ligand, acting with plexin co-receptors to regulate axon guidance (He and Tessier-Lavigne, Cell (1997) 90:739-51). It was later shown that NRP1 also binds members of the vascular endothelial growth factor (VEGF) ligand family to mediate vascular development (Soker et al, Cell (1998) 92:735-45; Kawasaki et al, Development (1999) 126:4895-902). Recent studies showed that NRP1 is expressed at high levels on nTreg cells and can be used to separate nTreg versus iTreg cells in certain physiological settings (Yadav et al., J Exp Med. 2012 Sep 24;209(10):1713-22, S1-19). In addition, iTreg cells generated through antigen delivery or converted under homeostatic conditions lack NRP1 expression. NRP1(lo) iTreg cells show similar suppressive activity to nTreg cells in controlling ongoing autoimmune responses under homeostatic conditions. In contrast, their activity might be compromised in certain lymphopenic settings. Thus, NRP1 provides a marker to distinguish distinct Treg subsets and can be useful in studying the role of nTreg versus iTreg cells in different disease settings.

Antibody Molecules

The fusion proteins described herein may comprise one or more antibody molecule. For example, the targeting moiety may comprise an antibody molecule. In embodiments, the antibody molecule binds to a cell surface protein on a regulatory T cell, *e.g.*, a human Treg antigen. For example, the antibody molecule binds specifically to an epitope, *e.g.*, linear or conformational epitope, on the Treg antigen.

In an embodiment, an antibody molecule is a monospecific antibody molecule and binds a single epitope, *e.g.*, a monospecific antibody molecule having a plurality of immunoglobulin variable domain sequences, each of which binds the same epitope.

In another embodiment, an antibody molecule is a multispecific antibody molecule, *e.g.*, it comprises a plurality of immunoglobulin variable domains sequences, wherein a first immunoglobulin variable domain sequence of the plurality has binding specificity for a first epitope and a second immunoglobulin variable domain sequence of the plurality has binding specificity for a second epitope. In an embodiment, the first and second epitopes are on the same antigen, *e.g.*, the same protein (or subunit of a multimeric protein). In an embodiment, the first and second epitopes overlap. In an embodiment, the first and second epitopes do not overlap. In an embodiment, the first and second epitopes are on different antigens, *e.g.*, the different proteins (or different subunits of a multimeric protein). In an embodiment, a multispecific antibody molecule comprises a third, fourth or fifth immunoglobulin variable domain. In an embodiment, a multispecific antibody molecule is a bispecific antibody molecule, a trispecific antibody molecule, or a tetraspecific antibody molecule.

In an embodiment, a multispecific antibody molecule is a bispecific antibody molecule. A bispecific antibody has specificity for no more than two antigens. A bispecific antibody molecule is characterized by a first immunoglobulin variable domain sequence which has binding specificity for a first epitope and a second immunoglobulin variable domain sequence that has binding specificity for a second epitope. In an embodiment, the first and second epitopes are on the same antigen, *e.g.*, the same protein (or subunit of a multimeric protein). In an embodiment, the first and second epitopes overlap. In an embodiment, the first and second epitopes do not overlap. In an embodiment, the first and second epitopes are on different antigens, *e.g.*, the different proteins (or different subunits of a multimeric protein). In an embodiment, a bispecific antibody molecule comprises a heavy chain variable domain sequence and a light chain variable domain sequence which have binding specificity for a first epitope and a heavy chain variable domain sequence and a light chain variable domain sequence which have binding specificity for a second epitope. In an embodiment, a bispecific antibody molecule comprises a half antibody having binding specificity for a first epitope and a half antibody having binding specificity for a second epitope. In an embodiment, a bispecific antibody molecule comprises a half antibody, or fragment thereof, having binding specificity for a first epitope and a half antibody, or fragment thereof, having binding specificity for a second epitope. In an embodiment, a bispecific antibody molecule comprises a scFv or a Fab, or fragment thereof, have binding specificity for a first epitope and a scFv or a Fab, or fragment thereof, have binding specificity for a second epitope.

In an embodiment, an antibody molecule comprises a diabody, and a single-chain molecule, as well as an antigen-binding fragment of an antibody (*e.g.*, Fab, F(ab')₂, and Fv). For example, an antibody molecule can include a heavy (H) chain variable domain sequence (abbreviated herein as VH), and a light (L) chain variable domain sequence (abbreviated herein as VL). In an embodiment, an antibody molecule comprises or consists of a heavy chain and a light chain (referred to herein as a half antibody). In another example, an antibody molecule includes two heavy (H) chain variable domain sequences and two light (L) chain variable domain sequence, thereby forming two antigen binding sites, such as Fab, Fab', F(ab')₂, Fc, Fd, Fd', Fv, single chain antibodies (scFv for example), single variable domain antibodies, diabodies (Dab) (bivalent and bispecific), and chimeric (*e.g.*, humanized) antibodies, which may be produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA technologies. These functional antibody fragments retain the ability to selectively bind with their respective antigen or receptor. Antibodies and antibody fragments can be from any class of antibodies including, but not limited to, IgG, IgA, IgM, IgD, and IgE, and from any subclass (*e.g.*, IgG1, IgG2, IgG3, and IgG4) of antibodies. A preparation of

antibody molecules can be monoclonal or polyclonal. An antibody molecule can also be a human, humanized, CDR-grafted, or *in vitro* generated antibody. The antibody can have a heavy chain constant region chosen from, *e.g.*, IgG1, IgG2, IgG3, or IgG4. The antibody can also have a light chain chosen from, *e.g.*, kappa or lambda. The term “immunoglobulin” (Ig) is used interchangeably with the term “antibody” herein.

Examples of antigen-binding fragments of an antibody molecule include: (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a diabody (dAb) fragment, which consists of a VH domain; (vi) a camelid or camelized variable domain; (vii) a single chain Fv (scFv), *see e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; (viii) a single domain antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

Antibody molecules include intact molecules as well as functional fragments thereof. Constant regions of the antibody molecules can be altered, *e.g.*, mutated, to modify the properties of the antibody (*e.g.*, to increase or decrease one or more of: Fc receptor binding, antibody glycosylation, the number of cysteine residues, target cell function, or complement function).

Antibody molecules can also be single domain antibodies. Single domain antibodies can include antibodies whose complementary determining regions are part of a single domain polypeptide. Examples include, but are not limited to, heavy chain antibodies, antibodies naturally devoid of light chains, single domain antibodies derived from conventional 4-chain antibodies, engineered antibodies and single domain scaffolds other than those derived from antibodies. Single domain antibodies may be any of the art, or any future single domain antibodies. Single domain antibodies may be derived from any species including, but not limited to mouse, human, camel, llama, fish, shark, goat, rabbit, and bovine. According to another aspect of the disclosure, a single domain antibody is a naturally occurring single domain antibody known as heavy chain antibody devoid of light chains. Such single domain antibodies are disclosed in WO 9404678, for example. For clarity reasons, this variable domain derived from a heavy chain antibody naturally devoid of light chain is known herein as a VHH or nanobody to distinguish it from the conventional VH of four chain immunoglobulins. Such a VHH molecule can be derived from antibodies raised in *Camelidae* species, for example in camel, llama, dromedary, alpaca and guanaco. Other species besides *Camelidae* may produce

heavy chain antibodies naturally devoid of light chain; such VHHs are within the scope of the present disclosure.

Antibody molecules can include non-antibody scaffolds and antibody mimetics. Exemplary non-antibody scaffolds include: lipocalins (*e.g.* anticalins), affibodies, fibronectin (5 *e.g.* monobodies or Adnectins), knottins, ankyrin repeats (*e.g.* DARPins), and A domains (*e.g.* avimers).

The VH and VL regions can be subdivided into regions of hypervariability, termed "complementarity determining regions" (CDR), interspersed with regions that are more conserved, termed "framework regions" (FR or FW).

10 The extent of the framework region and CDRs has been precisely defined by a number of methods (*see*, Kabat, E. A., *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Chothia, C. *et al.* (1987) *J. Mol. Biol.* 196:901-917; and the AbM definition used by Oxford Molecular's AbM antibody modeling software. *See*, generally, *e.g.*, *Protein Sequence and*
15 *Structure Analysis of Antibody Variable Domains*. In: Antibody Engineering Lab Manual (Ed.: Duebel, S. and Kontermann, R., Springer-Verlag, Heidelberg).

The terms "complementarity determining region," and "CDR," as used herein refer to the sequences of amino acids within antibody variable regions which confer antigen specificity and binding affinity. In general, there are three CDRs in each heavy chain variable region (HCDR1, 20 HCDR2, HCDR3) and three CDRs in each light chain variable region (LCDR1, LCDR2, LCDR3).

The precise amino acid sequence boundaries of a given CDR can be determined using any of a number of known schemes, including those described by Kabat *et al.* (1991), "Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National
25 Institutes of Health, Bethesda, MD ("Kabat" numbering scheme), Al-Lazikani *et al.*, (1997) *JMB* 273,927-948 ("Chothia" numbering scheme). As used herein, the CDRs defined according to the "Chothia" number scheme are also sometimes referred to as "hypervariable loops."

For example, under Kabat, the CDR amino acid residues in the heavy chain variable domain (VH) are numbered 31-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3); and the
30 CDR amino acid residues in the light chain variable domain (VL) are numbered 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3). Under Chothia, the CDR amino acids in the VH are numbered 26-32 (HCDR1), 52-56 (HCDR2), and 95-102 (HCDR3); and the amino acid residues in VL are numbered 26-32 (LCDR1), 50-52 (LCDR2), and 91-96 (LCDR3).

Each VH and VL typically includes three CDRs and four FRs, arranged from amino-
35 terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3,

FR4.

The antibody molecule can be a polyclonal or a monoclonal antibody.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal
5 antibody composition displays a single binding specificity and affinity for a particular epitope. A monoclonal antibody can be made by hybridoma technology or by methods that do not use hybridoma technology (*e.g.*, recombinant methods).

The antibody can be recombinantly produced, *e.g.*, produced by phage display or by combinatorial methods.

10 Phage display and combinatorial methods for generating antibodies are known in the art (as described in, *e.g.*, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.*
15 International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.*
20 (1991) *Nuc Acid Res* 19:4133-4137; and Barbas *et al.* (1991) *PNAS* 88:7978-7982, the contents of all of which are incorporated by reference herein).

In one exemplary phage display technique, antibody repertoires can be displayed on the surface of filamentous bacteriophage, and the constructed library may be screened for phages
25 that bind to the immunogen (*e.g.*, CD4, CD25, CD39, NRP1). Antibody phage is based on genetic engineering of bacteriophages and repeated rounds of antigen-guided selection and phage propagation. This technique allows *in vitro* selection of monoclonal antibodies. The phage display process begins with antibody-library preparation followed by ligation of the variable heavy (VH) and variable light (VL) PCR products into a phage display vector,
30 culminating in analysis of clones of monoclonal antibodies. The VH and VL PCR products, representing the antibody repertoire, are ligated into a phage display vector (*e.g.*, the phagemid pComb3X) that is engineered to express the VH and VL as an scFv fused to the pIII minor capsid protein of a filamentous bacteriophage of *Escherichia coli* that was originally derived from the M13 bacteriophage. However, the phage display vector pComb3X does not have all
35 the other genes necessary to encode a full bacteriophage in *E. coli*. For those genes, a helper

phage is added to the *E. coli* that are transformed with the phage display vector library. The result is a library of phages, each expressing on its surface a monoclonal antibody and harboring the vector with the respective nucleotide sequence within. The phage display can also be used to produce the monoclonal antibody itself (not attached to phage capsid proteins) in certain strains of *E. Coli*. Additional cDNA is engineered, in the phage display vector, after the VL and VH sequences to allow characterization and purification of the mAb produced. Specifically, the recombinant antibody may have a hemagglutinin (HA) epitope tag and a polyhistidine to allow easy purification from solution.

In one embodiment, the antibody is a fully human antibody (*e.g.*, an antibody made in a mouse which has been genetically engineered to produce an antibody from a human immunoglobulin sequence), or a non-human antibody, *e.g.*, a rodent (mouse or rat), goat, primate (*e.g.*, monkey), camel antibody. Preferably, the non-human antibody is a rodent (mouse or rat antibody). Methods of producing rodent antibodies are known in the art.

Human monoclonal antibodies can be generated using transgenic mice carrying the human immunoglobulin genes rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, *e.g.*, Wood *et al.* International Application WO 91/00906, Kucherlapati *et al.* PCT publication WO 91/10741; Lonberg *et al.* International Application WO 92/03918; Kay *et al.* International Application 92/03917; Lonberg, N. *et al.* 1994 *Nature* 368:856-859; Green, L.L. *et al.* 1994 *Nature Genet.* 7:13-21; Morrison, S.L. *et al.* 1994 *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman *et al.* 1993 *Year Immunol* 7:33-40; Tuailon *et al.* 1993 *PNAS* 90:3720-3724; Bruggeman *et al.* 1991 *Eur J Immunol* 21:1323-1326).

An antibody molecule can be one in which the variable region, or a portion thereof, *e.g.*, the CDRs, are generated in a non-human organism, *e.g.*, a rat or mouse. Chimeric, CDR-grafted, and humanized antibodies are within the present disclosure. Antibody molecules generated in a non-human organism, *e.g.*, a rat or mouse, and then modified, *e.g.*, in the variable framework or constant region, to decrease antigenicity in a human are within the present disclosure.

Chimeric antibodies can be produced by recombinant DNA techniques known in the art (see Robinson *et al.*, International Patent Publication PCT/US86/02269; Akira, *et al.*, European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.*, European Patent Application 173,494; Neuberger *et al.*, International Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent Application 125,023; Better *et al.* (1988 *Science* 240:1041-1043); Liu *et al.* (1987) *PNAS* 84:3439-3443; Liu

et al., 1987, *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *PNAS* 84:214-218; Nishimura *et al.*, 1987, *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.*, 1988, *J. Natl Cancer Inst.* 80:1553-1559).

A humanized or CDR-grafted antibody will have at least one or two but generally all three recipient CDRs (of heavy and or light immunoglobulin chains) replaced with a donor CDR. The antibody may be replaced with at least a portion of a non-human CDR or only some of the CDRs may be replaced with non-human CDRs. It is only necessary to replace the number of CDRs required for binding to the antigen. Preferably, the donor will be a rodent antibody, *e.g.*, a rat or mouse antibody, and the recipient will be a human framework or a human consensus framework. Typically, the immunoglobulin providing the CDRs is called the "donor" and the immunoglobulin providing the framework is called the "acceptor." In one embodiment, the donor immunoglobulin is a non-human (*e.g.*, rodent). The acceptor framework is a naturally-occurring (*e.g.*, a human) framework or a consensus framework, or a sequence about 85% or higher, preferably 90%, 95%, 99% or higher identical thereto.

As used herein, the term "consensus sequence" refers to the sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related sequences (See *e.g.*, Winnaker, *From Genes to Clones* (Verlagsgesellschaft, Weinheim, Germany 1987). In a family of proteins, each position in the consensus sequence is occupied by the amino acid occurring most frequently at that position in the family. If two amino acids occur equally frequently, either can be included in the consensus sequence. A "consensus framework" refers to the framework region in the consensus immunoglobulin sequence.

An antibody molecule can be humanized by methods known in the art (*see e.g.*, Morrison, S. L., 1985, *Science* 229:1202-1207, by Oi *et al.*, 1986, *BioTechniques* 4:214, and by Queen *et al.* US 5,585,089, US 5,693,761 and US 5,693,762, the contents of all of which are hereby incorporated by reference).

Humanized or CDR-grafted antibody molecules can be produced by CDR-grafting or CDR substitution, wherein one, two, or all CDRs of an immunoglobulin chain can be replaced. See *e.g.*, U.S. Patent 5,225,539; Jones *et al.* 1986 *Nature* 321:552-525; Verhoeyan *et al.* 1988 *Science* 239:1534; Beidler *et al.* 1988 *J. Immunol.* 141:4053-4060; Winter US 5,225,539, the contents of all of which are hereby expressly incorporated by reference. Winter describes a CDR-grafting method which may be used to prepare the humanized antibodies of the present present disclosure (UK Patent Application GB 2188638A, filed on March 26, 1987; Winter US 5,225,539), the contents of which is expressly incorporated by reference.

Also within the scope of the present disclosure are humanized antibody molecules in which specific amino acids have been substituted, deleted or added. Criteria for selecting amino

acids from the donor are described in US 5,585,089, *e.g.*, columns 12-16 of US 5,585,089, *e.g.*, columns 12-16 of US 5,585,089, the contents of which are hereby incorporated by reference. Other techniques for humanizing antibodies are described in Padlan *et al.* EP 519596 A1, published on December 23, 1992.

5 The antibody molecule can be a single chain antibody. A single-chain antibody (scFv) may be engineered (see, for example, Colcher, D. *et al.* (1999) *Ann N Y Acad Sci* 880:263-80; and Reiter, Y. (1996) *Clin Cancer Res* 2:245-52). The single chain antibody can be dimerized or multimerized to generate multivalent antibodies having specificities for different epitopes of the same target protein.

10 In yet other embodiments, the antibody molecule has a heavy chain constant region chosen from, *e.g.*, the heavy chain constant regions of IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgD, and IgE; particularly, chosen from, *e.g.*, the (*e.g.*, human) heavy chain constant regions of IgG1, IgG2, IgG3, and IgG4. In another embodiment, the antibody molecule has a light chain constant region chosen from, *e.g.*, the (*e.g.*, human) light chain constant regions of
15 kappa or lambda. The constant region can be altered, *e.g.*, mutated, to modify the properties of the antibody (*e.g.*, to increase or decrease one or more of: Fc receptor binding, antibody glycosylation, the number of cysteine residues, cell function, and/or complement function). For example, it is an isotype or subtype, fragment or other mutant, which does not support binding to an Fc receptor, *e.g.*, it has a mutagenized or deleted Fc receptor binding region.

20 Methods for altering an antibody constant region are known in the art. Antibodies with altered function, *e.g.* altered affinity for an effector ligand, such as FcR on a cell, or the C1 component of complement can be produced by replacing at least one amino acid residue in the constant portion of the antibody with a different residue (*see e.g.*, EP 388,151 A1, U.S. Pat. No. 5,624,821 and U.S. Pat. No. 5,648,260, the contents of all of which are hereby incorporated by
25 reference). Similar type of alterations could be described which if applied to the murine, or other species immunoglobulin would reduce or eliminate these functions.

 An antibody molecule can be derivatized or linked to another functional molecule (*e.g.*, a cytokine molecule as described herein or other chemical or proteinaceous groups). As used herein, a "derivatized" antibody molecule is one that has been modified. Methods of
30 derivatization include but are not limited to the addition of a fluorescent moiety, a radionucleotide, a toxin, an enzyme or an affinity ligand such as biotin. Accordingly, the antibody molecules of the present disclosure are intended to include derivatized and otherwise modified forms of the antibodies described herein, including immunoadhesion molecules. For example, an antibody molecule can be functionally linked (*e.g.*, by chemical coupling, genetic
35 fusion, noncovalent association or otherwise) to one or more other molecular entities, such as a

cytokine molecule, another antibody (*e.g.*, a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate association of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

5 One type of derivatized antibody molecule is produced by crosslinking an antibody molecule to one or more proteins, *e.g.*, a cytokine molecule, another antibody molecule (of the same type or of different types, *e.g.*, to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester) or
 10 homobifunctional (*e.g.*, disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, Ill.

In some embodiments, the targeting moiety of the FM includes an antibody molecule or a ligand that selectively binds to an immune regulatory cell surface target, *e.g.*, an immune cell surface receptor. In some embodiments, the immune regulatory cell surface target or receptor
 15 can have one, two, three or more of the following properties: (i) is abundantly present on the surface of an immune cell (*e.g.*, outnumbers the number of receptors for the cytokine molecule present on the immune cell surface); (ii) shows a slow downregulation, internalization, and/or cell surface turnover, *e.g.*, relative to the receptors activated by the cytokine of the FM; (iii) is present on the surface of the immune regulatory cell for a prolonged period of time, *e.g.*, relative
 20 to the receptors activated by the cytokine of the FM; or (iv) once internalized is substantially recycled back to the cell surface, *e.g.*, at least 25%, 50%, 60%, 70%, 80%, 90% or more of the immune regulatory cell surface target is recycled back to the cell surface. In some embodiments, the targeting moiety of the FM binds to a recycling cell surface receptor. In some embodiments, the targeting moiety of the FM binds to a receptor expressed on a cell (*e.g.*, an
 25 immune regulatory cell), *e.g.* the surface membrane of the cell, and further the cell also expresses a cytokine receptor (*e.g.*, a receptor to the cytokine molecule of the FM).

In some embodiments, the targeting moiety of the FM can be chosen from an antibody molecule or a ligand molecule that binds to an immune regulatory cell surface target, *e.g.*, a target chosen from CD45, CD4, CD3, CD2, CD25, CD127, CD197 (CCR7), CXCR3, CXCR4,
 30 CXCR5, CD38, CD27, CD8, CCR4, CCR5, CD137, CCR6 (CD196), CCR8, CCR10, OX40, GITR, NRP1, CTLA4, LAG3, CD73, CD103, CD62L, CCR2, CCR9, CD11a, or CD18. In some embodiments, the immune regulatory cell surface target is chosen from CD4, CD25, CD39, CD45, and/or NRP1.

In one embodiment, the targeting moiety comprises an antibody molecule or a ligand
 35 molecule that binds to CD4. In one embodiment, the targeting moiety comprises an antibody

molecule or a ligand molecule that binds to CD25. In one embodiment, the targeting moiety comprises an antibody molecule or a ligand molecule that binds to CD127. In one embodiment, the targeting moiety comprises a bispecific antibody molecule or a ligand molecule that binds to CD4 and CD25. In one embodiment, the targeting moiety comprises a bispecific antibody molecule or a ligand molecule that binds to CD127 and CD25 (*e.g.*, for negative enrichment of Tregs). In one embodiment, the targeting moiety comprises a bispecific antibody molecule or a ligand molecule that binds to CD127 and CD4 (*e.g.*, for negative enrichment of Tregs). The antibodies can be selected to fine tune the selective binding to the immune cell such as Treg.

In one embodiment, the targeting moiety comprises an antibody molecule or a ligand molecule that binds to CD45 (also interchangeably referred to herein as “CD45 receptor” or “CD45R”). In some embodiments, the target is CD45 (*e.g.*, a CD45 isoform chosen from CD45RA, CD45RB, CD45RC or CD45RO). In embodiments, CD45 is primarily expressed on T cells. For example, CD45RA is primarily expressed on naïve T cells; CD45RO is primarily expressed on activated and memory T cells. In one embodiment, the targeting moiety comprises an antibody molecule or a ligand molecule that binds to CD8. In one embodiment, the targeting moiety comprises a bispecific antibody molecule or a ligand molecule that binds to CD8 and CD45.

In other embodiments, the targeting moiety of the FM comprises an antibody molecule (*e.g.*, an antigen binding domain), a receptor molecule (*e.g.*, a receptor, a receptor fragment or functional variant thereof), or a ligand molecule (*e.g.*, a ligand, a ligand fragment or functional variant thereof), or a combination thereof, that binds to the Treg target or receptor.

In some embodiments, the antibody molecule of the targeting moiety of the FM comprises a full antibody (*e.g.*, an antibody that includes at least one, and preferably two, complete heavy chains, and at least one, and preferably two, complete light chains), or an antigen-binding fragment (*e.g.*, a Fab, F(ab')₂, Fv, a single chain Fv, a single domain antibody, a diabody (dAb), a bivalent antibody, or bispecific antibody or fragment thereof, a single domain variant thereof, or a camelid antibody)) that binds to the Treg target or receptor.

The heavy chain constant region of the antibody molecule can be chosen from IgG1, IgG2, IgG3, or IgG4, or a fragment thereof, and more typically, IgG1, IgG2 or IgG4. In some embodiments, the Fc region of the heavy chain can include one or more alterations, *e.g.*, substitutions, to increase or decrease one or more of: Fc receptor binding, neonatal-Fc receptor binding, antibody glycosylation, the number of cysteine residues, cell function, complement function, or stabilize antibody formation (*e.g.*, stabilize IgG4). For example, the heavy chain constant region for an IgG4, *e.g.*, a human IgG4, can include a substitution at position 228 (*e.g.*, a Ser to Pro substitution), as shown in, *e.g.*, SEQ ID NO: 67 (see *e.g.*, Angal, S, King, DJ, *et al.*

(1993) *Mol Immunol* 30:105-108 (initially described as S241P using a different numbering system); Owens, R, Ball, E, *et al.* (1997) *Immunotechnology* 3:107-116).

The light chain constant region of the antibody molecule can be chosen from the light chain constant regions of kappa or lambda, or a fragment thereof.

5 The antibody molecule of the targeting moiety of the FM can bind to the target antigen with a dissociation constant of less than about 100 nM, 50nM, 25nM, 10 nM, *e.g.*, less than 1 nM (*e.g.*, about 10 – 100 pM). In embodiments, the antibody molecule binds to a conformational or a linear epitope on the antigen. In certain embodiments, the antigen bound by the antibody molecule of the targeting moiety is stably expressed on the surface of the immune
10 cell. In embodiments, the antigen is a cell surface receptor that is more abundant on the cell surface relative to a receptor for the cytokine molecule of the FM on the cell surface.

In some embodiments, the targeting moiety is chosen from an antibody molecule (*e.g.*, a full antibody (*e.g.*, an antibody that includes at least one, and preferably two, complete heavy chains, and at least one, and preferably two, complete light chains), or an antigen-binding
15 fragment (*e.g.*, a Fab, F(ab')₂, Fv, a single chain Fv, a single domain antibody, a diabody (dAb), a bivalent antibody, or bispecific antibody or fragment thereof, a single domain variant thereof, or a camelid antibody)), or a non-antibody scaffold, or a ligand that binds to an immune cell surface target or ligand. In some embodiments, the targeting moiety is an antibody molecule or a ligand that binds to CD45, CD4, CD3, CD2, CD25, CD127, CD197 (CCR7), CXCR3,
20 CXCR4, CXCR5, CD38, CD27, CD8, CCR4, CCR5, CD137, CCR6 (CD196), CCR8, CCR10, OX40, GITR, NRP1, CTLA4, LAG3, CD73, CD103, CD62L, CCR2, CCR9, CD11a, or CD18, *e.g.*, a bispecific antibody that binds to two or more of the aforesaid targets.

In some embodiments, the antibody molecule (*e.g.*, mono- or bi-specific antibodies) binds to one or more of CD45, CD4, CD25, CD39, CD137, or NRP1, *e.g.*, it is an IgG, *e.g.*, human
25 IgG4, or an antigen binding domain, *e.g.*, a Fab, a F(ab')₂, Fv, a single chain Fv, that binds to CD45, CD4, CD25, CD39, CD137, or NRP1.

In some embodiments, the antibody molecule is a human, a humanized or a chimeric antibody. In embodiments, the antibody molecule is a recombinant antibody.

Also encompassed by the present disclosure are antibody molecules having the amino acid
30 sequences disclosed herein, or an amino acid sequence substantially identical thereof), nucleic acid molecules encoding the same, host cells and vectors comprising the nucleic acid molecules.

In certain embodiments, the targeting moiety can be a binding agent for CD45, such as an antibody or antigen-binding fragment thereof. In some embodiments, the anti-CD45 antibody is a human anti-CD45 antibody, a humanized anti-CD45 antibody, or a chimeric anti-
35 CD45 antibody. In some embodiments, the anti-CD45 antibody is an anti-CD45 monoclonal

antibody. Exemplary anti-CD45 antibodies include antibodies BC8, 4B2, GAP8.3 or 9.4. Antibodies against other immune cell surface targets are also disclosed, *e.g.*, anti-CD8 antibodies, such as OKT8 monoclonal antibodies, anti-CD18 antibodies, such as 1B4 monoclonal antibodies, and anti-CD11a antibodies, such as MHM24 antibodies.

5 In one embodiment, the antibody molecule that binds to CD45 is specific to one CD45 isoform or binds to more than one CD45 isoforms, *e.g.*, is a pan-CD45 antibody. In some embodiments, the anti-CD45 antibody molecule binds to CD45RA and CD45RO. In one embodiment, the anti-CD45 antibody molecule is a BC8 antibody. In some embodiments, the BC8 antibody binds to CD45RA and CD45RO. In other embodiments, the anti-CD45 antibody molecule is CD45RO-specific or is a pan-CD45 antibody molecule, *e.g.*, it binds to activated and memory T cells. Additional examples of anti-CD45 antibody molecules include, but is not limited to, GAP8.3, 4B2, and 9.4.

Exemplary Fusion Proteins

15 Exemplary formats for the FM include a cytokine molecule (*e.g.*, one or more cytokine molecules) coupled to an antibody molecule that binds to an immune cell surface target (*e.g.*, immunoglobulin moiety (Ig), for example an antibody (*e.g.*, a full antibody, IgG) or antibody fragment (Fab, scFv, a half antibody, or a single domain antibody and the like). In embodiments, the FM includes a fusion to the amino-terminus (N-terminus) or carboxy-terminus (C-terminus) of the antibody molecule, typically, the C-terminus. The cytokine molecule can be coupled to the antibody molecule, optionally, via a linker. In some embodiments, the cytokine or cytokine receptor, *e.g.*, receptor fragment (*e.g.*, sushi domain) is coupled to the N-terminus or the C-terminus of the antibody molecule. In embodiments, the cytokine or cytokine receptor, *e.g.*, receptor fragment (*e.g.*, sushi domain) is coupled to the N-terminus or the C-terminus of the light chain of the antibody molecule (*e.g.*, a full antibody or fragment thereof, *e.g.*, a Fab, a scFv, or a half antibody). Alternatively or in combination, the cytokine or cytokine receptor, *e.g.*, receptor fragment (*e.g.*, sushi domain) is coupled to the N-terminus or the C-terminus of the heavy chain of the antibody molecule (*e.g.*, a full antibody or fragment thereof, *e.g.*, a Fab, a scFv, a half antibody, or a single domain antibody (VH)). Examples of the formats are provided in, *e.g.*, FIG. 1, 2A, 3A-3D, 6A, 11A and 12A.

30 In some embodiments, the FM includes an antibody molecule or fragment thereof, *e.g.*, anti-CD45 (*e.g.*, BC8 antibody), anti-CD4, anti-CD25, anti-CD8, anti-CD39, or anti-NRP1, that is coupled to, *e.g.*, covalently linked, to a cytokine molecule, *e.g.*, IL-15, IL-2, IL-7, IL-21, IL-27, or a cytokine receptor, *e.g.*, IL-15Ralpha sushi domain, at either the N-terminal region or the C-terminal region of the light chain or heavy chain (as depicted in, *e.g.*, FIGS. 1B, 1G, 2A, 3A-

3D, 6A, 11A and 12A). Any combination of orientation of light chain- or heavy chain-cytokine molecule can be present in the FM.

For example, the FM can include a full antibody or a tetramer of two identical half-antibodies, *e.g.*, a first and second antibody, each having a light chain and a heavy chain (*e.g.*, a scFv) of the antibody molecule, *e.g.*, anti-CD45 (*e.g.*, BC8 antibody), anti-CD8, anti-CD4, anti-CD25, anti-CD39, or anti-NRP1, that is coupled to, *e.g.*, covalently linked, to a cytokine molecule, *e.g.*, IL-2 or a variant thereof, IL-15 or IL-15 sushi domain, at the N-terminal region of the light chain (*e.g.*, a depicted in FIGS. 3A and 3C).

In other embodiments, the FM can include a full antibody or a tetramer of two identical half-antibodies, *e.g.*, a first and second antibodies, each having a light chain and a heavy chain of the targeting antibody molecule, *e.g.*, anti-CD45 (*e.g.*, BC8 antibody), anti-CD8, anti-CD4, anti-CD25, anti-CD39, or anti-NRP1, that is coupled to, *e.g.*, covalently linked, to a cytokine molecule, *e.g.*, IL-2 or its variant, IL-15 or an IL-15 sushi domain, at the C-terminal region of the light chain (*e.g.*, a depicted in FIGS. 2A, 3A, 3C and 6A).

Alternatively, or in combination with the aforesaid formats, the FM can include a full antibody or a tetramer of two identical half-antibodies, *e.g.*, a first and second antibodies, each having a light chain and a heavy chain of the targeting antibody molecule, *e.g.*, anti-CD45 (*e.g.*, BC8 antibody), anti-CD8, anti-CD4, anti-CD25, anti-CD39, or anti-NRP1, that is coupled to, *e.g.*, covalently linked, to a cytokine molecule, *e.g.*, IL-2 or its variant, IL-15 or an IL-15 sushi domain, at the C-terminal region of the heavy chain (*e.g.*, a depicted in FIG. 6A).

Alternatively, or in combination with the aforesaid format, the FM can include a full antibody or a tetramer of two identical half-antibodies, *e.g.*, a first and second antibodies, each having a light chain and a heavy chain of the targeting antibody molecule, *e.g.*, anti-CD45 (*e.g.*, BC8 antibody), anti-CD8, anti-CD4, anti-CD25, anti-CD39, or anti-NRP1, that is coupled to, *e.g.*, covalently linked, to a cytokine molecule, *e.g.*, IL-2 or its variant, IL-15 or an IL-15 sushi domain, at the N-terminal region of the heavy chain.

Alternatively, or in combination with the aforesaid formats, the FM can include an antibody fragment (*e.g.*, a scFv, a Fab) having a light chain variable domain and a heavy chain variable domain of the targeting antibody molecule, *e.g.*, anti-CD45 (*e.g.*, BC8 antibody), anti-CD8, anti-CD4, anti-CD25, anti-CD39, or anti-NRP1, that is coupled to, *e.g.*, covalently linked, to a cytokine molecule, *e.g.*, IL-2 or its variants, IL-15 or an IL-15 sushi domain, at the N- or C-terminal region of the heavy chain variable domain (*e.g.*, a depicted in FIGS. 2A, 3B, 3D, 11A, and 12A).

Alternatively, or in combination with the aforesaid formats, the FM can include an antibody fragment (*e.g.*, a scFv, a Fab) having a light chain variable domain and a heavy chain

variable domain of the targeting antibody molecule, *e.g.*, anti-CD45 (*e.g.*, BC8 antibody), anti-CD8, anti-CD4, anti-CD25, anti-CD39, or anti-NRP1, that is coupled to, *e.g.*, covalently linked, to a cytokine molecule, *e.g.*, IL-2 or its variants, IL-15 or an IL-15 sushi domain, at the N- or C-terminal region of the light chain variable domain.

Alternatively, the FM includes a tetramer of two different half-antibodies, *e.g.*, a first and second antibodies, wherein the first antibody has a light chain and a heavy chain (or an fragment thereof, *e.g.*, scFv or Fab) of the targeting antibody molecule, *e.g.*, anti-CD45 (*e.g.*, BC8 antibody), anti-CD8, anti-CD4, anti-CD25, anti-CD39, or anti-NRP1, that is coupled to, *e.g.*, covalently linked, to a cytokine molecule, *e.g.*, IL-2 or its variants, IL-15 or an IL-15 sushi domain, at the N-terminal region of the light chain; and the second antibody has a light chain and a heavy chain (or an fragment thereof, *e.g.*, scFv or Fab) of the targeting antibody molecule, *e.g.*, anti-CD45 (*e.g.*, BC8 antibody), anti-CD8, anti-CD4, anti-CD25, anti-CD39, or anti-NRP1, that is coupled to, *e.g.*, covalently linked, to a cytokine molecule, *e.g.*, IL-2 or its variants, IL-15 or an IL-15 sushi domain, at the C-terminal region of the light chain or the heavy chain. Any pairing of antibodies to the same or different targets can be used, such as CD4:CD45, CD8:CD45, CD4:CD39, CD4:NRP1, and CD4:CD25. In some embodiments, while CD127 is a negative surface marker for Tregs, the pairing of CD127 with a positive Treg surface marker (*e.g.*, CD4:CD127, CD25:CD127) can be used to negatively enrich and/or sort Tregs. For example, a blocking antibody can be targeted to non-Treg cells (for example activated Tcons having CD25). The CD127 can target the antibody to the Tcons and the CD25 can then block binding of the IL-2 tethered fusion. Then, when a CD4/CD25-IL2 molecule is introduced, the binding sites for the CD25 targeting portion of the antibody can be blocked on Tcons. Alternatively, an anti-CD127 antibody can be attached to an inhibitor of CD25, such that even if an IL-2 tethered fusion bound the Tcon, it would not activate it. This improves enrichment of IL-2 on Tregs in mixed-cell populations.

In certain embodiments, the FM can be represented with the following formula in an N to C terminal orientation: R1-(optionally L1)-R2 or R2-(optionally L1)-R1; wherein R1 comprises a targeting moiety, L1 comprises a linker (*e.g.*, a peptide linker described herein), and R2 comprises a modulatory moiety, *e.g.*, a cytokine molecule.

In some embodiments, the modulatory moiety, *e.g.*, the cytokine molecule, is connected to, *e.g.*, covalently linked to, the targeting moiety.

In some embodiments, the modulatory moiety, *e.g.*, the cytokine molecule, is functionally linked, *e.g.*, covalently linked (*e.g.*, by chemical coupling, fusion, noncovalent association or otherwise) to the targeting moiety. For example, the modulatory moiety can be covalently coupled indirectly, *e.g.*, via a linker to the targeting moiety.

In embodiments, the linker is chosen from: a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, or a non-helical linker. In some embodiments, the linker is a peptide linker. The peptide linker can be 5-20, 8-18, 10-15, or about 8, 9, 10, 11, 12, 13, 14, or 15 amino acids long. In some embodiments, the peptide linker comprises Gly and Ser, *e.g.*, a linker comprising the amino acid sequence (Gly₄-Ser)_n, wherein *n* indicates the number of repeats of the motif, *e.g.*, *n*=1, 2, 3, 4 or 5 (*e.g.*, a (Gly₄Ser)₂ or a (Gly₄Ser)₃ linker). In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 36, 37, 38, or 39, or an amino acid sequence substantially identical thereto (*e.g.*, having 1, 2, 3, 4, or 5 amino acid substitutions). In one embodiment, the linker comprises an amino acid sequence GGGSGGGS (SEQ ID NO: 37). In another embodiment, the linker comprises amino acids from an IgG4 hinge region, *e.g.*, amino acids DKTHTSPSPAP (SEQ ID NO: 38).

In some embodiments, the linker can be a human serum albumin (HSA) linker. It has been surprisingly discovered that the HSA linker can improve IL-2 expression efficiency and increase overall stability of the FM. In some embodiments, the FM can have the formula R1-L1-R2 and can be constructed as a single genetic molecule for expression in cells.

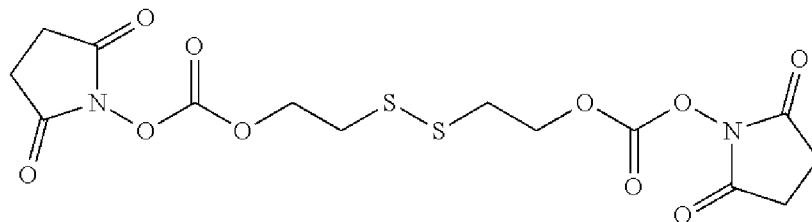
In other embodiments, the linker is a non-peptide, chemical linker. For example, the modulatory moiety is covalently coupled to the targeting moiety by crosslinking. Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) or homobifunctional (*e.g.*, disuccinimidyl suberate). In yet other embodiments, the modulatory moiety is directly covalently coupled to the targeting moiety, without a linker.

In some embodiments, the linker can be a biodegradable or cleavable linker. A cleavable linker allows for cleavage of the FM such that the immune stimulating moiety, *e.g.*, the cytokine molecule, can be released from the immune targeting moiety. The cleavage of the linker may be caused by biological activation within the relevant tissue or, alternatively, by external stimuli such as, *e.g.*, electromagnetic radiation *e.g.*, UV-radiation.

In one embodiment, the cleavable linker is configured for cleavage exterior to a cell, *e.g.*, to be cleaved in conditions associated with cell or tissue damage or disease. Such conditions include, for example, acidosis; the presence of intracellular enzymes (that are normally confined within cells), including necrotic conditions (*e.g.*, cleaved by calpains or other proteases that spill out of necrotic cells); hypoxic conditions such as a reducing environment; thrombosis (*e.g.*, a linker may be cleavable by thrombin or by another enzyme associated with the blood clotting cascade); immune system activation (*e.g.*, a linker may be cleavable by action of an activated complement protein); or other condition associated with disease or injury.

In one embodiment, a cleavable linker may include an S-S linkage (disulfide bond), or

may include a transition metal complex that falls apart when the metal is reduced. One embodiment of the S-S linker may have the following structure (as disclosed in U.S. Patent No. 9,603,944, incorporated herein by reference in its entirety:



5 Another example pH sensitive linkers which are cleaved upon a change in pH, e.g., at low pH, which will facilitate hydrolysis of acid (or base) labile moieties, e.g. acid labile ester groups etc. Such conditions may be found in the extracellular environment, such as acidic conditions which may be found near cancerous cells and tissues or a reducing environment, as may be found near hypoxic or ischemic cells and tissues; by proteases or other enzymes found
 10 on the surface of cells or released near cells having a condition to be treated, such as diseased, apoptotic or necrotic cells and tissues; or by other conditions or factors. An acid-labile linker may be, for example, a cis-aconitic acid linker. Other examples of pH-sensitive linkages include acetals, ketals, activated amides such as amides of 2,3 dimethylmaleamic acid, vinyl ether, other activated ethers and esters such as enol or silyl ethers or esters, imines, iminiums, orthoesters,
 15 enamines, carbamates, hydrazones, and other linkages known in the art (see, e.g., PCT Publication No. WO 2012/155920 and Franco et al. AIMS Materials Science, 3(1): 289-323, incorporated herein by reference). The linkers disclosed in U.S. Provisional Application Nos. 62/554,067 filed September 5, 2017 and 62/616,221 filed January 11, 2018 can also used and are incorporated herein by reference. The expression “pH sensitive” refers to the fact that the
 20 cleavable linker in question is substantially cleaved at an acidic pH (e.g., a pH below 6.0, such as in the range of 4.0-6.0).

In still another embodiment, the cleavable linker is configured for cleavage by an enzyme, such as a protease (e.g., pepsin, trypsin, thermolysine, matrix metalloproteinase (MMP), a disintegrin and metalloprotease (ADAM; e.g. ADAM-10 or ADAM-17)), a
 25 glycosidase (e.g., α -, β -, γ -amylase, α -, β -glucosidase or lactase) or an esterase (e.g. acetyl cholinesterase, pseudo cholinesterase or acetyl esterase). Other enzymes which may cleave the cleavable linker include urokinase plasminogen activator (uPA), tissue plasminogen activator (tPA), granzyme A, granzyme B, lysosomal enzymes, cathepsins, prostate-specific antigen, Herpes simplex virus protease, cytomegalovirus protease, thrombin, caspase, and interleukin 1
 30 beta converting enzyme.

Still another example is over-expression of an enzyme, e.g., proteases (e.g., pepsin,

trypsin), in the tissue of interest, whereby a specifically designed peptide linker will be cleaved in upon arrival at the tissue of interest. Illustrative examples of suitable linkers in this respect are Gly-Phe-Ser-Gly (SEQ ID NO: 97), Gly-Lys-Val-Ser (SEQ ID NO: 98), Gly-Trp-Ile-Gly (SEQ ID NO: 99), Gly-Lys-Lys-Trp (SEQ ID NO: 100), Gly-Ala-Tyr-Met (SEQ ID NO: 101).

5 In still another example, over-expression of an enzyme, e.g. of glycosidases (e.g. α -amylase), in the tissue of interest, causes a specifically designed carbohydrate linker to be cleaved upon arrival at the tissue of interest. Illustrative examples of suitable linkers in this respect are $-(\alpha\text{-1-4-D-Glucose})_n-$ where $n \geq 4$.

In still another example, the cleavable linker is configured for cleavage by
10 electromagnetic radiation, e.g., UV-radiation. UV-exposure of the tissue of interest resulting in cleavage of the linker B can facilitate drug release or facilitate nanoparticles uptake in the desired tissue.

The cleavable linker may include a total of from 2 to 60 atoms, such as from 2 to 20 atoms. The cleavable linker may include amino acid residues, and may be a peptide linkage, e.g.,
15 of from 1 to 30, or from 2 to 10, amino acid residues. In one variant, the cleavable linker B consists of from 1 to 30, such as from 2 to 10, or from 2 to 8, or from 3 to 9, or from 4-10, amino acids. For pH sensitive linkers, the number of atoms is typically from 2 to 50, such as from 2-30.

In some embodiments of the invention, the linker includes an aminocaproic acid (also
20 termed aminohexanoic acid) linkage or a linkage composed of from 1 to 30, or from 2 to 10 carbohydrate residues.

In one embodiment, the linker includes a peptide that can serve as a substrate of a matrix metalloproteinase. As the matrix metalloproteinase, for example, MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-3, MMP-7, MMP-9 (gelatinase B), and the like are
25 known, and a substrate peptide that can serve as a substrate of one or more kinds of matrix metalloproteinases among those mentioned above can be used. For matrix metalloproteinases, see for example, "Molecular mechanism of cancer metastasis", Ed. by Tsuruo T., pp. 92-107, Medical View Co., Ltd., published in 1993. As for the substrate peptide that can serve as a substrate of a matrix metalloproteinase, for example, the matrix metalloproteinases of particular
30 types and substrate peptides specifically recognized thereby are explained in Nature Biotechnology, 19, pp. 661-667, 2001. Therefore, by referring to this publication, a substrate peptide specifically cleaved by a particular type of matrix metalloproteinase can be chosen. For example, Val-Pro-Leu-Ser-Leu-Tyr-Ser-Gly (SEQ ID NO: 102) is known as a specific substrate for MMP-9, and it is preferable to use the aforementioned octapeptide as a substrate peptide that
35 can serve as a substrate of MMP-9. Illustrative examples of the substrate peptide that can serve

as a substrate of a matrix metalloproteinase include Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln (SEQ ID NO: 103), Val-Pro-Met-Ser-Met-Arg-Gly-Gly (SEQ ID NO: 104), Ile-Pro-Val-Ser-Leu-Arg-Ser-Gly (SEQ ID NO: 105), Arg-Pro-Phe-Ser-Met-Ile-Met-Gly (SEQ ID NO: 106), Val-Pro-Leu-Ser-Leu-Thr-Met-Gly (SEQ ID NO: 107), Ile-Pro-Glu-Ser-Leu-Arg-Ala-Gly (SEQ ID NO: 108), Arg-His-Asp, Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys (SEQ ID NO: 109), Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys (SEQ ID NO: 110), Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg (SEQ ID NO: 111), Pro-Leu-Gly-Ile-Ala-Gly-Arg (SEQ ID NO: 112), Gly-Pro-Leu-Gly-Pro (SEQ ID NO: 113), Gly-Pro-Ile-Gly-Pro (SEQ ID NO: 114), and the like.

In another embodiment, the linker includes a peptide that can serve as a substrate of a disintegrinase and metalloprotease (ADAM), an MMP, or a granzyme. For example, without limitation the linker could comprise a peptide substrate of ADAM-8, ADAM-10, ADAM-12, ADAM-17, MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, granzyme A, or granzyme B. Substrates of ADAMs, MMPs, and granzymes have been well-described (e.g. in Miller, MA, et al. Integrative Biology (2011) 3:422-438, Van Damme et al. Biol Chem (2010) 391:983-997, and Casciola-Rosen, L, et al. J Biol Chem (2007) 282:4545-4552) and one skilled in the art would readily be able to incorporate such peptides, or variants thereof, into a linker of interest.

The linker may, besides the substrate peptide, contain connectors, involved in the bond or bonds with the therapeutic protein. Such connectors may each consist of one amino acid residue or of an oligopeptide containing from 2 to 10, such as from 3 to 9, or from 4 to 8, or from 2 to 8, amino acid residues. The amino acid residue or oligopeptide as the connectors may, if present, bind to both ends of the substrate peptide, or may bind only to one end of the substrate peptide so as to represent one of the structures. Types of one amino acid usable as the connector(s), and amino acid residues constituting an oligopeptide usable as the connector(s) are not particularly limited, and one amino acid residue of an arbitrary type, or an arbitrary oligopeptide containing, e.g., from 2 to 8 of the same or different amino acid residues of arbitrary types can be used. Examples of the oligopeptide usable as the connector(s) include, for example, connectors that are rich in Gly amino acids. Other organic moieties can also be used as connectors.

In yet other embodiments, the modulatory moiety and the targeting moiety of the FM are not covalently linked, *e.g.*, are non-covalently associated.

Exemplary formats for fusion of a cytokine molecule to an antibody molecule, *e.g.*, an immunoglobulin moiety (Ig), for example an antibody (IgG) or antibody fragment (Fab, scFv and the like) can include a fusion to the amino-terminus (N-terminus) or carboxy-terminus (C-terminus) of the antibody molecule, typically, the C-terminus of the antibody molecule. In one embodiment, a cytokine-Ig moiety fusion molecule comprising a cytokine polypeptide,

cytokine-receptor complex, or a cytokine -receptor Fc complex joined to an Ig polypeptide, a suitable junction between the cytokine polypeptide chain and an Ig polypeptide chain includes a direct polypeptide bond, a junction having a polypeptide linker between the two chains; and, a chemical linkage between the chains. A typical junction is a flexible linker composed of small
 5 Gly4Ser linker (Gly–Gly–Gly–Gly–Ser)_N, where _N indicates the number of repeats of the motif. (Gly4Ser)₂ and (Gly4Ser)₃ are preferred embodiments of linkers for use in the fusion constructs of the present disclosure.

Exemplary fusion proteins described herein can comprise the amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5,
 10 SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55,
 15 SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83,
 20 SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to any of the aforesaid amino acid sequences).

Described herein are exemplary fusion proteins (or portion thereof) of the present disclosure. It should be noted that in certain scFv the arrangement is VH-linker-VL. However,
 25 the VL-linker-VH arrangement can also be used without affecting functionality. In addition, while specific linkers (Linker-1, Linker-2, etc.) were used in various constructs, other linkers disclosed herein can also be used interchangeably.

SEQ ID NO: 1

30 Name: LC-chBC8-sushi

Light-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from parental BC8 mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15R α -sushi domain genetically fused to antibody light-chain C-terminus using a flexible linker.

35 DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYLHWYQQKPGQPPLLIYLASNLES

GVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRELPTFTGSGTKLEIKRTVAAPSVFI
 FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL
 SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGGSITCPP
PMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTPSLKCI

5 R

From N-to C-terminus, a light chain portion of a chimeric BC8 Fab is shown fused via a peptide linker to an IL-15-binding sushi domain. The sequence of the light chain is shown in normal font; the location of the peptide linker is shown by italics and single underline; and the sushi domain is shown by the double underline. The kappa constant region is shown as SEQ ID

10 NO: 74.

In some embodiments, the chimeric BC8 antibody comprises the light chain variable amino acid sequence (optionally, further including a kappa light chain sequence) shown in SEQ ID NO:1, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 95% or higher identical to SEQ ID NO:1). In embodiments, the chimeric BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable region of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 1.

20 In embodiments, the FMs described herein can comprise the amino acid sequence selected from SEQ ID NO: 1, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO: 1, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 1.

25 In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 1.

SEQ ID NO: 2

Name: sushi-LC-chBC8

30 Light-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from parental BC8 mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15R α -sushi domain genetically fused to antibody light-chain N-terminus using a flexible linker.

ITCPPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTP
 35 SLKCIGGGGSGGGSGGGGSDIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYLHW

YQQKPGQPPKLLIYLASNLESGVPARFSGSGGTDFTLNIHPVEEEDAATYYCQHSRELP
 FTFGSGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNAL
 QSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE
 C

5 From N-to C-terminus, an IL-15-binding wild type sushi domain is fused via a peptide linker to a light chain portion of a chimeric BC8 Fab is shown. The sequence of the light chain is shown in normal font; the location of the peptide linker is shown by italics and single underline; and the sushi domain is shown by the double underline. The kappa constant region is shown as SEQ ID NO: 74.

10 In some embodiments, the chimeric BC8 antibody comprises the light chain variable amino acid sequence (optionally, further including a kappa light chain sequence) shown in SEQ ID NO:2, or an amino acid sequence substantially identical thereof (e.g., an amino acid sequence at least 85%, 90%, 95% or higher identical to SEQ ID NO:2). In embodiments, the BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable
 15 region of the BC8 antibody, e.g., according to the Kabat definition, or a closely related CDR, e.g., CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (e.g., substitutions, deletions, or insertions, e.g., conservative substitutions) from any of the CDR sequences of SEQ ID NO: 2.

In embodiments, the FMs described herein can comprise the amino acid sequence
 20 selected from SEQ ID NO: 2, or an amino acid sequence substantially identical thereto (e.g., an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO: 2, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (e.g., substitutions, deletions, or insertions, e.g., conservative substitutions) relative to SEQ ID NO: 2. In embodiments, the FM comprises no more than five, ten or fifteen alterations (e.g.,
 25 substitutions, deletions, or insertions, e.g., conservative substitutions) relative to SEQ ID NO: 2.

SEQ ID NO: 3

Name: LC-chBC8-IL15 (also referred to as LC-chBC8-L1-IL15)

Light-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from
 30 parental mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15 genetically fused to antibody light-chain C-terminus using a flexible linker.
 DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYLHWYQQKPGQPPKLLIYLASNLES
 GVPARFSGSGGTDFTLNIHPVEEEDAATYYCQHSRELPFTFGSGTKLEIKRTVAAPSVFI
 FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL
 35 SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGGSNWV

NVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELEQVISLESGDASIHDTVE
NLIILANNSLSSNGNVTESGCKECELEEKNIKEFLQSFVHIVQMFINTS

From N-to C-terminus, a light chain portion of a chimeric BC8 Fab is shown fused via a peptide linker to an IL-15 cytokine. The sequence of the light chain is shown in normal font; the location of the peptide linker is shown by italics and single underline; and the cytokine molecule is shown by the double underline. The kappa constant region is shown as SEQ ID NO: 74.

In some embodiments, the chimeric BC8 antibody comprises the light chain variable amino acid sequence (optionally, further including a kappa light chain sequence) shown in SEQ ID NO:3, or an amino acid sequence substantially identical thereof (e.g., an amino acid sequence at least 85%, 90%, 95% or higher identical to SEQ ID NO:3). In embodiments, the BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable domain of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 3.

In embodiments, the FMs described herein can comprise the amino acid sequence selected from SEQ ID NO: 3, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO: 3, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 3. In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 3.

SEQ ID NO: 4

Name: IL15-LC-chBC8

Light-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from parental mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15 genetically fused to antibody light-chain N-terminus using a flexible linker.

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELEQVISLESGDASIH
DTVENLIILANNSLSSNGNVTESGCKECELEEKNIKEFLQSFVHIVQMFINTSGGGGSGG
GGSGGGGSDIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYLHWYQQKPGQPPLLI
 YLASNLESGVPARFSGSGGTDFTLNIHPVEEDAATYYCQHSRELPTFTGSGTKLEIKR
 TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD
 SKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

From N-to C-terminus, an IL-15 cytokine fused via a peptide linker to a light chain

portion of a chimeric BC8 Fab is shown. The sequence of the light chain is shown in normal font; the location of the peptide linker is shown by italics and underline; and the cytokine molecule is shown by the double underline. The kappa constant region is shown as SEQ ID NO: 74.

In some embodiments, the chimeric BC8 antibody comprises the light chain variable amino acid sequence (optionally, further including a kappa light chain sequence) shown in SEQ ID NO:4, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 95% or higher identical to SEQ ID NO:4). In embodiments, the BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable domain of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from the any of the CDR sequences of SEQ ID NO: 4.

In embodiments, the FMs described herein can comprise the amino acid sequence selected from SEQ ID NO: 4, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO: 2, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 4. In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 4.

An exemplary corresponding heavy chain of the chimeric BC8 antibody molecule, *e.g.*, a Fab or IgG, is shown below as SEQ ID NO:5 or SEQ ID NO:6, respectively.

SEQ ID NO: 5

Name: HC-chBC8-Fab

Fab heavy-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from parental BC8 mouse monoclonal antibody and CH1 domain from human IgG1.

In some embodiments, the chimeric BC8 antibody comprises the heavy chain variable amino acid sequence (optionally, further including a CH1 domain sequence from human IgG1) shown in SEQ ID NO:5, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to SEQ ID NO:5). In embodiments, the BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the heavy chain variable domain of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more

than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from the CDR sequence of SEQ ID NO: 5.

SEQ ID NO: 6

5 Name: HC-chBC8-IgG4S228P

Heavy-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from parental BC8 mouse monoclonal antibody and constant region from human IgG4 containing S228P mutation (IgG4-S228P).

10 In some embodiments, the chimeric BC8 antibody comprises the heavy chain variable amino acid sequence (optionally, further including a constant domain sequence from human IgG4 containing S228P mutation (IgG4-S228P)) shown in SEQ ID NO:6, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to SEQ ID NO:6). In embodiments, the chimeric BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the heavy chain variable domain of the BC8
15 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 6.

20 SEQ ID NO: 7

Name: LC-chBC8

Light-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from parental BC8 mouse monoclonal antibody and human constant kappa domain.

25 In some embodiments, the BC8 antibody comprises the light chain variable amino acid sequence (optionally, further including a constant domain sequence from human kappa) shown in SEQ ID NO:7, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to SEQ ID NO:7). In embodiments, the BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable domain of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR,
30 *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 7.

SEQ ID NO: 8

35 Name: HC-chBC8-IgG4S228P-IL15

Heavy-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from parental BC8 mouse monoclonal antibody and constant region from human IgG4 containing S228P mutation (IgG4-S228P); contains wild-type IL-15 genetically fused to antibody heavy-chain C-terminus using a flexible linker.

5 QVQLVESGGGLVQPGGSLKLSCAASGFDPSRYWMSWVRQAPGKGLEWIGEINPTSSTI
 NFTP SLKDKVFISRDNAKNTLYLQMSKVRSEDTALYYCARGNYYRYGDAMDYWGQG
 TSVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF
 PAVLQSSGLYSLSSVVTVPSSSLGTKYTCNV DHKPSNTKVDKRVESKYGPPCPPCPAPE
 FLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKP
 10 REEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYT
 LPPS QEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSR
 LTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLKGGGGSGGGSGGGGSNWV
NVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFELLEQVISLESGDASIHTVE
NLIILANNSLSSNGNVTESGCKECELEEKNIKEFLQS FVHIVQMFINTS

15 From N-to C-terminus, a heavy chain portion of a chimeric BC8 IgG4 fused to an IL-15 cytokine fused via a peptide linker is shown. The sequence of the heavy chain is shown in normal font; the location of the peptide linker is shown by italics and underline; and the cytokine molecule is shown by the double underline.

In some embodiments, the BC8 antibody comprises the heavy chain variable amino acid
 20 sequence (optionally, further including human IgG4 constant sequence) shown in SEQ ID NO:8, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 95% or higher identical to SEQ ID NO:8). In embodiments, the BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the heavy chain variable domain of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which
 25 have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from the any of the CDR sequences of SEQ ID NO: 8.

In embodiments, the FMs described herein can comprise the amino acid sequence of
 30 SEQ ID NO: 8, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO: 8, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 8. In
 embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 8.

35

SEQ ID NO: 14

Name: HC-ch9.4-Fab

Fab heavy-chain of chimeric 9.4 anti-CD45 antibody; contains variable domain from parental 9.4 mouse monoclonal antibody and CH1 domain from human IgG1.

5 In some embodiments, the chimeric 9.4 antibody comprises the heavy chain variable amino acid sequence (optionally, further including a human IgG1 heavy chain sequence) shown in SEQ ID NO:14, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 95% or higher identical to SEQ ID NO:14). In embodiments, the 9.4 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the heavy chain variable domain of
10 the 9.4 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO:14.

15 SEQ ID NO: 15

Name: LC-ch9.4-IL15

Light-chain of chimeric 9.4 anti-CD45 antibody; contains variable domain from parental 9.4 mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15 genetically fused to antibody light-chain C-terminus using a flexible linker.

20 DIVMTQAAPSPVPTPGESLSISCRSSKSLHSSGITYLYWFLQRPQGSPQLLIYRMSNLAS
GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGGGTKLEIKRTVAAPSV
FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSTYS
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGGSNWV
NVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFELLEQVISLESGDASIHDTVE
25 NLIILANNSLSSNGNVTESGCKECELEEKNIKEFLOSFVHIVQMFINTS

From N-to C-terminus, a light chain portion of a chimeric 9.4 IgG1 fused to an IL-15 cytokine fused via a peptide linker is shown. The sequence of the light-chain is shown in normal font; the location of the peptide linker is shown by italics and underline; and the cytokine molecule is shown by the double underline. The kappa constant region is shown as SEQ ID NO:
30 74.

In some embodiments, the light chain variable amino acid sequence of the chimeric 9.4 antibody (optionally, further including a kappa light chain sequence) corresponding to the antibody portion of the amino acid sequence shown in SEQ ID NO:15, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or
35 higher identical to the antibody portion of SEQ ID NO:15). In embodiments, the 9.4 antibody

comprises one, two, or all three CDR1, CDR2 or CDR3 of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from the any of the CDR sequences of SEQ ID NO:

5 15.

In embodiments, the FMs described herein can comprise the amino acid sequence of SEQ ID NO: 15, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO: 15, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 15. In

10 In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 15.

SEQ ID NO: 16

15 Name: HC-ch4B2-Fab

Fab heavy-chain of chimeric 4B2 anti-CD45 antibody; contains variable domain from parental 4B2 mouse monoclonal antibody and CH1 domain from human IgG1.

In some embodiments, the chimeric 4B2 antibody comprises the heavy chain variable amino acid sequence (optionally, further including a human IgG1 heavy chain sequence) shown

20 in SEQ ID NO:16, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 95% or higher identical to SEQ ID NO:16). In embodiments, the 4B2 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the heavy chain variable domain of the 4B2 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations

25 (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO:16.

SEQ ID NO: 17

Name: LC-ch4B2-IL15

30 Light-chain of chimeric 4B2 anti-CD45 antibody; contains variable domain from parental 4B2 mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15 genetically fused to antibody light-chain C-terminus using a flexible linker.

DIVITQDELSNPVTSGESVSISCRSSKSLLYKDGKTYLNWFLQRPGQSPQLLIYLMSTRAS
GVSDRFSGSGSGTDFLTLEISRVKAEDVGVYYCQQLVEYPFTFGGGTKLEVKRTVAAPSV
35 FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS

LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGGSNWV
NVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFELLEQVISLESGDASIHDTVE
NLIILANNSLSSNGNVTESGCKECEEELEEKNIKEFLOSFVHIVQMFINTS

From N-to C-terminus, a light chain portion of a chimeric 4B2 IgG1 fused to an IL-15
 5 cytokine via a peptide linker is shown. The sequence of the light chain is shown in normal font;
 the location of the peptide linker is shown by italics and underline; and the cytokine molecule is
 shown by the double underline. The kappa constant region is shown as SEQ ID NO: 74.

In some embodiments, the light chain variable amino acid sequence of the chimeric 4B2
 antibody (optionally, further including a kappa light chain sequence) corresponding to the
 10 antibody portion of the amino acid sequence shown in SEQ ID NO:17, or an amino acid
 sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or
 higher identical to the antibody portion of SEQ ID NO:17). In embodiments, the 4B2 antibody
 comprises one, two, or all three CDR1, CDR2 or CDR3 of the 4B2 antibody, *e.g.*, according to
 the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid
 15 alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or
 insertions, *e.g.*, conservative substitutions) from the any of the CDR sequences of SEQ ID NO:
 17.

In embodiments, the FMs described herein can comprise the amino acid sequence of
 SEQ ID NO: 17, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid
 20 sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO: 17,
 or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions,
 deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 17. In
 embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions,
 deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 17.

25
 SEQ ID NO: 18

Name: HC-hBC8(23)-Fab

Fab heavy-chain of humanized BC8 anti-CD45 antibody; contains humanized variable
 domain and CH1 domain from human IgG1.

30 In some embodiments, the heavy chain variable amino acid sequence of a humanized
 BC8 antibody (optionally, further including a CH1 domain sequence from human IgG1) has the
 amino acid sequence shown in SEQ ID NO:18, or an amino acid sequence substantially identical
 thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody
 portion of SEQ ID NO:18). In embodiments, the BC8 antibody comprises one, two, or all three
 35 CDR1, CDR2 or CDR3 of the heavy chain variable domain of the BC8 antibody, *e.g.*, according

to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 18.

5 SEQ ID NO: 19

Name: LC-hBC8(23)-IL15

Light-chain of humanized BC8 anti-CD45 antibody; contains humanized variable domain and human constant kappa domain; contains wild-type IL-15 genetically fused to antibody light-chain C-terminus using a flexible linker.

10 EIVLTQSPATLSLGLGERATISCRASKSVSTSGYSYLHWYQQKPGQAPKLLIYLASNRAT
GVPARFSGSGPGTDFLTITSSLEPEDFATYYCQHSRELPTFGGQTKLEIKRTVAAPSVFIF
PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGGSNWVN
VISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLESGDASIHDVTEN
15 LIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

From N-to C-terminus, a light chain portion of a humanized BC8 IgG1 fused to an IL-15 cytokine via a peptide linker is shown. The sequence of the light chain is shown in normal font; the location of the peptide linker is shown by italics and underline; and the cytokine molecule is shown by the double underline. The kappa constant region is shown as SEQ ID NO: 74.

20 In some embodiments, the light chain variable amino acid sequence of the humanized BC8 antibody (optionally, further including a kappa light chain sequence) corresponding to the antibody portion of the amino acid sequence shown in SEQ ID NO:19, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody portion of SEQ ID NO:19). In embodiments, the BC8 antibody
25 comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable domain of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any the CDR sequences of SEQ ID NO: 19.

30 In embodiments, the FMs described herein can comprise the amino acid sequence of SEQ ID NO: 19, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO: 19, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 19. In
35 embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions,

deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 19.

SEQ ID NO: 20

Name: HC-hBC8(23)-null-Fab

- 5 Fab heavy-chain of humanized BC8 anti-CD45 antibody; contains humanized variable domain, an SGGGS (SEQ ID NO: 90) substitution in CDR-H3, and CH1 domain from human IgG1.

SEQ ID NO: 21

- 10 Name: LC-chBC8-L2-IL15

Light-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from parental mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15 genetically fused to antibody light-chain C-terminus using a flexible linker with sequence GGGSGGGS (SEQ ID NO: 37).

- 15 DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYLHWYQQKPGQPPKLLIYLASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRELPFTFGSGTKLEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNFPYFPAKVKQWKVDNALQSGNSQESVTEQDSKDYSL
SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC GGGSGGGSNWNVISDLKKI
EDLIQSMHIDATLYTESDVHPCKVTAMKCFLELQVISLESGDASIHDTVENLIILANNS
20 LSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

From N-to C-terminus, a light chain portion of a chimeric BC8 IgG1 fused to an IL-15 cytokine via a peptide linker (L2) is shown. The sequence of the light chain is shown in normal font; the location of the peptide linker is shown by italics and underline; and the cytokine molecule is shown by the double underline. The kappa constant region is shown as SEQ ID NO:

- 25 74.

- In some embodiments, the light chain variable amino acid sequence of the chimeric BC8 antibody (optionally, further including a kappa light chain sequence) corresponding to the antibody portion of the amino acid sequence shown in SEQ ID NO:21, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or
30 higher identical to the antibody portion of SEQ ID NO:21). In embodiments, the BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable domain of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any the CDR
35 sequences of SEQ ID NO: 21.

In embodiments, the FMs described herein can comprise the amino acid sequence of SEQ ID NO: 21, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO:21, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 21. In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 21.

SEQ ID NO: 22

10 Name: LC-chBC8-L3-IL15

Light-chain of chimeric BC8 anti-CD45 antibody; contains variable domain from parental mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15 genetically fused to antibody light-chain C-terminus using a linker related to the human IgG1 hinge (SEQ ID NO: 38).

15 DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYLHWYQQKPGQPPLLIYLASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRELPFTFGSGTKLEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNFPYAPREKVQWKVDNALQSGNSQESVTEQDSKDYSL
SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*DKTHTSPSPAPNWNVISD*
*LKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLES**GDASIHD**TVENLIIL*
20 *ANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS*

From N-to C-terminus, a light chain portion of a chimeric BC8 IgG1 fused to an IL-15 cytokine via a peptide linker (L3) is shown. The sequence of the light chain is shown in normal font; the location of the peptide linker is shown by italics and underline; and the cytokine molecule is shown by the double underline. The kappa constant region is shown as SEQ ID NO: 74.

In some embodiments, the light chain variable amino acid sequence of the chimeric BC8 antibody (optionally, further including a kappa light chain sequence) corresponding to the antibody portion of the amino acid sequence shown in SEQ ID NO:22, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody portion of SEQ ID NO:22). In embodiments, the BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable domain of the BC8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 22.

In embodiments, the FMs described herein can comprise the amino acid sequence of SEQ ID NO: 22, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO:22, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 22. In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 22.

SEQ ID NO: 25

10 Name: hBC8(23)-null heavy-chain variable domain

Heavy-chain variable domain of humanized BC8 anti-CD45 antibody; contains an SGGGS substitution in CDR-H3.

In embodiments, the FMs described herein can comprise the amino acid sequence of SEQ ID NO: 25, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO:25, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 25. In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 25.

20

SEQ ID NO: 26

Name: HC-chMHM24-Fab

Fab heavy-chain of chimeric MHM24 anti-CD11a antibody; contains variable domain from parental mouse monoclonal antibody and CH1 domain from human IgG1.

25 In some embodiments, the heavy chain variable amino acid sequence of a chimeric MHM24 antibody (optionally, further including a CH1 domain sequence from human IgG1) has the amino acid sequence shown in SEQ ID NO:26, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody portion of SEQ ID NO:26). In embodiments, the MHM24 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the heavy chain variable domain of the MHM24 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 26.

35

SEQ ID NO: 69

Name: HC-hMHM24-Fab

Fab heavy-chain of humanized MHM24 anti-CD11a antibody; contains variable domain
5 from a humanized MHM24 heavy-chain variable domain and CH1 domain from human IgG1.

In some embodiments, the heavy chain variable amino acid sequence of a humanized
MHM24 antibody (optionally, further including a CH1 domain sequence from human IgG1) has
the amino acid sequence shown in SEQ ID NO: 27, or an amino acid sequence substantially
identical thereto (e.g., an amino acid sequence at least 85%, 90%, 95% or higher identical to the
10 antibody portion of SEQ ID NO: 27). In embodiments, the MHM24 antibody comprises one,
two, or all three CDR1, CDR2 or CDR3 of the heavy chain variable domain of the MHM24
antibody, e.g., according to the Kabat definition, or a closely related CDR, e.g., CDRs which
have at least one amino acid alteration, but not more than two, three or four alterations (e.g.,
substitutions, deletions, or insertions, e.g., conservative substitutions) from any of the CDR
15 sequences of SEQ ID NO: 27.

SEQ ID NO: 27

Name: LC-chMHM24-IL15

Light-chain of chimeric MHM24 anti-CD11a antibody; contains variable domain from
20 parental mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-
15 genetically fused to antibody light-chain C-terminus using a flexible linker.

DVQITQSPSYLAASPGETISINCRASKTISKYLAWYQEKPGKTNKLLIYSGSTLQSGIPSRF
SGSGSGTDFTLTISSELPEDFAMYYCQQHNEYPLTFGTGTKLELKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNFPYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT
25 SKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGGSNWVNVISDL
KKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFELLEQVISLESGDASIHDVTENLIILA
NNSLSSNGNVTESGCKECELEEKNIKEFLQSFVHIVQMFINTS

From N-to C-terminus, a light chain portion of a chimeric MHM24 IgG1 fused to an IL-
15 cytokine via a peptide linker is shown. The sequence of the light chain is shown in normal
30 font; the location of the peptide linker is shown by italics and underline; and the cytokine
molecule is shown by the double underline. The kappa constant region is shown as SEQ ID NO:
74.

In some embodiments, the light chain variable amino acid sequence of the chimeric
MHM24 antibody (optionally, further including a kappa light chain sequence) corresponding to
35 the antibody portion of the amino acid sequence shown in SEQ ID NO:27, or an amino acid

sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody portion of SEQ ID NO:27). In embodiments, the chimeric MHM24 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable domain of the antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 27.

In embodiments, the FM described herein can comprise the amino acid sequence of SEQ ID NO: 27, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO:27, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 27. In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 27.

SEQ ID NO: 28

Name: HC-ch1B4-Fab

Fab heavy-chain of chimeric 1B4 anti-CD18 antibody; contains variable domain from parental mouse monoclonal antibody and CH1 domain from human IgG1.

In some embodiments, the heavy chain variable amino acid sequence of a chimeric 1B4 antibody (optionally, further including a CH1 domain sequence from human IgG1) has the amino acid sequence shown in SEQ ID NO:28, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody portion of SEQ ID NO:28). In embodiments, the chimeric 1B4 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the heavy chain variable domain of the 1B4 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 28.

SEQ ID NO: 29

Name: LC-ch1B4-IL15

Light-chain of chimeric 1B4 anti-CD18 antibody; contains variable domain from parental mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15 genetically fused to antibody light-chain C-terminus using a flexible linker.

DIVLTQSPASLAVSLGQRATISCRASESVDSYGNSFMHWYQQKPGQPPKLLIYRASNLES
 GIPARFSGSGSRTDFTLTINPVEADDVATYYCQQSNEDPLTFGAGTKLELKRTVAAPSVF
 IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSTYSL
 SSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGGSNWV
 5 NVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFELLEQVISLESGDASIHTVE
NLIILANNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINTS

From N-to C-terminus, a light chain portion of a chimeric 1B4 IgG1 fused to an IL-15 cytokine via a peptide linker is shown. The sequence of the light chain is shown in normal font; the location of the peptide linker is shown by italics and underline; and the cytokine molecule is shown by the double underline. The kappa constant region is shown as SEQ ID NO: 74.

In some embodiments, the light chain variable amino acid sequence of the chimeric 1B4 antibody (optionally, further including a kappa light chain sequence) corresponding to the antibody portion of the amino acid sequence shown in SEQ ID NO:29, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody portion of SEQ ID NO:29). In embodiments, the chimeric 1B4 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable domain of the antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 29.

In embodiments, the FMs described herein can comprise the amino acid sequence of SEQ ID NO: 29, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO:29, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 29. In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 29.

SEQ ID NO: 30

Name: HC-chOKT8-Fab

Fab heavy-chain of chimeric OKT8 anti-CD8 antibody; contains variable domain from parental mouse monoclonal antibody and CH1 domain from human IgG1.

In some embodiments, the heavy chain variable amino acid sequence of a chimeric OKT8 anti-CD8 antibody (optionally, further including a CH1 domain sequence from human IgG1) has the amino acid sequence shown in SEQ ID NO:30, or an amino acid sequence

substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody portion of SEQ ID NO:30). In embodiments, the chimeric OKT8 anti-CD8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the heavy chain variable domain of the OKT8 anti-CD8 antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from the CDR sequence of SEQ ID NO: 30.

SEQ ID NO: 31

10 Name: LC-chOKT8-IL15

Light-chain of chimeric OKT8 anti-CD8 antibody; contains variable domain from parental mouse monoclonal antibody and human constant kappa domain; contains wild-type IL-15 genetically fused to antibody light-chain C-terminus using a flexible linker.

DVQINQSPSFLAASPGETITINCRTSRISQYLAWYQEKPGKTNKLLIYSGSTLQSGIPSRF
 15 SGSGSGTDFTLTISGLEPEDFAMYYCQQHNENPLTFGAGTKLELKRTVAAPSVFIFPPSD
 EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSTLT
 LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGGSNWVNVISD
LKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIHDTVENLIIL
ANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

20 From N-to C-terminus, a light chain portion of a chimeric OKT8 IgG1 fused to an IL-15 cytokine via a peptide linker is shown. The sequence of the light chain is shown in normal font; the location of the peptide linker is shown by italics and underline; and the cytokine molecule is shown by the double underline. The kappa constant region is shown as SEQ ID NO: 74.

In some embodiments, the light chain variable amino acid sequence of the chimeric
 25 OKT8 antibody (optionally, further including a kappa light chain sequence) corresponding to the antibody portion of the amino acid sequence shown in SEQ ID NO:31, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody portion of SEQ ID NO:31). In embodiments, the chimeric OKT8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain
 30 variable domain of the antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences of SEQ ID NO: 31.

In embodiments, the FMs described herein can comprise the amino acid sequence of
 35 SEQ ID NO: 31, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid

sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO:31, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 31. In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 31.

SEQ ID NO: 32

Name: BC8scFv-IL15

IL-15 fused to the C-terminus of BC8-scFv using a short flexible linker and a hexahistidine tag.

QVQLVESGGGLVQPGGSLKLSCAASGFDPSRYWMSWVRQAPGKGLEWIGEINPTSSTI
 NFTP SLKDKVFISRDNAKNTLYLQMSKVRSEDTALYYCARGNYYRYGDAMDYWGQG
 TSVTVSGGGGSGGGGSGGGTGDIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYLH
 WYQQKPGQPPLLIY LASNLESGV PARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRE
 LPFTFGSGTKLEIK RSGSGGGGSLONWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCK
VTAMKCFLELEQVISLES GDASI HDTVENLIILANNSLSSNGNVTESGCKECEELEEKNIK
EFLOSFEVHIVQMFINTSAAAHHHHHH

From N-to C-terminus, a light chain portion of a BC8 scFv fused to an IL-15 cytokine via a peptide linker is shown. The scFv sequence is shown in normal font; the location of the peptide linker is shown by italics and underline; and the cytokine molecule is shown by the double underline.

In some embodiments, the light chain variable amino acid sequence of the BC8 scFv antibody corresponding to the antibody portion of the amino acid sequence shown in SEQ ID NO:31, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95% or higher identical to the antibody portion of SEQ ID NO:32). In embodiments, the BC8 antibody comprises one, two, or all three CDR1, CDR2 or CDR3 of the light chain variable domain of the antibody, *e.g.*, according to the Kabat definition, or a closely related CDR, *e.g.*, CDRs which have at least one amino acid alteration, but not more than two, three or four alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) from any of the CDR sequences within SEQ ID NO: 32.

In embodiments, the FMs described herein can comprise the amino acid sequence of SEQ ID NO: 32, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to SEQ ID NO:32, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 32. In

embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to SEQ ID NO: 32.

Additional sequences that can be included in the FM of the present disclosure are shown in Table 1 below. In some embodiments, the FM comprises a constant lambda or lamda region.

- 5 Exemplary constant lambda or lamda regions include SEQ ID NOS: 74-78. In various embodiments, the FMs described herein can comprise one or more of the amino acid sequences of SEQ ID NOS: 1-86, or an amino acid sequence substantially identical thereto (*e.g.*, an amino acid sequence at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher identity to any one of SEQ ID NOS: 1-86, or having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to any one of SEQ ID NOS: 1-86. In embodiments, the FM comprises no more than five, ten or fifteen alterations (*e.g.*, substitutions, deletions, or insertions, *e.g.*, conservative substitutions) relative to any one of SEQ ID NOS: 1-86.

15 **Table 1. Additional Sequences**

SEQ ID NO:	Name	Description/Comments
9	IL-15R α -sushi (also referred as sushi)	Sushi domain from wild-type IL-15R α .
10	IL15-WT	Wild-type IL-15, corresponds to mature polypeptide from full IL-15 sequence described in SEQ ID NO: 40.
11	IL15-N72D	IL-15 containing N72D mutation
12	sushiL77I-Fc	Sushi domain from IL-15R α fused to IgG1-Fc; sushi domain contains L77I mutation
13	sushi-Fc	Sushi domain from IL-15R α fused to IgG1-Fc
23	hBC8(23) heavy-chain variable domain	Humanized BC8(23) heavy-chain variable domain.
24	hBC8(23) light-chain variable domain	Humanized BC8(23) light-chain variable domain.
33	Leader-1	Leader sequence (<i>e.g.</i> signal sequence, signal peptide) used for antibody light-chain, IL-15, N-terminal IL-15 fusions, and N-terminal fusion of sushi to Fab-light-chain.
34	Leader-2	Leader sequence (<i>e.g.</i> signal sequence, signal peptide) used for antibody heavy-chain.
35	IL-15R α leader sequence	Leader sequence used for sushi and sushi-Fc constructs.
36	Linker-1 (L1)	(G4S)3 linker
37	Linker-2 (L2)	(G3S)2 linker
38	Linker-3 (L3)	Linker derived from IgG1 hinge
39	Linker-4 (L4)	Linker for fusing IL-15 to C-terminus of BC8-scFv
40	Human IL-15 full sequence	Genbank Accession No. CAA62616.1

SEQ ID NO:	Name	Description/Comments
41	Human IL-15R α full sequence	Genbank Accession No. AAI21141.1
42	IL-7 full sequence	Genbank Accession No. AAA59156, AAC63047, and NP_000871, and UniProtKB/Swiss-Prot- P13232
43	IL-7 mature sequence	
44	IL-21 full sequence	Genbank Accession No. AAG29348, AAH66262, AAH69124, and EAX05226
45	IL-21 mature sequence	
46	IL-12A full sequence (also referred to as IL-12p35)	Genbank Accession No. P29459
47	IL-12A mature sequence	
48	IL-12B full sequence (also referred to as IL-12p40)	Genbank Accession No. P29460
49	IL-12B mature sequence	
50	scIL-12p70-BA	Synthetic sequence; IL-12B and IL-12A joined by flexible linker
51	scIL-12p70-AB	Synthetic sequence; IL-12A and IL-12B joined by flexible linker
52	Minimal sushi domain	
53	IgG1-Fc	Fc domain (CH2 and CH3 domains) from human IgG1
54	IgG2-Fc	Fc domain (CH2 and CH3 domains) from human IgG2
55	IgG2Da-Fc	Fc domain from human IgG2 containing two point mutations
56	sushi-IgG2Da-Fc (also referred to as sushi-Fc2Da)	Sushi domain from IL-15R α fused to IgG2Da-Fc
57	BC8 heavy-chain variable domain	
58	BC8 light-chain variable domain	
59	9.4 heavy-chain variable domain	
60	9.4 light-chain variable domain	
61	4B2 heavy-chain variable domain	
62	4B2 light-chain variable domain	
70	Linker-5 (L5)	(G ₃ S) ₄ linker, e.g., as described above in SEQ ID NOs: 50 and 51
71	Linker-6 (L6)	(G ₄ S) ₄ linker
72	scIL-12p70-BA-L6	Synthetic sequence; IL-12B and IL-12A joined by linker L5.
73	scIL-12p70-AB-L5	Synthetic sequence; IL-12A and IL-12B joined by linker L5.
74	human immunoglobulin kappa constant domain	

SEQ ID NO:	Name	Description/Comments
75	human immunoglobulin lambda constant 1	
76	human immunoglobulin lambda constant 2	
77	human immunoglobulin lambda constant 3	
78	human immunoglobulin lambda constant 7	
79	LC-h9.4Fab	Fab light chain of a humanized anti-CD45 antibody; contains humanized 9.4 (h9.4) light-chain variable domain and human constant kappa domain
80	Human IL-2 mature sequence	
81	HC-h9.4Fab-IL2	Heavy-chain of a humanized anti-CD45 antibody; contains variable domain from h9.4 heavy-chain and the CH1 domain from human IgG1; contains a human IL-2 (SEQ ID NO: 80) genetically fused to antibody heavy-chain C-terminus using a flexible linker (Linker-1, SEQ ID NO: 36).
82	HSA	Human Serum Albumin (HSA)
83	scFv-h9.4-HSA-IL2	An scFv comprising a humanized 9.4 antibody; heavy-chain and light-chain variable domains are genetically fused using a flexible linker (Linker-6; SEQ ID NO: 101); contains an HSA peptide (SEQ ID NO: 82) genetically fused to the c-terminus of the scFv domain using a flexible linker (Linker-1, SEQ ID NO: 36); contains a human IL-2 (SEQ ID NO: 80) genetically fused to the HSA c-terminus with a flexible linker (GGGGS (SEQ ID NO: 88))
84	FcgR3B	
85	scFv-h9.4-FcgR3B-IL2	An scFv comprising a humanized 9.4 antibody; heavy-chain and light-chain variable domains are genetically fused using a flexible linker (Linker-6; SEQ ID NO: 101); contains an FcgR3B peptide (SEQ ID NO: 84) genetically fused to the c-terminus of the scFv domain using a flexible linker (Linker-1, SEQ ID NO: 36); contains a human IL-2 (SEQ ID NO: 80) genetically fused to the FcgR3B c-terminus with a flexible linker (GGGSGGGGS (SEQ ID NO: 89)).
86	HC-h9.4Fab-HSA-IL2	Heavy-chain of a humanized anti-CD45 antibody; contains variable domain from h9.4 heavy-chain and the CH1 domain from human IgG1; contains an HSA peptide (SEQ ID NO: 82) genetically fused to the antibody heavy-chain C-terminus using a flexible linker (Linker-1, SEQ ID NO: 36); contains a human IL-2 (SEQ ID NO: 80) genetically fused to the c-terminus of the HSA peptide using a flexible linker (GGGGS, SEQ ID NO: 88)

Protein variants

Full length polypeptides and variants thereof are described below. Full-length IL-15 sequence (SEQ ID NO: 40) is taken from Genbank Accession No. CAA62616.1; mature IL-15 is devoid of the signal sequence and is defined in SEQ ID NO: 10. Full-length IL-15R α (SEQ ID NO: 41) is taken from Genbank Accession No. AAI21141.1. The sushi domain of IL-15R α (IL-15R α -sushi) is given by SEQ ID NO: 9. A minimal sushi domain encompassing the first and fourth cysteines and the intervening amino acids (SEQ ID NO: 52) have also been described elsewhere and are plausible substitutes. Similarly, optional N-terminal additions to the minimal sushi domain comprising the native Thr or Ile-Thr and/or optional C-terminal additions to the minimal sushi domain comprising Ile or Ile-Arg residues are also plausible.

Protein variants described below specify protein subunit names and SEQ ID NOs corresponding to the mature proteins. Each protein subunit was recombinantly expressed with an N-terminal signal peptide to facilitate secretion from the expressing cell. The native IL-15R α signal peptide (SEQ ID NO: 35) was used for sushi, sushi-L77I-Fc, and sushi-Fc. The leader sequence in SEQ ID NO: 33 was used to support secretion of antibody light-chains, IL15-WT, N-terminal IL-15 fusions, IL15-N72D, and N-terminal fusion of sushi to Fab-light-chain. The leader sequence in SEQ ID NO: 34 was used to support secretion of antibody heavy chains.

For “heavy-chain” and “light-chain” nomenclature we use the standard naming system for antibodies. For example, in an antibody Fab fragment both chains have approximately the same molecular mass, but we refer to the heavy-chain as the chain of the Fab fragment corresponding to the heavy-chain in the full-length antibody (*e.g.* containing the variable heavy-chain and CH1 domains). Further, in the case of cytokine fusions to the light-chain of a Fab fragment, the light-chain would actually have a larger molecular mass than the heavy-chain due to the cytokine fusion; for consistency, however, we maintain the standard naming convention in which the variable light-chain domain and constant kappa domain and cytokine fusion comprise the “light-chain” while the variable heavy-chain domain and CH1 domain comprises the “heavy-chain”.

Protein name: chBC8-IL15/sushi Fab (Also referred to as chBC8-L1-IL15/sushi Fab).

This protein was made by coexpression of three subunits: HC-chBC8-Fab (SEQ ID NO: 5), LC-chBC8-IL15 (SEQ ID NO: 3), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of wild-type IL-15 to the C-terminus of the chimeric BC8 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association with IL-15R α -sushi (via interaction with IL-15). The chimeric BC8 Fab is an anti- human CD45R antibody Fab

fragment comprising variable-heavy and variable-light chain domains (VH and VL) from the parental mouse monoclonal antibody (mAb) and constant domains from human (human constant kappa domain and human IgG1-CH1 domain).

5 Protein name: IL15-chBC8/sushi Fab

This protein was made by coexpression of three subunits: HC-chBC8-Fab (SEQ ID NO: 5), IL15-LC-chBC8 (SEQ ID NO: 4), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of IL-15 to the N-terminus of the chimeric BC8 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association between IL-15 and IL-15R α -sushi.

Protein name: chBC8-sushi/IL15-N72D Fab

This protein was made by coexpression of three subunits: HC-chBC8-Fab (SEQ ID NO: 5), LC-chBC8-sushi (SEQ ID NO: 1), and IL15-N72D (SEQ ID NO: 11). The resulting protein comprises a fusion of IL-15R α -sushi to the C-terminus of the chimeric BC8 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association between IL15-N72D and IL-15R α -sushi.

Protein name: sushi-chBC8/IL15-N72D Fab

This protein was made by coexpression of three subunits: HC-chBC8-Fab (SEQ ID NO: 5), sushi-LC-chBC8 (SEQ ID NO: 2), and IL15-N72D (SEQ ID NO: 11). The resulting protein comprises a fusion of IL-15R α -sushi to the N-terminus of the chimeric BC8 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association between IL15-N72D and IL-15R α -sushi.

Protein name: chBC8-IL15/sushi IgG

This protein was made by coexpression of three subunits: HC-chBC8-IgG4S228P (SEQ ID NO: 6), LC-chBC8-IL15 (SEQ ID NO: 3), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of wild-type IL-15 to the C-terminus of the chimeric BC8 IgG-S228P light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association with IL-15R α -sushi (via interaction with IL-15). The chimeric BC8 IgG4-S228P is an anti-human CD45R antibody comprising variable-heavy and variable-light chain domains (VH and VL) from the parental mouse mAb and constant domains from human (human constant kappa domain and human IgG4 containing an S228P point mutation, which reduces susceptibility of Fab-arm-exchange of

IgG4).

Protein name: IL15-chBC8/sushi IgG

This protein was made by coexpression of three subunits: HC-chBC8-IgG4S228P (SEQ ID NO: 6), IL15-LC-chBC8 (SEQ ID NO: 4), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of wild-type IL-15 to the N-terminus of the chimeric BC8 IgG4-S228P light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association between IL-15 and IL-15R α -sushi.

10 Protein name: chBC8-sushi/IL15-N72D IgG

This protein was made by coexpression of three subunits: HC-chBC8-IgG4S228P (SEQ ID NO: 6), LC-chBC8-sushi (SEQ ID NO: 1), and IL15-N72D (SEQ ID NO: 11). The resulting protein comprises a fusion of IL-15R α -sushi to the C-terminus of the chimeric BC8 IgG4-S228P light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association with between IL-15R α -sushi and IL-15.

Protein name: sushi-chBC8/IL15-N72D IgG

This protein was made by coexpression of three subunits: HC-chBC8-IgG4S228P (SEQ ID NO: 6), sushi-LC-chBC8 (SEQ ID NO: 2), and IL15-N72D (SEQ ID NO: 11). The resulting protein comprises a fusion of IL-15R α -sushi to the N-terminus of the chimeric BC8 IgG4-S228P light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association with between IL-15R α -sushi and IL-15.

Protein name: chBC8-(HC)-IL15/sushi IgG

25 This protein was made by coexpression of three subunits: HC-chBC8-IgG4S228P-IL15 (SEQ ID NO: 8), LC-chBC8 (SEQ ID NO: 7), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of IL-15 to the C-terminus of the chimeric BC8 IgG4-S228P heavy-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association with between IL-15 and IL-15R α -sushi.

30 Protein name: chBC8-IL15/sushi scFv

This protein was made by coexpression of two subunits: BC8scFv-IL15 (SEQ ID NO: 32) and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of IL-15 to the C-terminus of BC8-scFv using linker-L1 (SEQ ID NO: 36), and a noncovalent association with

between IL-15 and IL-15R α -sushi. BC8-scFv comprises the variable domains from the parental mouse mAb joined by a flexible linker.

Protein name: IL15-N72D/sushiL771-Fc

5 This protein was made by coexpression of two subunits: IL15-N72D (SEQ ID NO: 11) and sushi-L771-Fc (SEQ ID NO: 12). The resulting protein comprises IL-15R α -sushi containing an L771 mutation fused to the N-terminus of human IgG1-Fc region, and a noncovalent association with between IL15-N72D and IL-15R α -sushi.

10 Protein name: IL15-WT/sushi-Fc

This protein was made by coexpression of two subunits: IL15-WT (SEQ ID NO: 10) and sushi-Fc (SEQ ID NO: 13). The resulting protein comprises a fusion of IL-15R α -sushi to the N-terminus of human IgG1-Fc region, and a noncovalent association with between IL15-WT and IL-15R α -sushi.

15

Protein name: IL15-WT/sushi-IgG2Da-Fc

This protein was made by coexpression of two subunits: IL15-WT (SEQ ID NO: 10) and sushi-IgG2Da-Fc (SEQ ID NO: 56). The resulting protein comprises a fusion of IL-15R α -sushi to the N-terminus of human IgG2Da-Fc region, and a noncovalent association with IL15-WT (via
20 interaction with IL-15R α -sushi).

Protein name: ch9.4-IL15/sushi Fab

This protein was made by coexpression of three subunits: HC-ch9.4-Fab (SEQ ID NO: 14), LC-ch9.4-IL15 (SEQ ID NO: 15), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein
25 comprises a fusion of wild-type IL-15 to the C-terminus of the chimeric 9.4 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association between IL-15 and IL-15R α -sushi. The chimeric 9.4 Fab is an anti-CD45R antibody Fab fragment comprising variable-heavy and variable-light chain domains (VH and VL) from the parental mouse mAb (corresponding to ATCC hybridoma HB-10508) and constant domains from human (human
30 constant kappa domain and human IgG1-CH1 domain).

Protein name: ch4B2-IL15/sushi Fab

This protein was made by coexpression of three subunits: HC-ch4B2-IL15 (SEQ ID NO: 16), LC-ch4B2-IL15 (SEQ ID NO: 17), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein

comprises a fusion of wild-type IL-15 to the C-terminus of the chimeric 4B2 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association between IL-15 and IL-15R α -sushi. The chimeric 4B2 Fab is an anti-CD45R antibody Fab fragment comprising variable-heavy and variable-light chain domains (VH and VL) from the parental mouse mAb
5 (corresponding to ATCC hybridoma HB-11186) and constant domains from human (human constant kappa domain and human IgG1-CH1 domain).

Protein name: hBC8(23)-IL15/sushi Fab

This protein was made by coexpression of three subunits: HC-hBC8(23)-Fab (SEQ ID NO: 18),
10 LC-hBC8(23)-IL15 (SEQ ID NO: 19), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of wild-type IL-15 to the C-terminus of the humanized BC8 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association between IL-15 and IL-15R α -sushi. The humanized BC8(23) Fab is an anti-CD45R antibody Fab fragment comprising variable-heavy and variable-light chain domains (VH and VL) humanized
15 from the parental mouse mAb and constant domains from human (human constant kappa domain and human IgG1-CH1 domain).

Protein name: hBC8-null-IL15/sushi Fab

This protein was made by coexpression of three subunits: HC-chBC8(23)-null-Fab (SEQ ID
20 NO: 20), LC-hBC8(23)-IL15 (SEQ ID NO: 19), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of wild-type IL-15 to the C-terminus of the humanized BC8 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association between IL-15 and IL-15R α -sushi. This protein differs from hBC8(23)-IL15/sushi Fab in that it's heavy chain contains a GGGS substitution within the CDR-H3 loop, which results in ablated
25 binding affinity towards CD45R (hence, the designation as "null" binding variant). This IL15 fusion protein thus serves as a negative control for the effect of CD45R engagement.

Protein name: chBC8-L2-IL15/sushi Fab

This protein was made by coexpression of three subunits: HC-chBC8-Fab (SEQ ID NO: 5), LC-
30 chBC8-L2-IL15 (SEQ ID NO: 21), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of IL-15 to the N-terminus of the chimeric BC8 Fab fragment light-chain using linker-L2 (SEQ ID NO: 37), and a noncovalent association between IL-15 and IL-15R α -sushi.

Protein name: chBC8-L3-IL15/sushi Fab

This protein was made by coexpression of three subunits: HC-chBC8-Fab (SEQ ID NO: 5), LC-chBC8-L3-IL15 (SEQ ID NO: 22), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of IL-15 to the N-terminus of the chimeric BC8 Fab fragment light-chain using linker-L3 (SEQ ID NO: 38), and a noncovalent association between IL-15 and IL-15R α -sushi.

Protein name: chMHM24-IL15/sushi Fab

This protein was made by coexpression of three subunits: HC-chMHM24-Fab (SEQ ID NO: 26), LC-MHM24-IL15 (SEQ ID NO: 27), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of wild-type IL-15 to the C-terminus of the chimeric MHM24 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association with IL-15R α -sushi (via interaction with IL-15). The chimeric MHM24 Fab is an anti-human CD11a antibody Fab fragment comprising variable-heavy and variable-light chain domains (VH and VL) from the parental mouse mAb and constant domains from human (human constant kappa domain and human IgG1-CH1 domain).

Protein name: ch1B4-IL15/sushi Fab

This protein was made by coexpression of three subunits: HC-ch1B4-Fab (SEQ ID NO: 28), LC-ch1B4-IL15 (SEQ ID NO: 29), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of wild-type IL-15 to the C-terminus of the chimeric 1B4 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association with IL-15R α -sushi (via interaction with IL-15). The chimeric 1B4 Fab is an anti-human CD18 antibody Fab fragment comprising variable-heavy and variable-light chain domains (VH and VL) from the parental mouse mAb and constant domains from human (human constant kappa domain and human IgG1-CH1 domain).

Protein name: chOKT8-IL15/sushi Fab

This protein was made by coexpression of three subunits: HC-chOKT8-Fab (SEQ ID NO: 30), LC-chOKT8-IL15 (SEQ ID NO: 31), and IL-15R α -sushi (SEQ ID NO: 9). The resulting protein comprises a fusion of wild-type IL-15 to the C-terminus of the chimeric OKT8 Fab fragment light-chain using linker-L1 (SEQ ID NO: 36), and a noncovalent association with IL-15R α -sushi (via interaction with IL-15). The chimeric OKT8 Fab is an anti-human CD8 antibody Fab fragment comprising variable-heavy and variable-light chain domains (VH and VL) from the

parental mouse mAb and constant domains from human (human constant kappa domain and human IgG1-CH1 domain).

Protein name: Ab-HSA-Linker-IL-2

5 This protein was made by expression of a fusion protein comprising three subunits: the targeting antibody (*e.g.*, anti-CD45, anti-CD4 and/or anti-CD25), human serum albumin (SEQ ID NO: 79, no underline), and IL-2 (SEQ ID NO: 80). The three subunits are operably linked together via a peptide linker comprising G and S (SEQ ID NO: 79, single underline).

10 Protein name: h9.4Fab-IL2

This protein is made by coexpression of two subunits: LC-h9.4Fab (SEQ ID NO: 79), and HC-h9.4Fab-IL2 (SEQ ID NO: 81). The resulting protein comprises a fusion of a human IL-2 to the C-terminus of h9.4 Fab fragment heavy-chain using Linker-1 (SEQ ID NO: 36).

15 Protein name: h9.4Fab-HSA- IL2

This protein is made by coexpression of two subunits: LC-h9.4Fab (SEQ ID NO: 79), and HC-h9.4Fab-HSA-IL2 (SEQ ID NO: 81). The resulting protein comprises a fusion of HSA to the C-terminus of h9.4 Fab fragment heavy-chain using Linker-1 (SEQ ID NO: 36), and IL-2 to the C-terminus of HSA using a flexible linker (GGGGS) (SEQ ID NO: 88).

20

Immune regulatory cells

In embodiments, the FMs disclosed herein can be used to target an immune cell such as a nucleated cell. In some embodiments, the targeting moiety is capable of binding to an immune cell surface target, thereby targeting the modulatory moiety (*e.g.*, cytokine) to the immune cell,
 25 *e.g.*, an immune regulatory cell (*e.g.*, a regulatory T cell). In certain embodiments, the FMs, once targeted to an immune cell via binding between the targeting moiety and its corresponding immune regulatory cell surface target, can be transpresented to neighboring cells such as neighboring immune effector cells.

Without wishing to be bound by theory, binding of the immune targeting moiety to the
 30 immune cell surface target is believed to increase the concentration, *e.g.*, the concentration over time, of the immune modulatory moiety, *e.g.*, cytokine molecule, with its corresponding receptor, *e.g.*, a cytokine receptor, on the surface of the immune cell, *e.g.*, relative to the association of the free cytokine molecule with its cytokine receptor. This can result in an immune effect on the immune cell itself bound by the FMs (autocrine signaling), or on a
 35 neighboring immune cell (paracrine signaling).

In some embodiments, the immune regulatory cell surface target is abundantly present on the surface of an immune regulatory cell (*e.g.*, outnumbers the number of receptors for the cytokine molecule present on the immune cell surface). In some embodiments, the targeting moiety can be an antibody molecule or a ligand molecule that binds to an immune cell surface target, *e.g.*, CD45, CD4, CD25, CD39, or Neuropilin 1 (NRP1) on Tregs. In some

5 embodiments, the immune cell surface receptor is selected from one or more of CD4, CD45, CD3, CD2, CD25, CD127, CD197 (CCR7), CXCR3, CXCR4, CXCR5, CD38, CD27, CCR4, CCR5, CD137, CD39, CCR4, CCR5, CCR6 (CD196), CCR8, CCR10, OX40, GITR, CTLA4, LAG3, CD73, CD103, CD62L, CCR2, CCR9, Neuropilin 1 (NRP1), CD8, CD11a, or CD18, or

10 variants thereof.

In one embodiment, the targeting moiety comprises an antibody molecule or a ligand molecule that binds to CD45. In other embodiments, the targeting moiety binds bispecifically to CD4 and CD25. In embodiments, the targeting moiety is believed to specifically deliver and/or increase the concentration of the cytokine molecule (*e.g.*, IL-2) to the surface of an immune cell,

15 thereby resulting in one or more of increased localization, altered distribution and/or enhanced cell surface availability of the cytokine molecule. In embodiments, the FM does not substantially interfere with the signaling function of the cytokine molecule. Such targeting effect results in localized and prolonged stimulation of proliferation and activation of the immune cells, thus inducing the controlled expansion and activation of an immune response.

In some embodiments, the immune cell is a regulatory T cells. In embodiments, the immune regulatory cell, *e.g.*, a regulatory T cell acquired from a patient, *e.g.*, a patient's blood. In other embodiments, the immune regulatory cell is acquired from a healthy donor. Regulatory T cells express the transcription factor Foxp3 and restrain immune responses to self and foreign antigens. Treg cells express abundant amounts of the interleukin 2 receptor α -chain (IL-2R α ;

25 CD25), but are unable to produce IL-2. IL-2 binds with low affinity to IL-2R α or the common γ -chain (γ c)-IL-2R β heterodimers, but receptor affinity increases $\sim$ 1,000 fold when these three subunits together interact with IL-2. IL-2 and STAT5, a key IL-2R downstream target, are indispensable for Foxp3 induction and differentiation of Treg cells in the thymus. IL-2R β and γ c are shared with the IL-15 receptor, whose signaling can also contribute to the induction of

30 Foxp3. IL-2, in cooperation with the cytokine TGF- β , is also required for extrathymic Treg cell differentiation.

CD4⁺ T cells are commonly divided into regulatory T (Treg) cells and conventional T helper (Th) cells. Th cells control adaptive immunity against pathogens and cancer by activating other effector immune cells. Treg cells are defined as CD4⁺ T cells in charge of suppressing

35 potentially deleterious activities of Th cells. This review briefly summarizes the current

knowledge in the Treg field and defines some key questions that remain to be answered.

Functions for Treg cells include:

1. Prevention of autoimmune diseases by establishing and maintaining immunologic self-tolerance.
- 5 2. Suppression of allergy and asthma.
3. Induction of tolerance against dietary antigens, i.e. oral tolerance.
4. Induction of maternal tolerance to the fetus.
5. Suppression of pathogen-induced immunopathology.
6. Regulation of the effector class of the immune response.
- 10 7. Suppression of T-cell activation triggered by weak stimuli.
8. Feedback control of the magnitude of the immune response by effector Th cells.
9. Protection of commensal bacteria from elimination by the immune system.
10. Prevention of T cells that have been stimulated by their true high-affinity agonist ligand from killing cells that only express low-affinity T-cell receptor (TCR)
- 15 ligands such as the self peptide-major histocompatibility complex (MHC) molecule that positively selected the T cell.

The most widely used markers for Treg cells are:

- CD25
- cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)
- 20 • glucocorticoid-induced tumour necrosis factor receptor family-related gene (GITR)
- lymphocyte activation gene-3 (LAG-3)
- CD127 (negative marker)
- forkhead/winged-helix transcription factor box P3 (Foxp3)

25

Human Treg cells, in some embodiments, can be identified by the CD4+CD25+CD127- markers.

Exemplary Treg includes:

- tTreg (nTreg previously): FoxP3+; develop in the thymus; control central tolerance
- 30 • pTreg (iTreg previously): FoxP3+; develop in the periphery; important for peripheral tolerance
- iTreg: Foxp3+; induced *in vitro* via IL-10 and TGF- β stimulation
- Tr1: FoxP3-, CD49B+, Lag3+, CD226+; functions primarily through IL-10 secretion
- Th3: Involved in oral allergy tolerance, TGF- β mediated suppression

- Tr35: FoxP3-; makes IL-35 inducing anergy in neighboring cells; induced by IL-35; target of infectious tolerance

In some embodiments, the Tregs can be *ex vivo* induced Tregs. For example, CD4 cells can be isolated and induced to become Tregs by activation and culturing with, e.g., TGF-B, IL-10. In some embodiments, the Tregs can be antigen trained (specific) Tregs, which can be used in, e.g., organ or cell transplant rejection. In some embodiments, the Tregs can be CAR-Tregs or transgenic TCR Tregs.

In some embodiments, the FMs can be used for the *in vitro* expansion of Treg cells. For example, the FMs can be used as a cell culturing agent, e.g., in place of free stocks of cytokines currently used in Treg manufacturing and expansion during *ex vivo* culturing prior to injection. FMs can provide greater persistence than natural form of cytokine. In addition, cell specific targeting is also possible with FMs for *ex vivo* expansion of sorted Tregs as well as selective expansion of Tregs from a population of mixed PBMCs.

In certain embodiments, the FMs disclosed herein can be directly administered *in vivo* as an immune therapy such as immunosuppressive therapy. By targeting specific cell surface receptors (e.g., CD4, CD25, CD39, Neuropilin 1), the FMs can facilitate cell specific targeting, e.g., to Tregs. In some embodiments, the FMs can be designed to target to cell surface receptors nonselectively expressed (e.g., CD45, CD2, CD11a, CD18) and to increase the pharmacokinetics and/or alter cytokine biodistribution.

In some embodiments, the FMs disclosed herein can be pre-loaded to immune cells prior to a cell therapy, e.g., autologous cell therapy. For example, FMs can be pre-loaded onto a Treg or CAR-T cell.

Nucleic Acids/Vectors/Cells

The disclosure also features nucleic acids comprising nucleotide sequences that encode the fusion proteins described herein. Further provided herein are vectors comprising the nucleotide sequences encoding an FM described herein. The vectors include, but are not limited to, a virus, plasmid, cosmid, lambda phage or a yeast artificial chromosome (YAC). Numerous vector systems can be employed. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as, for example, bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (Rous Sarcoma Virus, MMTV or MOMLV) or SV40 virus. Another class of vectors utilizes RNA elements derived from RNA viruses such as Semliki Forest virus, Eastern Equine Encephalitis virus and Flaviviruses.

Additionally, cells which have stably integrated the DNA into their chromosomes may

be selected by introducing one or more markers which allow for the selection of transfected host cells. The marker may provide, for example, prototrophy to an auxotrophic host, biocide resistance (*e.g.*, antibiotics), or resistance to heavy metals such as copper, or the like. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or
5 introduced into the same cell by cotransformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcriptional promoters, enhancers, and termination signals.

Once the expression vector or DNA sequence containing the constructs has been prepared for expression, the expression vectors may be transfected or introduced into an
10 appropriate host cell. Various techniques may be employed to achieve this, such as, for example, protoplast fusion, calcium phosphate precipitation, electroporation, retroviral transduction, viral transfection, gene gun, lipid based transfection or other conventional techniques. In the case of protoplast fusion, the cells are grown in media and screened for the appropriate activity.

Methods and conditions for culturing the resulting transfected cells and for recovering
15 the antibody molecule produced are known to those skilled in the art, and may be varied or optimized depending upon the specific expression vector and mammalian host cell employed, based upon the present description.

In another aspect, the application features host cells and vectors containing the nucleic acids described herein. The nucleic acids may be present in a single vector or separate vectors
20 present in the same host cell or separate host cell. The host cell can be a eukaryotic cell, *e.g.*, a mammalian cell, an insect cell, a yeast cell, or a prokaryotic cell, *e.g.*, *E. coli*. For example, the mammalian cell can be a cultured cell or a cell line. Exemplary mammalian cells include lymphocytic cell lines (*e.g.*, NSO), Chinese hamster ovary cells (CHO), COS cells, oocyte cells, and cells from a transgenic animal, *e.g.*, mammary epithelial cell.

The disclosure also provides host cells comprising a nucleic acid encoding an antibody
25 molecule as described herein. In one embodiment, the host cells are genetically engineered to comprise nucleic acids encoding the antibody molecule. In one embodiment, the host cells are genetically engineered by using an expression cassette. The phrase "expression cassette," refers to nucleotide sequences, which are capable of affecting expression of a gene in hosts compatible
30 with such sequences. Such cassettes may include a promoter, an open reading frame with or without introns, and a termination signal. Additional factors necessary or helpful in effecting expression may also be used, such as, for example, an inducible promoter. The present disclosure also provides host cells comprising the vectors described herein. The cell can be, but is not limited to, a eukaryotic cell, a bacterial cell, an insect cell, or a human cell. Suitable
35 eukaryotic cells include, but are not limited to, Vero cells, HeLa cells, COS cells, CHO cells,

HEK293 cells, BHK cells and MDCKII cells. Suitable insect cells include, but are not limited to, Sf9 cells.

Compositions and modified cells

5 Compositions, including pharmaceutical compositions, comprising the fusion proteins are provided herein. A composition can be formulated in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. The term “pharmaceutically acceptable” means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients (*e.g.*, biologically-active proteins of the nanoparticles). Such
10 compositions may, in some embodiments, contain salts, buffering agents, preservatives, and optionally other therapeutic agents. Pharmaceutical compositions also may contain, in some embodiments, suitable preservatives. Pharmaceutical compositions may, in some embodiments, be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. Pharmaceutical compositions suitable for parenteral administration, in some
15 embodiments, comprise a sterile aqueous or non-aqueous preparation of the nanoparticles, which is, in some embodiments, isotonic with the blood of the recipient subject. This preparation may be formulated according to known methods. A sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent.

 Additional compositions include modified cells, such as modified Tregs having a
20 plurality of the fusion proteins tethered on cell surface. This can be useful for *ex vivo* Treg expansion as well as *in vivo* expansion.

 For example, conventional Treg manufacturing additionally requires long and costly *ex vivo* expansion. In contrast, as shown in FIG. 1D, sorted Tregs or unsorted PBMC can be loaded with backpacks (*e.g.*, CD3/CD28/IL-2) followed by direct infusion to patient, whereby the Tregs
25 are expanded *in vivo*, without *in vitro* culturing. Furthermore, loaded Tregs (*e.g.*, with IL-2 backpacks), prepared either from *ex vivo* expansion or without culturing, can provide pro-survival signal *in vivo*, while increasing expansion and engraftment (FIG. 1E).

Therapeutic Uses and Methods

30 The fusion proteins and compositions containing such fusions have numerous therapeutic utilities, including, *e.g.*, the treatment of conditions or diseases where the immune system displays an excessive or overactive response. The present disclosure provides, *inter alia*, methods for reducing or suppressing an immune response in a subject with conditions in which the immune system is overactive, such as autoimmune diseases, allergies and/or bone marrow or
35 organ transplant. Exemplary methods comprise administering to the subject a therapeutically

effective amount of any of the fusion proteins and/or modified cells (e.g., Treg-targeted fusion proteins) described herein to provide, e.g., an immunosuppressive therapy.

Exemplary applications of immunosuppressive therapy include allo-immune diseases, auto-immune diseases, allergy, and other inflammatory diseases (FIG. 1H). Allo-immune diseases include organ transplant rejection, graft versus host disease (GVHD) (e.g., post allogeneic hematopoietic stem cell transplant, HSCT) and GVHD post allogeneic stem cell transplantation (SCT). Treg number has been shown to correlate with GVHD control and organ transplant tolerance.

For allergy or allergic diseases, expansion of antigen-specific Tregs has been shown to associate with loss of milk allergy, and Treg depletion enhances allergy. Allergic rhinitis is the most common of these diseases, affecting 15-20% of the population. The allergic reaction is triggered by allergen-mediated cross-linking of specific IgE on the surface of mast cells and basophils, leading to release of histamine and other mediators, thus causing an acute allergic reaction, followed by a late-phase reaction characterized by an influx of eosinophils, neutrophils and Th2 cells producing IL-4, IL-5 and IL-13.

Autoimmune diseases are diseases in which the immune system attacks its own proteins, cells, and tissues, or in which immune effector T cells are autoreactive to endogenous self peptides and cause destruction of tissue. Thus an immune response is mounted against a subject's own antigens, referred to as self antigens. A comprehensive listing and review of autoimmune diseases can be found in *The Autoimmune Diseases* (Rose and Mackay, 2014, Academic Press). Autoimmune diseases are generally considered to be Th1 biased. As a result, induction of a Th2 immune response or Th2 like cytokines can be beneficial. Such cytokines include IL-4, IL-5 and IL-10. Exemplary auto-immune diseases that can be treated include Type 1 diabetes, Multiple Sclerosis and alopecia. These diseases have functional defects in Tregs, and decreased number of Tregs. Autoimmune diseases include but are not limited to rheumatoid arthritis, Crohn's disease, multiple sclerosis, systemic lupus erythematosus (SLE), autoimmune encephalomyelitis, myasthenia gravis (MG), Hashimoto's thyroiditis, Goodpasture's syndrome, pemphigus (e.g., pemphigus vulgaris), Grave's disease, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, scleroderma with anti-collagen antibodies, mixed connective tissue disease, polymyositis, pernicious anemia, idiopathic Addison's disease, autoimmune-associated infertility, glomerulonephritis (e.g., crescentic glomerulonephritis, proliferative glomerulonephritis), bullous pemphigoid, Sjogren's syndrome, insulin resistance, and autoimmune diabetes mellitus.

Inflammatory diseases can be used to broadly define a vast array of disorders and conditions that are characterized by inflammation. Examples include allergy, asthma,

autoimmune diseases, coeliac disease, glomerulonephritis, hepatitis, inflammatory bowel disease, preperfusion injury and transplant rejection.

More specifically, inflammatory diseases can include Inflammatory Bowel Disease, Rheumatoid Arthritis and Lupus. Inflammatory Bowel Disease (IBD) includes two major types, namely Crohn's Disease (CD) and Ulcerative Colitis (UC). These diseases have increased Treg number, but decreased Treg function. Tregs have been shown to control/prevent these diseases in mouse models. For example, both CD and UC as inflammatory disorders have long been considered as a breakdown in immunoregulation in the tissues of the intestinal mucosa, representing the most immunologically active sites of the human body. The interaction between luminal flora and the adaptive immune system is considered critical to disease pathogenesis. Exuberant Teff (proinflammatory) activity is observable in both animal models and human disease alike, and has been attributed in recent years to a breakdown in Treg-mediated (anti-inflammatory) homeostatic mechanisms. Thus, in certain embodiments, the compositions and cells (e.g., modified and/or expanded Tregs) disclosed herein can be used as an anti-inflammatory agent to treat various inflammatory diseases.

In embodiments, the fusion proteins or pharmaceutical composition can be administered to the subject parenterally. In embodiments, the modified cells can be administered to the subject intravenously, subcutaneously, intratumorally, intranodally, intramuscularly, intradermally, or intraperitoneally. In embodiments, the cells are administered, e.g., injected, directly into a tumor or lymph node. In embodiments, the cells are administered as an infusion (e.g., as described in Rosenberg et al., New Eng. J. of Med. 319:1676, 1988) or an intravenous push. In embodiments, the cells are administered as an injectable depot formulation.

In embodiments, the subject is a mammal. In embodiments, the subject is a human, monkey, pig, dog, cat, cow, sheep, goat, rabbit, rat, or mouse. In embodiments, the subject is a human. In embodiments, the subject is a pediatric subject, e.g., less than 18 years of age, e.g., less than 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 or less years of age. In embodiments, the subject is an adult, e.g., at least 18 years of age, e.g., at least 19, 20, 21, 22, 23, 24, 25, 25-30, 30-35, 35-40, 40-50, 50-60, 60-70, 70-80, or 80-90 years of age.

The fusion proteins disclosed herein can be used in combination with a second therapeutic agent or procedure.

In some embodiments, the FM is administered in conjunction with a cell therapy, e.g., a cell therapy chosen from an adoptive cell therapy, CAR-T cell therapy, engineered TCR T cell therapy, a tumor infiltrating lymphocyte therapy, an antigen-trained T cell therapy, or an enriched antigen-specific T cell therapy.

In embodiments, the FM and the second therapeutic agent or procedure are

administered/performed after a subject has been diagnosed with a condition in which the immune system is overactive, *e.g.*, before the condition has been eliminated from the subject. In embodiments, the FM and the second therapeutic agent or procedure are administered or performed simultaneously or concurrently. For example, the delivery of one treatment is still occurring when the delivery of the second commences, *e.g.*, there is an overlap in administration of the treatments. In other embodiments, the FM and the second therapeutic agent or procedure are administered or performed sequentially. For example, the delivery of one treatment ceases before the delivery of the other treatment begins.

In embodiments, combination therapy can lead to more effective treatment than monotherapy with either agent alone. In embodiments, the combination of the first and second treatment is more effective (*e.g.*, leads to a greater reduction in symptoms and/or cancer cells) than the first or second treatment alone. In embodiments, the combination therapy permits use of a lower dose of the first or the second treatment compared to the dose of the first or second treatment normally required to achieve similar effects when administered as a monotherapy. In embodiments, the combination therapy has a partially additive effect, wholly additive effect, or greater than additive effect.

EXAMPLES

Example 1. Constructing antibody/IL-15 fusion molecules with increased biological persistence

To demonstrate the ability to generate FMs with improved properties we constructed a series of FMs comprising multiple formats: (i) FMs comprising an IgG, Fab fragment, or scFv fragment, (ii) fusion to the N- or C-terminus of the antibody or antibody fragment; (iii) fusion of either IL-15 or the IL-15R α sushi domain to the antibody or antibody fragment (*e.g.*, **FIG. 1** and **FIGS. 2A-2C**); and (iv) FMs comprising varied linker composition between the antibody and cytokine.

A. IL-15 FMs comprising multiple different antibody formats

To explore the potential for fusion molecules of IL-15 and an immune-targeted antibody to improve IL-15 biological activity we constructed FMs comprising IL-15 and mAb clone BC8, which targets human CD45, an abundant receptor on the surface of immune cells (Cyster et al., EMBO Journal, Vol 10, no4, 893-902, 1991). The FMs comprised three different antibody formats: scFv, Fab, and full-length IgG. Briefly, wild-type IL-15 was genetically fused to the C-terminus of the Fab or IgG light-chain using an amino acid linker consisting of (GGGGS)₃ (SEQ ID NO: 36) or to the C-terminus of the scFv using the amino acid linker RSGSGGGGSLQ

(SEQ ID NO: 39). The Fab and IgG antibody formats comprised human constant regions and variable domains from the mouse monoclonal antibody BC8 (e.g. chBC8 Fab or chBC8 IgG). The scFv comprised BC8 heavy- and light-chain variable domains genetically fused with a (GGGGS)₃ linker. The IL-15/antibody fusions were co-expressed with IL-15R α -sushi; the high-affinity interaction between IL-15 and sushi resulted in an IL-15-antibody complex comprising a noncovalent association with IL-15R α -sushi. Together, this resulted in three FM variants: chBC8-IL15/sushi scFv, chBC8-IL15/sushi Fab, and chBC8-IL15/sushi IgG (**FIG. 2A**; see Protein Variants section for more details).

The effects of the FMs on CD8 T cell expansion were evaluated using a pulse bioassay in which the cytokine is incubated with cells for a fixed amount of time followed by removal of unbound cytokine by washing. As compared with a “static” stimulation assay, in which the cytokine is not washed away, the pulse bioassay provides better characterization of the persistence of a biological effect. It also accommodates confounding factors associated with ligand-depletion, which is a well-appreciated challenge for quantitative analysis of high-affinity receptor/ligand interactions (Hulme, Tevethick, Ligand binding assays at equilibrium: validation and interpretation Br J Pharm 2010, 161:1219-1237). The same principles apply to quantitative cell-based assays of cytokines and growth factors that mediate their effects by binding to cellular receptors. We compared the effects of the FMs to an IL15/sushi-Fc protein comprising a sushi domain containing a conservative L77I mutation fused to a human IgG1-Fc domain, and noncovalently associated (via interaction with the sushi domain) with an IL-15 variant containing an N72D mutation: IL15^{N72D}/sushiL77I-Fc. The N72D mutation is purported to improve agonistic properties of IL-15 (Zhu, Marcus, Xu, Lee, et al. Novel Human Interleukin-15 Agonists. Journal of Immunology 2009). As a negative control we also evaluated chBC8 alone (without fusion to IL-15). FMs and controls were incubated with CD8 T cells for 1 hr at 37 °C, followed by three washes to remove unbound protein. Cells were then plated in full media, allowed to grow for three days, and then were assessed for proliferation using both CellTiter Blue (**FIG. 2B**) and flow cytometry counting beads (**FIG. 2C**). Both of these methods yielded consistent results, and demonstrated that all three antibody formats were capable of producing FMs with greater persistence than the IL-15^{N72D}/sushiL77I-Fc protein. There was a trend of increasing potency of FMs containing scFv, Fab, or IgG (i.e. potency: IgG FM > Fab FM > scFv FM). The negative control of the chBC8 antibody alone did not induce T cell expansion indicating the presence of IL-15 is necessary for the proliferative effects of the FMs. The foregoing provides a non-limiting example of a fusion of IL-15 to an anti-CD45 antibody or antibody fragment that improves biological persistence of IL-15. Multiple antibody formats, e.g., as described herein, are capable of producing such FMs with improved potency.

B. FMs comprising varied fusion strategies of IL-15 to anti-CD45

To explore alternative fusion strategies between the cytokine and the antibody of the FM we constructed six additional FMs comprising fusion of IL-15 to the N- or C-terminus of chimeric BC8 Fab or IgG antibodies. The first two FMs were constructed by genetically fusing IL-15 to the N-terminus of chBC8 Fab or chBC8 IgG light-chains and the antibody fusions were co-expressed with IL-15R α to result in the FMs: IL15-chBC8/sushi Fab and IL15-chBC8/sushi IgG (**FIGS. 3A-3B**; see “Protein Variants” section for more details). We use a naming convention of chBC8-IL15 in Example 1A above and IL15-chBC8 here to refer to fusion of IL-15 to the antibody C- or N-terminus, respectively. The four additional FMs were constructed by fusing wild-type IL-15R α -sushi to the N- or C-terminus of the chBC8 Fab or IgG light-chain, and co-expressed these antibody fusions with IL-15^{N72D} (**FIGS. 3C-3D**): chBC8-sushi/IL15^{N72D} Fab, sushi-chBC8 /IL15^{N72D} Fab, chBC8-sushi/IL15^{N72D} IgG, sushi-chBC8 /IL15^{N72D} IgG (see Protein Variants section for more details). Along with the two Fab and IgG FMs in Example 1A (chBC8-IL15/sushi Fab and chBC8-IL15/sushi IgG) these eight FMs allowed us to test a variety of antibody fusion strategies, genetic fusion of IL-15 vs. genetic fusion of sushi to the immune-targeting moiety, and FMs comprising either wild-type or mutated IL-15 variants.

In a first experiment we evaluated the FMs comprising IL-15^{N72D} in the pulse bioassay format and found that for both the Fab and IgG fusions, as well as the N- and C-terminal fusions, the FMs exhibited improved activity as compared to the IL-15 format comprising a complex between IL-15^{N72D} and a sushi-Fc variant (**FIGS. 4A-4B**). In an additional experiment comparing all eight FM variants we observed similar potencies for either IL-15 or sushi fusion to the antibody, and also observed similar potencies for protein variants containing wild-type IL-15 and IL15-N72D (**FIGS. 5A-5B**). We observed a trend in which fusion of IL-15 or sushi to the C-terminus of the chBC8 IgG or Fab resulted in greater CD8 T cell expansion than fusion to the antibody N-terminus (**FIGS. 4A-4B** and **FIGS. 5A-5B**). However, each of the FM variants (including N- -terminal fusion to the IgG or Fab) displayed greater potency than the control protein, IL15-N72D/sushiL77I-Fc, which does not contain CD45 receptor binding functionality (**FIGS. 3C-3D** and **FIGS. 4A-4B**). As a control, chimeric BC8 or the parental BC8 mouse mAb antibodies alone did not induce T cell expansion (**FIGS. 4A-4B** and **FIGS. 5A-5B**), consistent with our observations above. We also evaluated the eight FMs on CD8 T cell proliferation in a “static” assay format. After three days of incubation (without washing) each of the FMs resulted in similar activity as IL15^{N72D}/sushiL77I-Fc, with the exception of the FM comprising N-terminal fusion of IL-15 to the Fab fragment (IL15-chBC8/sushi Fab), which resulted in slightly weaker potency in this assay format (**FIGS. 5C-5D**). The IgG fusions displayed similar potency

to the IL-15 Fab fusions (**FIG. 3B**). We conclude that multiple fusions strategies of the IL-15/sushi complex to an antibody targeting the CD45 receptor result in FMs with increased biological persistence, and that the FM fusion strategy improves activity of both wild-type and mutated forms of IL-15.

5

C. FMs comprising IL-15 fused to the C-terminus of an antibody heavy chain or light chain

We constructed an additional chBC8-cytokine fusion to evaluate the ability to fuse the cytokine to the antibody heavy- or light-chain C-terminus. Wild-type IL-15 was genetically fused to the C-terminus of the chBC8 heavy-chain (HC) and this construct was co-expressed with IL15R α -sushi to yield the FM variant chBC8(HC)-IL15/sushi IgG (**FIG. 6A**). We compared chBC8(HC)-IL15/sushi IgG to the chBC8-IL15/sushi IgG described in the previous example, which comprises IL-15 fused to the light-chain C-terminus; for clarity we refer to this latter protein here as chBC8(LC)-IL15/sushi IgG in order to differentiate from the FM comprising fusion to the heavy-chain C-terminus. Activated human CD8 T cells were pulsed with seven serial five-fold dilutions spanning 400 nM to 25.6 pM of chBC8(HC)-IL15/sushi IgG or chBC8-IL15(LC)/sushi IgG for 1 hr at 37 °C, and then washed three times with full media and plated at 500,000 cells/mL. Serial dilution of media only, the parental BC8 antibody (which does not contain a fusion with IL-15), or IL-15^{WT}/sushi-Fc (which is devoid of immune-targeting function) were used as controls. Following 5 days of culture we measured CD8 T cell expansion using CellTiter Blue. The chBC8(HC)-IL15/sushi IgG and chBC8-IL15(LC)/sushi IgG FMs, but not IL15^{WT}/sushi-Fc or the parental BC8 antibody, each induced T cell expansion and exhibited similar dose-response characteristics (**FIG. 6B**). We conclude that fusion of IL-15 to the C-terminus of the antibody light- or heavy-chain are each capable of producing FMs with increased persistence.

25

D. Functional antibody binding is required for improved potency of FMs.

We have demonstrated above that binding to CD45 alone (e.g. with a BC8 antibody that does not comprise a fusion with IL-15) does not explain the increased activity of the FMs (**FIGS. 3C-3D, FIGS. 4A-4B, and FIGS. 5A-5D**). This supports the requirement of the cytokine for the potency of the FM. We conducted two additional controls to demonstrate the functional requirement of the antibody binding to its cognate cell surface receptor. In the first experiment we conducted a pulse assay on activated primary human CD8 T cells for each of the eight FMs described in Example 1A in the presence or absence of 1 μ M BC8 as a soluble competitor to the CD45 cell surface receptor. Following a 1 hr pulse incubation at 37 °C cells were washed three times and then plated in full RPMI medium at a density of 300,000 cells/mL.

35

Following 5 days at 37 °C cell expansion was measured using CellTiter Blue; the presence of the BC8 competitor ablated the activity of all eight FMs in the pulse bioassay (**FIGS. 7A-7B**).

In a second experiment we generated two FMs comprising either a functional or non-functional BC8 binding region. The FM with the functional BC8 region comprised a Fab
 5 antibody fragment with humanized BC8 variable domain, in which CDRs from the parental BC8 antibody were grafted onto human variable domains, and a fusion of IL-15^{WT} to the humanized BC8 Fab light-chain. This complex was co-expressed with IL-15R α -sushi to result in the FM variant hBC8(23)-IL15/sushi Fab (see “Protein Variants” section for more details). The humanized BC8 antibody (hBC8(23)) possesses similar binding affinity as the chimeric BC8
 10 antibody (**FIG. 10A**). The FM with the nonfunctional BC8 region (hBC8-null-IL15/sushi Fab) comprised the same Fab fragment, with the exception of five amino acids of CDR3 in the antibody heavy-chain, which were replaced with the residues SGGGS. These amino acid substitutions resulted in a humanized BC8 antibody that did not exhibit detectable binding to cellular CD45 for the concentrations tested (**FIG. 10A**). Each of these proteins were pulsed with
 15 activated human CD8 T cells (seven serial five-fold dilutions spanning a concentration range of 400 nM to 25.6 pM), cells were then washed and plated at 500,000 cells/mL in 96-well plates. Following three days at 37 °C hBC8(23)-IL15/sushi Fab, but not hBC8-null-IL15/sushi Fab, supported CD8 T cell expansion (**FIG. 7C**). Taken together, these data demonstrate functional cell surface receptor binding is required for the improved potency of the FMs.

E. Fusion to anti-CD45 increases loading and cell surface persistence of IL-15

To explore whether the improved biological persistence of the FMs correlates to increased loading and persistence of IL-15 onto cells we analyzed cell surface FM levels over time. CD8 T cells at a density of 5×10^7 cells/mL were pulsed with 0.75 mg/mL hBC8(23)-
 25 IL15/sushi Fab, IL-15/sushi-Fc2Da, or IL-15^{N72D}/sushiL77I-Fc for 1 hr in a 1:1 mixture of PBS and HBSS, and then washed three times with full media. Sushi-Fc2Da is a fusion of wild-type IL-15R α -sushi to a variant of the human IgG2-Fc domain. A mock pulse control in which cells were incubated in a 1:1 mixture of PBS and HBSS was used as a negative control. Cells were then stained for IL-15 using a fluorescently-labeled anti-IL-15 antibody or for IgG domains (Fab
 30 or Fc fragments) using a fluorescently-labeled polyclonal antibody that recognizes human IgG light and heavy chains. These antibodies provided detection of both IL-15 or the associated Fab or Fc fragments on the T cell surface. The hBC8(23)-IL15/sushi Fab protein yielded substantially higher levels of IL-15 staining than the IL15/sushi-Fc2Da or IL15-
 N72D/sushiL77I-Fc proteins indicating a greater abundance of IL-15 on the cell surface (**FIG.**
 35 **8A**). We observed consistent results for IgG staining in which the FMs resulted in a higher

levels of staining than the IL15/sushi-Fc constructs (**FIG. 8A**). As a positive control for proliferative effects of IL-15 we added approximately 10 nM of IL15/sushi-Fc2Da to a mock pulse control (a “mock then saturating” condition); this corresponds to a concentration above the EC90 for IL-15/sushi-containing variants (see **FIGS. 3A-3D**) and thus serves as a positive control for a maximal proliferative effect by IL-15. The cells were propagated in culture and monitored for IL-15 surface levels for three days following the pulse incubation. IL-15 detection remained above background for hBC8(23)-IL15/sushi Fab, while both of the IL15/sushi-Fc variants were indistinguishable from background one day after the pulse incubation (**FIG. 8B**). The pulse of hBC8(23)-IL15/sushi Fab further resulted in similar cell expansion to the saturating amount of the IL15/sushi-Fc variant (**FIG. 8C**).

We reason that the IL-15 antibody that we used (clone no. 34559, R&D Systems) likely binds to an epitope on IL-15 that at least partially overlaps with IL-15R β , and thus may detect IL-15 that is not already productively engaged with IL-15 signaling receptors. This antibody has been described as a blocking/neutralizing antibody by several groups (see e.g., Neely, GG, Robbins, Amankwah, et al. (2001) *J Imm* 167:5011-5017; Krutzik, Hewison, Liu, Robles, et al (2008) *J Imm* 181:7115-7120; Schlaepfer, Speck (2008) *PLoS ONE* 3:e1999; and Correia, Cardoso, Pereira, Neves, et al., *J Imm* (2009) 182: 6149-6159). However, it does not appear to mediate its neutralizing effects through competitive IL-15 binding with IL-15R α : IL-15 is presented on the dendritic cell surface via its interaction with IL-15R α , and this antibody has been used previously to detect such a presentation of IL-15 on dendritic cells (Ferlazzo, Pack, Thomas, Paludan, et al *PNAS* 2004). Consistent with these observations, we are able to detect hBC8(23)-IL15/sushi Fab and IL15/sushi-Fc variants on the T cell surface using this antibody (**FIGS. 8A-8B**; each protein variant comprises an association between IL-15R α -sushi and IL-15). The antibody's neutralizing activity may therefore derive from competitive binding with one of the other two cell surface receptors for IL-15, IL-2/IL-15R β or the common γ chain. As such, detection of IL-15 with this antibody may reflect an excess loading of IL-15 onto the T cell surface beyond the levels that saturate its cell surface receptors, e.g. IL-2/IL-15R β or the common γ chain. This may also explain the low levels of cell surface IL-15 detected for the IL-15/sushi-Fc constructs (**FIGS. 8A-8B**), which would only interact with T cells through the IL-15 receptors, though the dimeric IL-15 that results from association with the sushi-Fc fusion could result in a slight abundance of the cytokine. Overall, we conclude that FMs both facilitate greater initial loading of IL-15 on the T cell surface and support elevated levels of biologically available IL-15 on the cell surface over time.

F. Multiple linker compositions support enhanced FM potency

To evaluate flexibility around linkage between IL-15 and the antibody of the FM we explored multiple different polypeptide linker compositions. The FMs explored in Example 1 each comprised a flexible 15 amino acid linker between IL-15 or sushi and the antibody consisting of three tandem repeats of (GGGGS)₃; Linker-1, SEQ ID NO: 36). Here, we explored two additional linker compositions including a flexible eight amino acid linker ((GGGS)₂; Linker-2, SEQ ID NO: 37) and a moderately flexible 12 amino acid linker derived from the human IgG1 hinge region (DKTHTSPPSPAP; Linker-3, SEQ ID NO: 38, underlined positions denote residues of the IgG1 hinge region that were mutated from cysteine to serine for this linker). IL-15^{WT} was fused to the C-terminus of the chBC8 Fab fragment light-chain using Linker-2 or Linker-3 and was co-expressed with IL-15R α -sushi to generate chBC8-L2-IL15/sushi Fab and chBC8-L3-IL15/sushi Fab, respectively (**FIG. 9A**). We previously described chBC8-IL15/sushi Fab in Example 1; this FM variant comprises a (GGGGS)₃ linker (Linker-1, SEQ ID NO: 36) between IL-15 and the chBC8 light-chain; for consistency we refer to this construct here as chBC8-L1-IL15/sushi Fab (**FIG. 9A**). These protein constructs were evaluated in the pulse bioassay with CD8 T cells as described in Example 1. Incubation with a mock serial dilution comprising media only or hBC8-null-IL15/sushi Fab, which comprises a BC8 binding domain mutated to ablate affinity to CD45 (**FIG. 10A**), were used as negative controls. Three days after the pulse incubation we analyzed cell proliferation using CellTiter Blue and found all three linker compositions were capable of inducing T cell expansion (**FIG. 9B**). We conclude that multiple linker compositions between the cytokine and the antibody are capable of generating FMs with improved potency.

Methods

Protein expression. Proteins were produced from suspension adapted HEK 293 cells in serum-free media. An exception was the parental mouse monoclonal antibody BC8, which was purified from the BC8 hybridoma culture obtained from American Type Cell Culture (ATCC, cat. no. HB-10507). Proteins were then purified by either protein A resin (for Fc fusions and full-length (IgG) antibodies), KappaSelect resin (GE Healthcare; for fusions to antibody Fab fragments), or histidine affinity resin (e.g. nickel-nitrilotriacetic acid, Ni-NTA; for 6-His-tagged scFv fusions) as appropriate. Purified proteins were buffer exchanged into phosphate buffered saline (PBS). Approximate molecular weights were confirmed by reducing and non-reducing SDS-PAGE and aggregation and multimeric status was determined by size-exclusion chromatography (SEC). Based on aggregation or multimeric status, proteins were optionally purified by SEC using a HiLoad Superdex 200 prep-grade columns (GE Life Sciences) on an

AKTA Pure chromatography system (GE Life Sciences).

Static bioassay. CD8 T-cells isolated from human blood (from Biospecialty Corp.) were activated with CD3/CD28 beads (Dynabeads cat. No. 11132D, ThermoFisher Scientific, Inc.) according to the manufacturer's instructions and in the presence of 10 ng/mL human IL-2 (cat. no. 202-IL-050/CF, R&D Systems) according to the manufacturer's instructions, plated in 6 well 9.5 cm² plates in full RPMI media containing RPMI 1640 (cat. No. 30-2001, American Type Cell Culture), supplemented with 10% heat inactivated fetal bovine serum (FBS-HI, ThermoFisher Scientific, Inc.), penicillin/streptomycin, and 1% Glutamax (cat. no. 35050-061, ThermoFisher Scientific, Inc.), and cultured at 37°C and 5% CO₂. After 3 days of stimulation, the CD3/CD28 beads were removed, and cells were allowed to rest in full RPMI media for 24 hours. The T-cells were then seeded into 96-well plates at 0.5x10⁶ cells/mL in the presence of six serial four-fold dilution of FMs or control proteins spanning a concentration range of 20 nM to 4.9 pM, unless otherwise indicated, or with media only as a negative control. Following three days at 37 °C and 5% CO₂ cell expansion was analyzed via CellTiter-Blue (cat. no. G8081, Promega).

Pulse Bioassay. Human CD8 T-cells (from Biospecialty Corp.) were stimulated with CD3/CD28 beads (Dynabeads, ThermoFisher Scientific, Inc.) in the presence of 10 ng/mL IL-2 as described for the static bioassay. After 3 days of stimulation, the CD3/CD28 beads were removed, and cells allowed to rest in full RPMI media for 24 hours. Unless otherwise indicated, the activated T-cells were incubated at a cell density of 500,000 cells/mL with FMs or control proteins at concentration range of 80 nM to 5 nM (three serial four-fold dilutions), unless otherwise indicated, or with media only (0 nM) as a negative control. Following 1 hour at 37°C and 5% CO₂ cells were washed three times with full RPMI media to remove unbound cytokine and then plated in full RPMI media (in the absence of additional cytokines) at 0.5x10⁶ cells/mL in 96-well plates unless otherwise indicated. Additional controls optionally included either the parental BC8 antibody or chBC8 IgG antibody. Following three days at 37 °C and 5% CO₂ T cell expansion was analyzed via CellTiter-Blue or Flow cytometry. For flow cytometry, 7-AAD (cat. no. A9400-5MG, Sigma) was used to stain for (and subsequently exclude dead cells during the analysis) and CountBright Absolute Counting Beads (cat. no. C36950, Thermo Fisher Scientific, Inc.) were used to quantify viable cell densities according the manufacturer's instructions.

Measurement of FM on cell surface. Cells were analyzed by immunofluorescent staining to detect cell surface FMs following 0, 1, and 3 days in culture following pulse incubation and washes. Briefly, cells were washed with 1x phosphate buffered saline (PBS) containing 1 mg/mL bovine serum albumin (PBS/BSA) and then stained with a 1:100 dilution of PE-

conjugated anti-IL15 antibody (clone no. 34559; R&D Systems cat. no. IC2471P) and a 1:100 dilution of DyLight650-conjugated anti-human IgG H+L polyclonal antibody (ThermoFisher cat. no. Sa5-10129) in PBS/BSA. Following 20-30 min incubation at 4°C, cells were washed one time with PBS/BSA, and then resuspended in PBS/BSA for analysis on a FACSCelesta
 5 using Diva software (BD Biosciences). Data were analyzed using Cytobank (Cytobank, Inc) and GraphPad Prism (GraphPad Software, Inc).

Example 2. Fusion of IL15 to alternative anti-CD45 antibodies

To evaluate whether anti-CD45 antibodies other than BC8 are capable of generating FMs
 10 with improved potency, we evaluated two additional anti-CD45 monoclonal antibodies (mAb clones 9.4 and 4B2). We first evaluated the binding affinities of chimeric IgG forms of these antibodies to CD45 expressed on the surface of activated CD8 T cells. The chimeric antibodies each comprise mouse variable domains and human constant region (human constant kappa domain and human IgG4 containing an S228P mutation to stabilize the IgG4 hinge region). For
 15 comparison with previous results we evaluated these antibodies alongside chBC8, hBC8(23), and a non-binding variant hBC8-null. The humanized BC8 antibody consists of variable domains containing CDR regions grafted from BC8, and the hBC8-null variant contains an SGGGS substitution in the heavy-chain CDR3, which ablates binding to CD45 (**FIG. 10A**). The ch4B2 and ch9.4 antibodies exhibited the highest affinity to cellular CD45, with apparent
 20 dissociation constants ($K_{D,app}$) of 0.44 and 0.84 nM, respectively, while the chBC8 and hBC8(23) antibodies exhibited $K_{D,app}$ of 6.68 and 7.8 nM, respectively (**FIG. 10A**). The hBC8-null antibody did not exhibit significant binding at the highest concentration examined (500 nM; **FIG. 10A**). We use the term apparent dissociation constant ($K_{D,app}$) to distinguish from a true equilibrium dissociation constant: the bivalent nature of the IgG antibody introduces an avidity
 25 effect that complicates the calculation of a true equilibrium dissociation constant for these type of assays. Nevertheless, the $K_{D,app}$ is a useful metric for scoring relative affinities of the antibodies to the cellular receptor in its native context.

We next evaluated the potency of FMs comprising each of these antibodies. Briefly, IL-15 was fused to the C-terminus of ch9.4 or ch4B2 Fab fragment light-chain and co-expressed
 30 with IL-15R α -sushi to generate ch9.4-IL15/sushi Fab and ch4B2-IL15/sushi Fab, respectively. Construction of chBC8-IL15/sushi Fab, hBC8(23)-IL15/sushi Fab, and hBC8-null-IL15/sushi Fab were described in Example 1. We compared the potency of these FMs in the pulse bioassay, and included a series of negative controls: hBC8-null-IL15/sushi Fab (an FM with ablated CD45 binding), IL-15^{WT}/sushi-Fc, BC8 antibody, and media only. Each of the FMs induced greater T
 35 cell expansion than the negative controls as measured three days after the pulse incubation (**FIG.**

10B). The FMs comprising 4B2 or 9.4 possessed increased potency compared to FMs comprising chBC8 or hBC8(23). This is consistent with the increased affinity of 4B2 and 9.4 compared to chBC8 and hBC8(23), and is consistent with the idea that increased affinity of the antibody to the cell surface receptor results in increased potency of the FM. Overall, we
5 conclude that generation of FMs with improved biological persistence is not restricted to the BC8 antibody, and that increased affinity to the cell surface receptor can improve the potency of the FM.

Methods

10 Cell binding assay. Primary human CD8 T-cells were activated with CD3/CD28 beads and 10 ng/mL IL-2 as described in Example 1. Following activation and an overnight rest in full RPMI media (see Example 1 methods) the T-cells were incubated with seven serial five-fold dilutions of antibodies spanning 500 nM to 6.4 pM, or with media only control at 4°C for 1 hr. Cells were washed with MACS buffer (PBS, 2% FBS, 1mM EDTA) three times, and antibody
15 binding was detected using a PE-conjugated anti-human IgG-Fc antibody (BioLegend, cat. no. HP6017) for 20 min. at 4°C. Cells were washed two times with MACS buffer, and the resuspended in MACS buffer with live-dead stain (7-AAD). Antibody binding was quantified using the median fluorescence intensity (MFI) of the live cell population (via 7-AAD exclusion) within the PE signal. Curves were generated by plotting mean fluorescent units against antibody
20 concentration, and apparent dissociation constants ($K_{D,app}$) were determined by non-linear regression using a four-parameter logistic equation using GraphPad Prism (GraphPad Software, Inc.).

Pulse bioassay. The pulse bioassay was conducted as described in Example 1.

Protein expression. Proteins were produced as described in Example 1.

Example 3. FMs targeting additional cell surface receptors facilitate improved potency and specificity

A. Additional cell surface receptors other than CD45 facilitate improved cytokine potency.

To explore whether FMs targeting cell surface receptors other than CD45 are capable of
30 resulting in increased biological potency we constructed FMs comprising antibodies targeting additional cell surface receptors. The alternative receptors can either be abundant receptors on the cell surface or exhibit persistence at the protein level (e.g. display slow receptor internalization or strong recycling back to the cell surface upon receptor internalization). Ideally the receptor is both abundant and persistent. Based on these considerations we selected FMs
35 targeting CD8, CD11a, or CD18 for evaluation. CD8 is a co-receptor for the T cell receptor that

is expressed on cytotoxic T cells. CD8 can also be expressed in cortical thymocytes, dendritic cells, and natural killer cells. In cytotoxic T cells, CD8 helps maintain the activated T cell receptor/MHC complex on the cell surface (Wooldridge, van den berg, Glick, et al. JBC 2005), and once the T cell receptor is internalized the CD8 co-receptor is preferentially maintained on the cell surface in comparison to the T cell receptor (1989 J Imm Boyer, Auphan, Gabert, Schmitt-Verhulst et al Comparison of phosphorylation and internalization of the antigen receptor/CD3 complex, CD8, and Class I MHC-encoded proteins on T cells). Lymphocyte function-associated antigen 1 (LFA-1) is a heterodimeric cell surface receptor composed of integrin alpha-L (CD11a) and integrin beta-2 (CD18) and is involved in intercellular adhesion and lymphocyte costimulation. Integrins generally display persistence at the protein level: they typically either exhibit slow internalization from the cell surface or are recycled back to the cell surface upon internalization (2012 J Cell Sci Integrins at a Glance and 1989 EMBO J Endocytosis and recycling of the Fn receptor in CHO). LFA-1 is present at high levels on several different types of leukocytes and has been shown to exhibit slow internalization in multiple different cell types, including monocytic U937 cells, the promyelocytic leukemia cell line HL-60, and the lymphoblastoid cell line JY (1992 EMBO J Circulating Integrins Bretscher). In addition, preclinical studies of the CD11a antibody efalizumab demonstrated that CD11a exhibits persistence at the protein level in human and mouse T cells isolated from blood; efalizumab can, however, induce receptor internalization and degradation in the presence of a secondary cross-linking antibody (2004 Coffey, Pippig et al J Pharm Exp Ther).

We constructed FMs targeting CD8 (antibody clone OKT8), CD11a (antibody clone MHM24), or CD18 (antibody clone 1B4) by genetically fusing IL-15 to the C-terminus of the respective antibody fragment light-chain. The IL-15-antibody fusions were co-expressed with IL-15R α -sushi to generate three FMs: chMHM24-IL15/sushi Fab, ch1B4-IL15/sushi Fab, and chOKT8-IL15/sushi Fab (**FIG. 11A**). Each Fab fragment was composed of chimeric human and mouse regions with mouse variable region and human constant domains (kappa and CH1). The potency of the FMs on CD8 T cells was analyzed in the pulse bioassay as described in Example 1; after three days in culture following the pulse incubation cells were analyzed for proliferation using CellTiter Blue. The three FMs each supported similar levels of T cell proliferation to that of chBC8-IL15/sushi Fab (**FIG. 11B**). We have demonstrated above that forms of IL-15 that do not contain an immune-targeting functionality exhibit minimal effects in the pulse bioassay (**FIGS. 2A-2C, 4A-4B, 5A-5D, 6A-6B, and 8A-8C**). Together this demonstrates that additional cell surface receptors are capable of generating FMs with improved biological persistence.

B. FMs that facilitate improve potency towards CD8 T cells

To evaluate whether cell surface receptor targeting can facilitate improved potency towards individual cell types we compared the potency of CD8-targeted FM on CD4 and CD8 T cells. CD4 or CD8 cells were treated with chOKT8-IL15/sushi Fab in the pulse bioassay format, and cell proliferation was measured three days after the pulse incubation. The CD8-targeted FM more potently stimulated CD8 T cells compared to CD4 T cells (**FIGS. 12A-12B**), supporting the ability to target specific cell types based on the antibody of the FM.

Methods

Pulse bioassay on CD4 or CD8 T cells. Primary human CD3 T cells, which contain a mixture of cells expressing CD4, CD8, or CD4 and CD8 coreceptors, were activated using CD3/CD28 activation beads in the presence of 10 ng/mL IL-2 as described in Example 1. Following three days of activation the beads were removed and cells were rested overnight in full RPMI media. We then purified individual populations of CD4 and CD8 T cells using magnetic activated cell sorting (MACS) using (cat. no. 130-045-101, and no. 130-045-201, Miltenyi Biotec, Inc.) according the manufacturer's protocol. CD4 or CD8 T cells were then analyzed in the pulse bioassay as described in Example 1.

Example 4. Protein nanogels comprising IL-15 FMs support long-term cell expansion

Drug-loaded nanoparticles (also referred to herein as “backpacks”) hold potential for supporting cell viability and function. We therefore explored the ability to form nanoparticles comprising an FM and the effect of these nanoparticles on T cell viability and expansion. We crosslinked hBC8(23)-IL15/sushi Fab into a protein nanogel using a reversible, amine-reactive, homobifunctional cross-linker. Protein nanogels comprising IL15^{N72D}/sushi^{L77I}-Fc were used as a comparator. Both of these proteins efficiently crosslinked into protein nanogels: by SEC analysis the protein nanogels traveled with a faster elution time than the free protein, consistent with a larger species (**FIGS. 13A and 13B**). Comparison of HPLC traces of the protein nanogel with the free (noncrosslinked) protein (**FIGS. 13A-13B**) revealed a minimal amount of free protein remaining in the cross-linked nanogels, suggesting high conversion of the proteins into nanogels. The protein nanogels were coated with a co-block polymer of polyethylene glycol and polylysine and incubated with CD8 T cells. Both protein nanogels supported long term cell expansion in comparison to non-crosslinked protein and unstimulated controls (**FIG. 13C**).

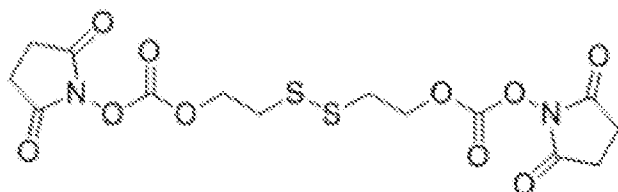
Methods

Protein nanogel synthesis. Protein nanogels comprising a crosslinked protein nanoparticle were formed as follows. The chBC8-IL15/sushi Fab fusion protein was crosslinked

into protein nanogels by incubating the protein at a concentration of 17 mg/mL with a 27-fold molar excess of a degradable crosslinker (Formula I) in the presence of a final concentration of 2.5% polyethylene glycol with an average molecular weight of 400 Dalton (PEG-400, Spectrum Chemical Mfg. Corp.) and 10% glycerol (Sigma). The IL15^{N72D}/sushiL77I-Fc protein was

crosslinked into protein nanogels by incubating the protein at a concentration of 17 mg/mL with a 27-fold molar excess of a degradable crosslinker (Formula I). The cross-linker used in this study, Bis[2-(N-succinimidyl-oxycarbonyloxy)ethyl] disulfide, contains two N-hydroxysuccinimide (NHS) ester groups joined together by a flexible disulfide-containing linker as shown in Formula I.

Formula I:



After 30 minutes incubation at room temperature, the reactions were diluted with Dulbecco's phosphate buffered saline (DPBS) to a final cytokine concentration of 1.5 mg/mL. Protein nanogels were then purified from linker leaving groups (which comprise molecular fragments of the linker that are removed as part of the cross-linking reaction) and unreacted linker by buffer exchange into DPBS using a Zeba column (40,000 MW cut-off, Thermo-Fisher). Zeba columns were used according to the manufacturer's instructions, including equilibrating the column in DPBS by three consecutive washes with DPBS to facilitate buffer exchange, followed by application of the reaction products. Buffer-exchanged protein nanogels were analyzed by size exclusion chromatography (SEC) using a BioSepTM SEC-s4000 column (Phenomenex Inc.) with PBS (pH 7.2) as eluent (flow rate 0.5 mL/min) on a Prominence HPLC system equipped with a photodiode array (Shimadzu Corp.).

Protein nanogels at a cytokine concentration of approximately 1 - 1.5 mg/mL were conjugated with a polyethylene glycol-polylysine (PEG-polyK) block co-polymer: PEG5k-polyK30 (Alamanda Polymers cat. no. 050-KC030), which is a block co-polymer comprising a polyethylene glycol polymer of 5 kiloDalton (kD) average molecular weight and a 30 amino acid polylysine polymer (polylysine30 or polyK30). PEG5k-polyK30 or were reconstituted to 1 mg/mL in DPBS and added to 1-1.5 mg/mL of protein nanogels at a final block copolymer concentration of 50 ug/mL and incubated at room temperature for 30 min.

T cell expansion analysis. Protein nanogels comprising PEG5k-polyK30 at a protein

concentration of 1-1.5 mg/mL were mixed in equal volume with 1×10^6 CD8 T-cells in HBSS at a cell concentration of 100×10^6 cells/mL and incubated at 37°C for 1 hr. T-cells were then washed three times with RPMI media containing 10% FBS, penicillin/ streptomycin, and Glutamax (all from ThermoFisher Scientific, Inc.), seeded at a cell density of approximately 1×10^6 cells/mL, and cultured for 9 days in 24-well tissue culture plates. Cells were split into fresh medium at a ratio of 1:5 on three days after cell attachment of backpacks. Cell proliferation was measured by live-dead cell stain (7-AAD) and counting beads (CountBright Absolute Counting Beads, Thermo Fisher Scientific, Inc.) by flow cytometry on Days 0, 1, 2, 3, 6, and 9.

Example 5: Versatility of immunotargeted surface receptor

FIG. 14 illustrates various combinations of immunotargeting moieties (e.g., antibodies against various cell surface receptors) and cytokine molecules. Multiple receptors can facilitate cytokine immunotargeting. For example, tethered fusions can be made with various cytokines tethered to antibodies against CD11a, CD18, CD45 and/or CD2 which may be present on multiple cell types. Cytokines can also be immunotargeted to specific cells via cell type-specific surface receptors such as CD4, CD8 or CD56.

Example 6: Tethered fusion platform enables selective cell targeting

CD8-targeted IL-7 delivers selective loading and expansion of CD8 T cells. FIGS. 15A-15B shows cell-specific targeting, in which CD8-targeted-IL-7 selectively targets CD8 T cells. FIG. 15C shows selective expansion of CD8 T cells, but not CD4 T cells, in cultures of activated total CD3 human T cells by pulse incubation with CD8-targeted IL-7. Briefly, activated human T cells were prepared by stimulating total CD3 human T cells (comprising both CD4 and CD8 T cells) for three days using CD3/CD28 Dynabead Activator beads (Thermo) according to the manufacturer's instructions and in the presence of 20 ng/mL IL-7 and 100 ng/mL IL-21. The CD3/CD28 beads were then removed and cells were incubated in full medium (RPMI 1640 containing 10% FBS) overnight in the presence of 20 ng/mL IL-7 and 100 ng/mL IL-21 at 37C and 5% CO₂. Cells were then washed and incubated with 500 nM of a CD8-targeted IL-7 construct (chOKT8Fab-IL7). After 1 hr at 37C cells were washed two times with full medium, plated at a density of 200,000 cells/mL, and incubated at 37C and 5% CO₂. Separately, cells were analyzed for loading onto CD8 and CD4 T cells by staining the T cells with DyLight 650-conjugated anti-human IgG polyclonal antibody (Thermo cat. no. SA5-10129; DyLight 650 fluorophore can be read on the APC channel for flow cytometry, see FIG. 15B), FITC-conjugated anti-CD4 antibody (BioLegend cat. no. 344604), and APC/Cy7-conjugated anti-CD8 antibody (BioLegend cat. no. 344715). The anti-human IgG polyclonal antibody

detects the Fab region of the CD8-targeted FM. The anti-CD4 and anti-CD8 antibodies can differentiate the CD4 and CD8 T cell populations (**FIG. 15A**). **FIG. 15B** shows that the CD8-targeted IL-7 construct selectively loaded to greater levels on CD8 T cells when incubated with a mixed population of CD4 and CD8 T cells. **FIG. 15C** shows selective expansion of the CD8 T cells by the CD8-targeted IL-7 construct.

To further explore selective cell loading by different antibody clones and antibody configurations we compared the loading onto CD4 and CD8 T cells by two different anti-CD8 monoclonal antibodies. Briefly, we recombinantly produced anti-human CD8 antibody clones OKT8 and 51.1 as Fab fragments (chOKT8Fab and ch51.1Fab). We additionally constructed two IL-15 FMs: one comprising the chOKT8Fab (chOKT8Fab-IL15/sushi) and a second comprising the chOKT8Fab and an anti-CD45 scFv (chOKT8Fab-BC8scFv-IL15/sushi). This latter construct contains antibody fragments against two different cell surface receptors, CD8 and CD45, and we reasoned it may give stronger cell loading of CD8 T cells through an avidity effect. We evaluated the ability of these four antibody and FM constructs to load onto CD4 and CD8 T cells using total CD3 T cells (comprising mixtures of CD4 and CD8 T cells). **FIG. 15D** shows strong selective loading onto CD8 T cells for each construct. In **FIG. 15E** we similarly show selective loading of IL-15 onto CD8 T cells using anti-IL15 staining (Fab constructs that do not contain IL-15, chOKT8Fab and ch51.1Fab, are of course negative in this assay).

To compare relative affinities against CD8 T cells, we compiled the binding titrations against CD8 T cells from **FIG. 15D** into a single plot in **FIG. 15F**. The chOKT8Fab-BC8scFv antibody configuration delivered the tightest binding to the CD8 T cells. This FM also resulted in loading onto CD4 T cells when used at higher concentrations (**FIG. 15D** and **FIG. 15E**). We reason that this is due to the BC8scFv, which binds to the CD45 receptor, which is present on both CD4 and CD8 T cells. The relative affinity of chOKT8Fab-BC8scFv-IL15/sushi for CD4 T cells observed in **FIG. 15D** is similar to the affinity of monomeric BC8scFv against T cells (**FIG. 15G**, the BC8scFv was constructed using a hexahistidine tag, which is used here as a handle for detection by flow cytometry); together with the minimal binding of chOKT8Fab and chOKT8Fab-IL15/sushi to CD4 T cells, we conclude that binding of chOKT8Fab-BC8scFv-IL15/sushi to CD4 T cells is driven by the BC8scFv. A dimeric form of BC8scFv results in higher affinity to T cells, likely due to the avidity effect from bivalent binding. We further reason that the higher binding affinity of chOKT8Fab-BC8scFv-IL15/sushi to CD8 vs CD4 T cells – as well as the higher binding to CD8 T cells as compared with the monovalent chOKT8Fab-IL15/sushi – is similarly due to an avidity effect that results from bivalent binding to CD8 T cells. We thus conclude that FMs can be constructed using antibody clones that are specific for different cell surface receptors to improve cellular affinity and loading. Furthermore, one or more of the

selected antibodies can be specific for a particular cell type in order to enable improved affinity while still retaining cell-selective loading. Using such a method, we show that it is further feasible to construct heterospecific antibodies with varied loading efficiencies on different cell types (e.g. in this case, stronger loading onto CD8 T cells and weaker – but nonzero – loading onto CD4 T cells).

Example 7: Cytokine engineering improves tethered fusion potency

We evaluated the ability to further augment FM potency by engineering cytokine mutants with modulated biological activity. Based on the structure-function understanding of the interaction between IL-15 and its cellular signaling receptors (Chirifu et al. Nat Immunol. 2007 Sep;8(9):1001-7; Ring et al. Nat Immunol. 2012 Dec;13(12):1187-95), we constructed three IL-15 variants comprising mutations to amino acid side-chains believed to interact with the IL-15/IL-2 receptor beta (specifically, IL-15-D8N, IL-15-D61N, and IL-15N72A), and three additional IL-15 variants comprising mutations to amino acid side-chains believed to interact with the gamma chain (IL-15-K10Q, IL-15-Q101N, and IL-15-Q108N). We constructed the IL-15 mutants as fusions to the chBC8Fab antibody fragment and evaluated their binding and activity on human T cells. Briefly, primary human T cells were activated as described in Example 1, and then incubated with five-fold serial dilutions of FMs comprising the IL-15 variants (without washing after FM incubation, e.g. a ‘constant’ incubation assay format). An FM comprising wild-type IL-15 and an IL-15/sushi-Fc construct were used as controls for comparison. After incubation with the IL-15 constructs for three days at 37C and 5% CO2 cell density was analyzed using CountBright Absolute Counting Beads (Thermo) by flow cytometry following the manufacturer’s instructions. The FM containing wild-type IL-15 (chBC8Fab-IL15/sushi) exhibited similar cellular potency as the IL-15/sushi-Fc construct (FIG. 16A and FIG. 16B). In addition, FIG. 16A and FIG. 16B shows that three different IL-15 variants result in stronger cellular potency than wild-type IL-15 (IL-15-K10Q, IL-15-Q101N, and IL-15-N72A), two IL-15 variants resulted in reduced cellular potency (IL-15-D61N and IL-15-Q108N), and a single IL-15 variant did not display cellular activity under the range of concentrations evaluated (IL-15-D8N). In a separate experiment we evaluated the surface persistence of the two attenuated IL-15 variants (IL-15-D61N and IL-15-Q108N) in the pulse assay format as described in Example 1. We found that the FMs comprising the attenuated variants exhibited stronger persistence on the cell surface (FIG. 16C). Activation of growth factor and cytokine signaling receptors induces negative regulatory feedback mechanisms within the cell. One such mechanism involves internalization and degradation of activated cytokine and growth factor receptors (reviewed in Jones et al. Trends Biotechnol. 2008 Sep;26(9):498-505).

Without wishing to be bound by theory, we reason that the elevated surface persistence from cytokine variants having attenuated activity may be due to weaker induction of such negative regulatory feedback mechanisms within the cell.

To further explore the relationship between cytokine activity and FM surface persistence and potency, we designed four additional IL-15 variants comprising mutations at the interface with IL-15 and its IL-15/IL-2 receptor beta. In particular, we constructed CD8-targeted FMs comprising the IL-15 variants IL-15-D61H, IL-15E64H, IL-15-D65H, or IL-15-N72H.

Activated human T cells were prepared by stimulating CD3 human T cells for three days using CD3/CD28 Dynabead Activator beads (Thermo) according to the manufacturer's instructions, beads were then removed and cells were incubated in full medium (RPMI 1640 containing 10% FBS) overnight in the presence of 20 ng/mL IL-7 and 100 ng/mL IL-21 at 37C and 5% CO₂.

The activated T cells were then incubated with a serial five-fold dilution of FMs spanning a concentration range of 10 – 0.0032 nM and plated at a density of 200,000 cells/mL into 96-well plates (without washing after FM incubation, e.g. a 'constant' incubation assay format). After three days at 37C and 5% CO₂ cells were harvested and evaluated for cell expansion and FM surface levels. For analysis of FM surface levels, cells were stained with BV421-conjugated anti-CD4 antibody (BioLegend cat. no. 344632), BV786-conjugated anti-CD8 antibody (BioLegend cat. no. 344740), and Alexa-Fluor-647-conjugated anti-human kappa (light-chain) antibody. FIG. 16D demonstrates high loading of the CD8-targeted IL-15 constructs onto CD8 T cells, and minimal loading onto CD4 T cells. The limited loading of the CD8-targeted FMs onto CD4 T cells enabled a controlled evaluation of the effects of IL-15 mutations on cellular responses. Expansion of CD4 T cells in response to incubation with CD8-targeted IL-15 constructs comprising D61H, E64H, N65H, or N72H point mutations demonstrated attenuating activity of D61H, E64H, and N65H mutations (FIG. 16E).

We further evaluated activity of these constructs on CD8 and CD4 T cells in the pulse assay format. Briefly, activated total CD3 human T cells were incubated with the CD8-targeted IL-15 variants for 1 hr at 37C, washed to remove unbound FM, plated at a density of 200,000 cells/mL, and incubated at 37C and 5% CO₂. To control for effects of induced cytokine secretion or tethered fusion unbinding and rebinding, each of which may support further T cell survival or expansion, we refreshed the cell culture medium one and three days after pulse incubation; cells were additionally split using a five-fold dilution ratio into fresh medium three days after pulse incubation to allow for further cell expansion. Each CD8-targeted construct selectively loaded to higher degrees onto CD8 T cells as compared with CD4 T cells (FIG. 17A). In addition, each of the histidine mutants comprising attenuated cellular activity (D61H, E64H, and N65H) exhibited higher surface persistence one day following the pulse incubation

onto mixed CD4 and CD8 T cells (FIG. 17A). To evaluate the ability of the CD8-targeted constructs to selectively expand CD8 vs CD4 T cells we compared their activity with a nonselectively-loaded, CD45-targeted IL-15 FM constructed with the same antibody configuration as the CD8-targeted IL-15 FMs (h9.4Fab-BC8scFv-IL15/sushi, which contains a Fab-scFv format consistent with the antibody format for the CD8-targeted constructs). Each CD8-targeted IL-15 construct delivered selective expansion of CD8 T cells, and the CD8-targeted IL-15 constructs comprising the D61H and E64H mutations supported greater CD8 T cell expansion than the CD8-targeted construct comprising wild-type IL-15 (FIG. 17B). Notably, in this pulse assay format each of the CD8-targeted IL-15 constructs only weakly expanded the CD4 T cells (FIG. 17B), consistent with their design for CD8-specificity and consistent with their specificity for loading onto CD8 T cells. For comparison, a full expansion time course is shown for the CD8-targeted constructs comprising wild-type IL-15, IL-15-D61H, or IL-15-E64H (FIG. 17C).

We additionally evaluated the attenuated IL-15 variant IL-15-D61H within the context of a CD45-targeted FM (h9.4Fab-BC8scFv-IL15-D61H). Consistent with the data observed for the CD8-targeted IL-15-D61H FM, the CD45-tethered construct delivered stronger cell surface persistence as compared both with a monovalent and bivalent CD45-tethered IL-15 FMs (FIG. 17D). Collectively, we conclude that FMs comprising attenuated IL-15 variants can result in higher cell surface persistence and induce stronger cell proliferation over time.

Example 8. Sorting of Tregs

FoxP3 positivity defines the Treg cell subset; however, because FoxP3 is an intracellular protein, it cannot be used to sort and isolate live cells using currently available technologies. CD25 is a surface marker for Tregs, but it is also expressed on activated T cells. The IL-7 receptor (CD127) can be used to distinguish between Tregs and activated T cells based on its higher expression on activated T cells. Magnetic and flow cytometry-based sorting methods that enrich for the surface markers CD4⁺CD25⁺ and deplete CD127⁺ cells are used to enrich and isolate Tregs from PBMCs or CD3 T cells.

Frozen CD3 T cells were sorted into Treg-enriched and Treg-depleted CD4 populations by magnetic sorting. The Treg-enriched fraction was positively selected for CD4 and CD25, and negatively selected for CD127 (CD4⁺/CD25⁺/CD127⁻) using magnetic sorting reagents from Miltenyi and following the manufacturer's protocols. Treg -enriched and -depleted cells were activated with CD3/CD28 beads and expanded with IL-2 for 5 days. After 5 days of culture, the Treg-enriched cells maintained significantly more FoxP3⁺ cells, and significantly fewer CD127⁺ cells (FIG. 18). As shown in FIG. 18, enrichment via CD4⁺/CD25⁺/CD127⁻ magnetic

selection gives 30% FoxP3-positive cells, a 2-fold enrichment over Treg-depleted.

Example 9. IL-2 containing fusion molecules

An exemplary IL-2 containing FM can have the following sequence (SEQ ID NO: 79)

5 that can be linked to an antibody or fragment thereof at one terminus:

GGGSGGGSGGGSDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKL
VNEVTEFAKTCVADESAENCDSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNE
CFLQHKDDNPPLRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAK
10 RYKAAFTECCQAADKAACLLPKLDEL RDEGKASSAKQRLKCASLQKFGERAFAKAWAV
ARLSQRFPAEFAEVSKLVTDLT KVHTECCHGDLLECADDRADLAKYICENQDSISSKL
KECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYE
YARRHPDYSVVLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQN
CELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKHPEAKRMPCA
15 EDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFT
FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKKADDKET
CFAEEGKKLV AASQAALGLGGGSAPTSSTKKTQLQLEHLLLDLQMLNGINNYKNP
KLTRMLTFKFYMPKKATELKHLCLEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVL
ELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT*

20 Double underline: IL-2

Single underline: Linker

No underline: HSA (human serum albumin)

This FM was made by expression of a fusion protein comprising three proteins: the
25 targeting antibody (e.g., anti-CD45, anti-CD4 and/or anti-CD25), human serum albumin (SEQ
ID NO: 79, no underline), and IL-2 (SEQ ID NO: 80). The three subunits are operably linked
together via a peptide linker comprising G and S (SEQ ID NO: 79, single underline). Details of
nucleic acid construct preparation, protein expression and use can be found in Examples 1-4.

30 Example 10: Suppressive cell therapy using IL-2 fusion

While Treg therapies have shown promise in clinical trials for Graft vs. Host Disease,
Type 1 Diabetes, and Crohn's Disease, one common failure point in all of these trials has been a
rapid loss of detectable Tregs. Multiple trials have suggested that 90-95% of injected Tregs are
lost within the first two weeks after reinjection into the human subject. It is widely believed that
35 a lack of cytokine support for the *in vitro*-expanded Tregs leads to Activation-Induced Cell
Death (AICD) and a rapid decrease in cell numbers. To support Treg survival *in vivo*, some
studies have incorporated systemic low-dose IL-2 into the Treg adoptive cell therapy treatment
regimen. IL-2 is a pan-T cell pro-proliferative and survival cytokine. A form of IL-2
(aldesleukin) is FDA-approved for treatment of renal cell carcinoma; however, it possesses
40 severe dose-limiting toxicities. Because Tregs express high levels of the IL-2 receptor alpha

subunit (IL2R α or CD25), it is possible to obtain a degree of selective Treg expansion by using IL-2 doses well below toxic levels and with minimal effects on pro-inflammatory T cells. In this Example, we evaluate FMs comprising IL-2 on adoptively transferred Tregs. By directly tethering IL-2 to the surface of adoptively transferred Tregs, this approach can provide strong
5 IL-2 signaling to the Tregs while minimizing effects on other pro-inflammatory cells. This approach can improve Treg survival and engraftment, and promote stronger and more durable treatment of various immune-related conditions, such as recovery of gut homeostasis in Inflammatory Bowel Disease (IBD), including refractory Crohn's Disease and Ulcerative Colitis.

10 The technology as disclosed in, *e.g.*, U.S. Publication No. 2017/0080104, U.S. Patent No. 9,603,944, U.S. Publication No. 2014/0081012, and PCT Application No. PCT/US2017/037249, each of which is incorporated herein by reference in its entirety, is built upon the concept of attaching protein nanogel and fusion molecules to the surface of immune cells immediately prior to adoptive transfer. These non-covalently attached proteins allow for
15 non-genetic control of cell behavior after transfer into the patient. When applied in Treg therapies, IL-2 loading improves on state-of-the-art cell therapy approaches by providing the cells with their own pro-survival/proliferation microenvironment that they carry with them into the patient. Because cytokine is attached directly to the target cells, delivery is non-systemic and a comparable on-target dose is given with far-lower total protein administration, leading to a
20 significantly improved safety profile. Furthermore, because it is non-genetic, the cell production time is reduced, genome insertion related concerns are eliminated, and the cell process is significantly less expensive. Together this can provide to a cell product with improved efficacy and lower cost to the patient and insurers. The proposed mode of action (MOA), as compared to conventional Treg adoptive cellular therapies (ACT), is shown in FIG. 19.

25 We first evaluated the effects of an FM comprising IL-2 on Treg-enriched CD4 T cells using the pulse assay format. Briefly, Treg-enriched CD4 T cells were obtained as described in Example 8. The Treg-enriched CD4 T cells were activated as described in Example 8. Cells were then pulse incubated with an IL-2 FM (h9.4Fab-IL2 for 1 hr at 37C, washed two times, and then plated at a density of 500,000 cells/mL in full medium (containing RPMI 1640 and 10%
30 FBS). IL-2 surface levels following pulse incubation with the IL-2 FM were analyzed by flow cytometry. FIG. 20 shows IL-2 surface levels after titration of an FM comprising IL-2 pulse-loaded onto Treg-enriched CD4s. The IL-2 FM shows titratable surface loading at Day 0 and persistence on the cell surface for at least 3 days. In addition, the cells show dose-dependent proliferation, and all concentrations of the IL-2 FM evaluated (concentration range 1.5 – 100
35 nM) were capable of maintaining cell viability above those of the negative control (FIG. 20).

To evaluate effects of FMs comprising IL-2 on Tregs we designed and produced multiple FMs comprising human IL-2 and a variety of human CD45-targeting moieties and linker configurations including Fab-IL2 (h9.4Fab-IL2), Fab-HSA-IL2 (h9.4Fab-HSA-IL2), scFv-HSA-IL2 (scFv-h9.4-HSA-IL2), scFv-FcγR3B-IL2 (scFv-h9.4-FcγR3B-IL2). *In vitro* tests of these molecules on human T cells demonstrated strong binding and surface persistence, as well as strong expansion of both Treg-enriched CD4s and total CD3s. Briefly, Treg-enriched cells were loaded by incubating with 100nM tethered fusions (TF) or HBSS (mock), washed to removed unbound TF, plated, and then analyzed for cell expansion over time. After 18 hours, soluble recombinant IL2 (sIL2) was added to one of the mock wells at a concentration of 100 ng/mL to serve as a positive control for expansion by saturating levels of IL-2. As shown in FIG. 21, left panel, at 18 hours, strong surface staining was observed for all IL-2 TFs, with intensity of Fab-IL2 > Fab-HSA-IL2 > scFv-HSA-IL2. Cell expansion at day 3 was correlated with surface staining observed on day 1 (FIG. 21); cell viability, however, was consistent across all TFs and sIL2 (FIG. 21, right panel), and exhibited a 2-fold increase over mock. We conclude that multiple configurations of the targeting moiety are capable of facilitating IL-2 loading and persistence on the cell surface and subsequent cellular expansion and viability.

To examine the ability of IL-2-containing FMs to support cell proliferation and survival over longer time courses, CD4 cells were pulsed with vehicle or IL-2 FMs and cultured for 9 days. A separate mock-treated condition was supplemented with constant incubation with 100 ng/mL recombinant IL-2 to serve as a positive control. The IL-2 TFs expanded Treg-enriched CD4 T cells and increased viability over time (FIG. 22). Cells loaded with each of the TF constructs showed equal or better expansion compared to constant stimulation with sIL2 through 6 days post-pulse incubation; Fab-IL2 had the highest surface stain and best growth and survival effects. Mock pulsed cells showed reduced viability and cell number by day 3, and retained only 5% viability by day 6. Conversely, the IL-2 TFs supported sustained cell expansion and had >75% viability 6 days post-BP. We conclude that multiple configurations of IL-2 TFs are capable of promoting expansion and survival of Treg-enriched CD4s for at least 9 days *in vitro*.

To evaluate the ability for IL-2 TFs to promote expansion and survival *in vivo*, tethered fusion-pulsed cells were injected into NOD scid gamma (NSG) mice. 5 million human CD4 T cells were loaded with with Fab-HSA-IL2 as described above and injected into NSG mice (d0). 1 million activated human CD8s were injected on d3 in order to mimic the clinical application of HSCT and measure off-target effects. Blood was drawn on d4, 7, 10, 13, 20, 27, 30, etc. to measure Treg-enriched CD4s and CD8s. As shown in FIG. 23, adoptively transferred cells were detectable in the blood starting d7, and loading of Fab-HSA-IL2 significantly increased cell numbers at early and late time points. Peak increased expansion was detected at d10 with >5-

fold more Fab-HSA-IL2 - loaded cells in the blood stream. Furthermore, FIG. 24 shows that the CD4:CD8 ratio was higher in the mice treated with the FM-pulsed CD4 cells. This indicates that there were greater effects of the IL-2 on pulsed cells than on bystander CD8s, and that pulse loading of an IL-2 FM provides pro-survival and proliferative signals preferentially to the pulsed cells.

In summary, all four TFs show strong binding to unsorted CD3s or Treg-enriched CD4s. Binding is concentration-dependent and saturates between 25 and 100nM for all constructs. All TFs promote survival and expansion similar to soluble IL-2 at saturating concentrations *in vitro*. *In vivo*, Fab-HSA-IL2 promotes early expansion and prolonged engraftment of Treg-enriched CD4s (5 fold at d10).

It should be noted that with antibody-tethered cytokine fusions (TFs), a certain amount of activity on non-pulsed cells has been observed. This can result from either TF dissociation from the loaded cell, or through trans or paracrine effects on neighboring cells. The next step is to reduce these off-target effects, giving maximal selectivity of IL-2 activity for the Tregs. This can be accomplished by engineering TFs for (a) reduced IL-2 affinity for its receptor and (b) enhanced strength, stability and selectivity of Treg binding. Because Tregs have extremely high expression of the IL-2 receptor and the cytokine will be held in close proximity to the receptor via antibody targeting, reduced affinity IL-2 will still drive signaling on loaded cells without off target effects on nearby cells or in the circulation. By increasing antibody specificity for Tregs, it can be ensured that any antibody that dissociates from loaded cells will not bind and initiate signaling on off-target cells. Together, these modifications can create a highly specific molecule with maximal Treg on-target signaling, without off-target toxicities.

Specifically, IL-2 affinity modulated mutants can be engineered to increase Treg-specific signaling. This can increase specificity of activation by reducing paracrine off-target IL-2 signaling on non-loaded cells. Reduced affinity human and mouse IL-2 molecules can be made based on structure of receptor binding and analogous work with IL-15 (see Example 7). Optimal mutant can be selected *in vitro* using mixed loaded/non-loaded cell assay and maximizing ratio of on-target to off-target activity. Reduced off-target effects of affinity modulated IL-2 *in vivo* can be validated.

Potency and Treg selectivity of antibody binding motifs can also be optimized. This can increase specificity and strength of IL-2 tethering to Tregs to reduce off target effects, and enable selective loading of stabler and more active Treg subsets. Affinity matured, humanized CD45 antibody and humanized anti-CD4 (OKT4) can be produced. Antibody campaigns for CD25 (general Treg) and CD39 (active subset specific) can also be conducted. Optimal antibody binding motifs can be selected using the following criteria: (a) Stable and

selective binding to Tregs; (b) Maximized stability of binding to Tregs. With highly stable binding, selectivity is less important since clinical flow-based sorting and *ex vivo* expansion results in a 90-95% pure Treg population; and (c) Treg selectivity is more important if tethered fusions come off the target cell and enter circulation. In that case, Treg-specificity will prevent re-binding and activation of off-target cell types.

Next, affinity-modulated IL-2 with optimized tethering domains can be combined to produce highly Treg-selective lead molecules. Optimized lead molecules can include bi-specific antibody tethering (e.g., in a design similar to FIG. 3E), which allows for increased persistence and cell type selectivity, and/or complex surface marker selectivity. Human leads and mouse surrogates can then be validated using *in vitro* binding, expansion and off-target mixed cell assays. Efficacy, off-target cell effects and toxicity can be validated using mouse surrogates in Treg-controllable disease models, e.g., Naïve T cell model of colitis (IBD model). Expansion, survival, engraftment and homing of final human molecule can be validated in the NSG human intestinal graft model.

Without wishing to be bound by theory, it is believed that the mono-specific TFs bind through a single, non-specific targeting motif (FIG. 25, upper panel). IL-2 signaling occurs primarily on loaded cells (1). However, trans signaling (2), dissociation and soluble signaling (3) and rebinding and signaling (4) result in low levels of off-target cell activation. As shown in FIG. 25, lower panel, by increasing antibody targeting potency and specificity (e.g., by using a bi-valent or multi-specific binding moiety), as well as reducing IL-2 affinity for its receptor, loaded cells can continue to receive maximal IL-2 signal while eliminating off-target effects. This can result in specific survival, expansion and engraftment of transferred Tregs, leading to improved outcomes for patients.

Modifications and variations of the described methods and compositions of the present disclosure will be apparent to those skilled in the art without departing from the scope and spirit of the disclosure. Although the disclosure has been described in connection with specific embodiments, it should be understood that the disclosure as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the disclosure are intended and understood by those skilled in the relevant field in which this disclosure resides to be within the scope of the disclosure as represented by the following claims.

INCORPORATION BY REFERENCE

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference. The PCT International Application having Attorney Docket No. 174285-010900/PCT titled “POLYNUCLEOTIDES ENCODING
5 IMMUNOSTIMULATORY FUSION MOLECULES AND USES THEREOF” filed July 3, 2018 and the PCT International Application having Attorney Docket No. 174285-010800/PCT titled “IMMUNOSTIMULATORY FUSION MOLECULES AND USES THEREOF” filed July 3, 2018 are both incorporated herein by reference in its entirety.

CLAIMS

1. A fusion molecule, comprising:
 - (a) a cytokine or growth factor molecule; and
 - 5 (b) a regulatory T cell (Treg) targeting moiety comprising an antigen-binding fragment of an antibody having an affinity to an antigen on the surface of a Treg;

wherein the cytokine or growth factor molecule is operatively linked to the antigen-binding fragment.
- 10 2. The fusion molecule of claim 1, wherein the antigen is one or more of CD45, CD4, CD25, CD39, Neuropilin 1 (NRP1), or a variant of any of the foregoing.
3. The fusion molecule of claim 1, wherein the targeting moiety comprises a bispecific molecule having an antigen-binding fragment specific for CD4 and an antigen-binding fragment

15 specific for CD25.
4. The fusion molecule of claim 1, wherein the antigen is one or more of CD4, CD45, CD3, CD2, CD25, CD127, CD197 (CCR7), CXCR3, CXCR4, CXCR5, CD38, CD27, , CCR4, CCR5, CD137, CD39, CCR4, CCR5, CCR6 (CD196), CCR8, CCR10, OX40, GITR, CTLA4,

20 LAG3, CD73, CD103, CD62L, CCR2, CCR9, Neuropilin 1 (NRP1), CD8, CD11a, CD18, or a variant of any of the foregoing.
5. The fusion molecule of claim 1, wherein the cytokine or growth factor molecule is one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, or TGF- β , or a variant of

25 any of the foregoing.
6. The fusion molecule of claim 1, wherein the cytokine or growth factor molecule is one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, TGF- β , IL-7, or IL-21, or a variant of any of the foregoing.

30
7. The fusion molecule of claim 1, wherein the cytokine or growth factor molecule is one or more of IL-15, IL-2, IL-18, IL-27, IL-10, IL-35, Amphiregulin, IL-33, TGF- β , IL-7, IL-21, IL-6, IL-12, or IL-23, or a variant of any of the foregoing.
- 35 8. The fusion molecule of any one of claims 1-7, wherein the Treg is a healthy and/or non-

malignant Treg.

9. The fusion molecule of any one of the preceding claims, further comprising a linker for operably linking the cytokine or growth factor molecule and the targeting moiety.

5

10. The fusion molecule of claim 9, wherein the linker is selected from one or more of a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, or a non-helical linker.

10 11. The fusion molecule of claim 10, wherein the peptide linker is a human serum albumin or a variant thereof.

12. The fusion molecule of claim 9 or 10, wherein the linker, at either end, is fused to the cytokine or growth factor molecule and the targeting moiety via a peptide comprising Gly and
15 Ser.

13. The fusion molecule of claim 12, wherein the peptide is a (GGGS)_N (SEQ ID NO: 87) or (GGGGS)_N (SEQ ID NO: 88) linker, where N indicates the number of repeats of the motif and is an integer selected from 1-10.

20

14 The fusion molecule of claim 1, wherein the antigen-binding fragment is a Fab fragment comprising a light chain and a heavy chain fragment linked by a disulfide bond, and wherein the cytokine or growth factor molecule is operably linked to the Fab fragment at a C-terminus of the light chain, an N-terminus of the light chain, a C-terminus of the heavy chain fragment, or an N-
25 terminus of the heavy chain fragment.

15. The fusion molecule of any one of the preceding claims, wherein the targeting moiety localizes the cytokine or growth factor molecule to the Treg and enhances its concentration, distribution, duration of exposure and/or availability on a cell surface of the Treg.

30

16. An isolated nucleic acid molecule engineered to encode the fusion molecule of any one of claims 1-15.

17. A vector comprising the nucleic acid molecule of claim 16.

35

18. A host cell comprising the nucleic acid molecule of claim 16 or the vector of claim 17.

19. A method of making the fusion molecule of any one of claims 1-15, comprising culturing the host cell of claim 18 under suitable conditions.

5

20. A pharmaceutical composition comprising the fusion molecule of any one of claims 1-15 and a pharmaceutically acceptable carrier, excipient, or stabilizer.

21. A modified Treg, comprising a healthy and/or non-malignant Treg and the fusion molecule of any one of claims 1-15 bound or targeted thereto.

10

22. A method for *ex vivo* expansion of Tregs, comprising providing a population of PBMCs from a subject, and selectively expanding Tregs therein in the presence of a plurality of fusion molecules of any one of claims 1-15, thereby producing a plurality of expanded Tregs.

15

23. A method for providing immunosuppressive therapy, comprising administering to a subject in need thereof a plurality of fusion molecules of any one of claims 1-15, a plurality of the modified Tregs of claim 21, or the plurality of expanded Tregs of claim 22.

24. The method of claim 23, wherein the immunosuppressive therapy is used to treat diseases selected from allo-immune diseases, auto-immune diseases, allergy, and inflammatory diseases.

20

25. The method of claim 24, wherein the allo-immune diseases include organ transplant rejection, graft versus host disease (GVHD) (*e.g.*, post allogeneic hematopoietic stem cell transplant, HSCT) and GVHD post allogeneic stem cell transplantation (SCT).

25

26. The method of claim 24, wherein the auto-immune diseases include Type 1 diabetes, Multiple Sclerosis and Alopecia.

30

27. The method of claim 24, wherein the inflammatory diseases include Inflammatory Bowel Disease, Rheumatoid Arthritis and Lupus.

28. A fusion molecule, comprising:

(a) a cytokine or growth factor molecule; and

35

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

5 wherein the cytokine or growth factor molecule is operably linked to the antibody at the C-terminus of the light chain, the N-terminus of the light chain, or the N-terminus of the heavy chain portion.

29. The fusion molecule of claim 28, wherein the antigen is a CD4 or a CD25 receptor
10 expressed on the cell surface of the Treg.

30. The fusion molecule of claim 28, wherein the cytokine or growth factor molecule comprises an IL-2, or a variant thereof.

15 31. The fusion molecule of claim 28, wherein the cytokine or growth factor molecule is operably linked to the antibody by a linker.

32. The fusion molecule of claim 31, wherein the linker is selected from a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, and a non-
20 helical linker.

33. The fusion molecule of claim 31, wherein the linker is a peptide linker comprising a Gly and a Ser.

25 34. The fusion molecule of claim 33, wherein the peptide linker is a (GGGS)_N (SEQ ID NO: 87) or (GGGGS)_N (SEQ ID NO: 88) linker, wherein _N indicates the number of repeats of the motif and is an integer selected from 1-10.

35. A modified Treg comprising:

- 30 (a) a fusion molecule comprising
- (i) a cytokine or growth factor molecule; and
 - (ii) a Treg targeting moiety having an affinity to a Treg cell surface antigen; and
- (b) a Treg, wherein the fusion molecule is bound to the surface of Treg through interaction with the cell surface antigen.

35. A modified Treg comprising a Treg and the fusion molecule of any one of claims 1-15 and 28-33 bound thereto.

36. A method of preparing modified Tregs, comprising:

- 5 (a) providing a population of Tregs; and
(b) incubating the fusion molecule of any one of claims 1-15 and 28-33 with the population of Tregs so as to permit targeted binding of the fusion molecule thereto, thereby producing a population of Tregs having fusion molecules bound on the cell surface.

10 37. A composition for use in cell therapy, the composition comprising:

- (a) a plurality of fusion molecules, each fusion molecule comprising
(i) a cytokine or growth factor molecule; and
(ii) a Treg targeting moiety having an affinity to a cell surface antigen of a Treg;
(b) a population of Tregs, wherein the plurality of fusion molecules are bound to the
15 surface of the Treg through interaction with the cell surface antigen; and
(c) a pharmaceutically acceptable carrier, excipient, or stabilizer.

38. A pharmaceutical composition comprising the fusion molecule of any one of the claims 1-15 and 28-30 and a pharmaceutically acceptable carrier, excipient, or stabilizer.

20

39. A method for the suppressing or preventing an immune response in a human subject, the method comprising administering to the human subject a cell therapeutic composition, the composition comprising:

- (a) a plurality of fusion molecules, each fusion molecule comprising
25 (i) a cytokine or growth factor molecule; and
(ii) a Treg targeting moiety having an affinity to a cell surface antigen of a Treg;
and
(b) a population of Tregs,
wherein the plurality of fusion molecules are bound to the surface of the Tregs, and
30 wherein the cytokine or growth factor molecule acts *in vivo* upon the population of Tregs in the human subject to suppress or prevent an immune response in the human subject.

40. The fusion molecule of any one of claims 1-15 and 28-30, further comprising a nanoparticle.

35

41. The fusion molecule of any one of claims 1-15 and 28-30, further comprising a liposome.
42. The fusion molecule of claims 1-15 and 28-30, wherein the nanoparticle comprises a protein nanogel, a nucleotide nanogel, a polymer nanoparticle, or a solid nanoparticle.
43. The fusion molecule of claim 40, wherein the nanoparticle comprises a protein nanogel.
44. The fusion molecule of claim 43, wherein the nanoparticle comprises at least one polymer, cationic polymer, or cationic block co-polymer on the nanoparticle surface.
45. The fusion molecule of claim 44, wherein the nanoparticle comprises a nanogel that is cross linked by a reversible linker that is sensitive to redox (disulfide) or pH (hydrolysable groups) or enzymes (proteases).
46. The fusion molecule of any one of claims 1-15 and 28-30, further comprising a biodegradable polymer.
47. A composition comprising:
- (a) a fusion molecule comprising
 - (i) a cytokine or growth factor molecule; and
 - (ii) an immune cell targeting moiety having an affinity to a Treg cell surface antigen;
 - (b) a Treg expressing or otherwise displaying the cell surface antigen,
- wherein the fusion molecule is bound to the surface of the Treg through interaction with the cell surface antigen; and
- (c) a nanoparticle, nanogel, or liposome.

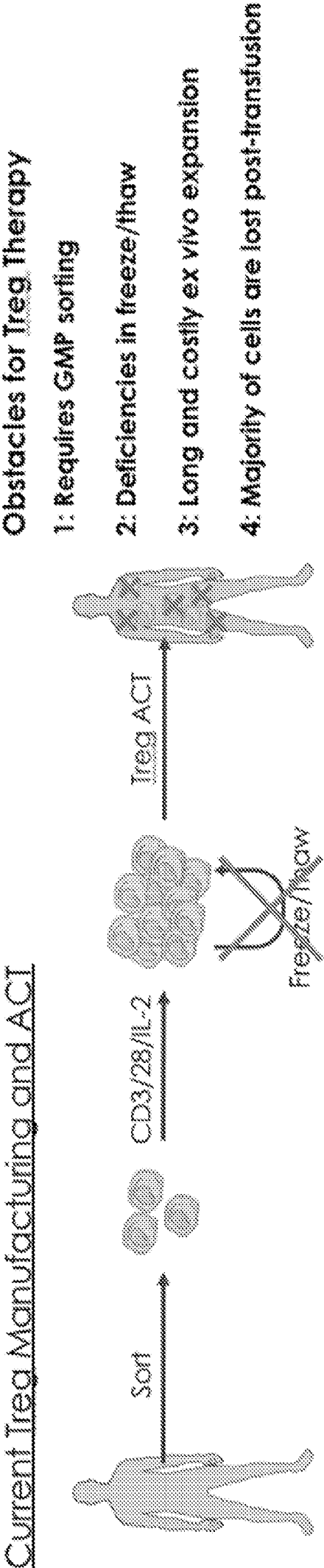


FIG. 1A

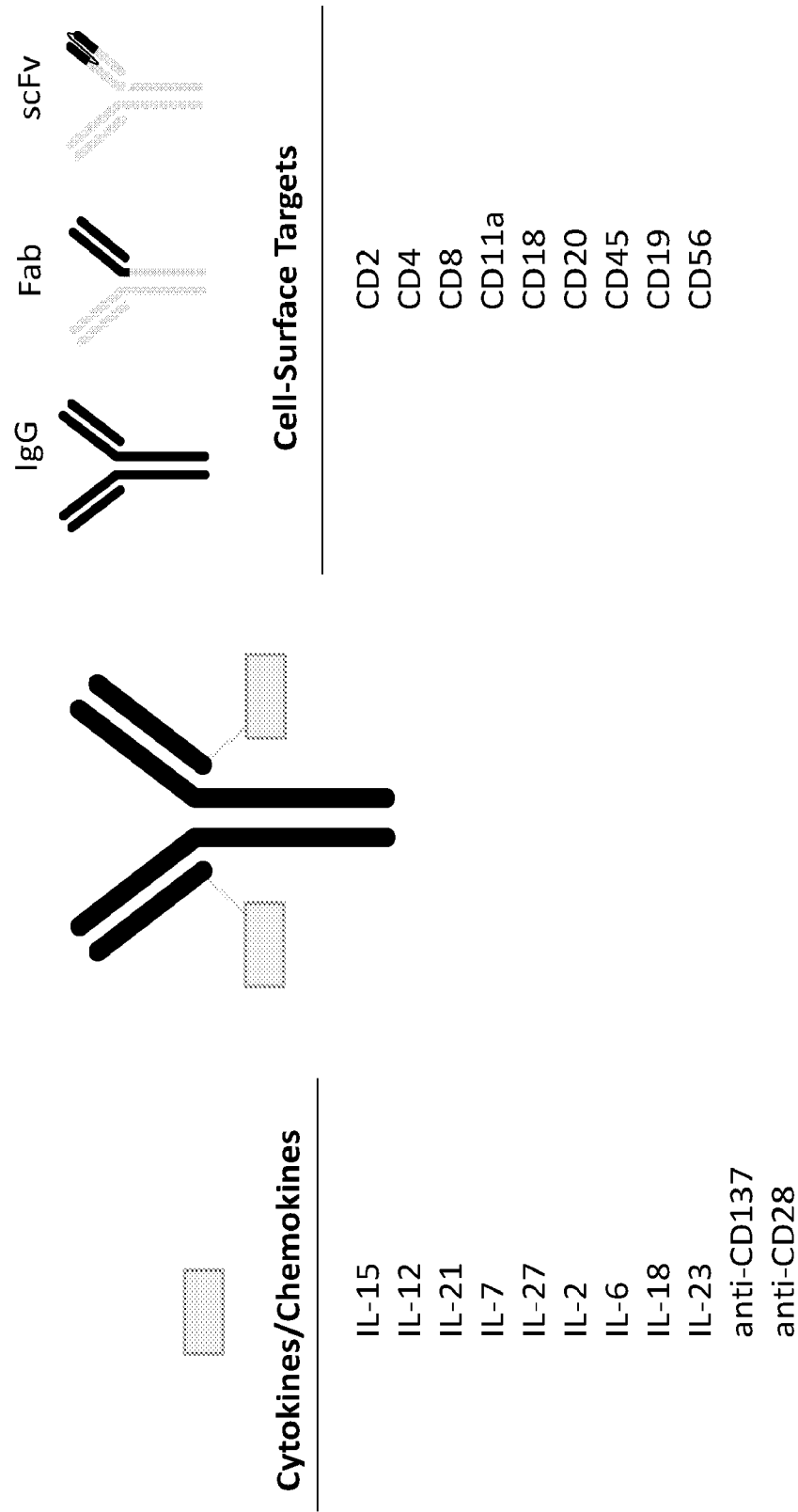


FIG. 1B

Manufacturing: Ex vivo expansion

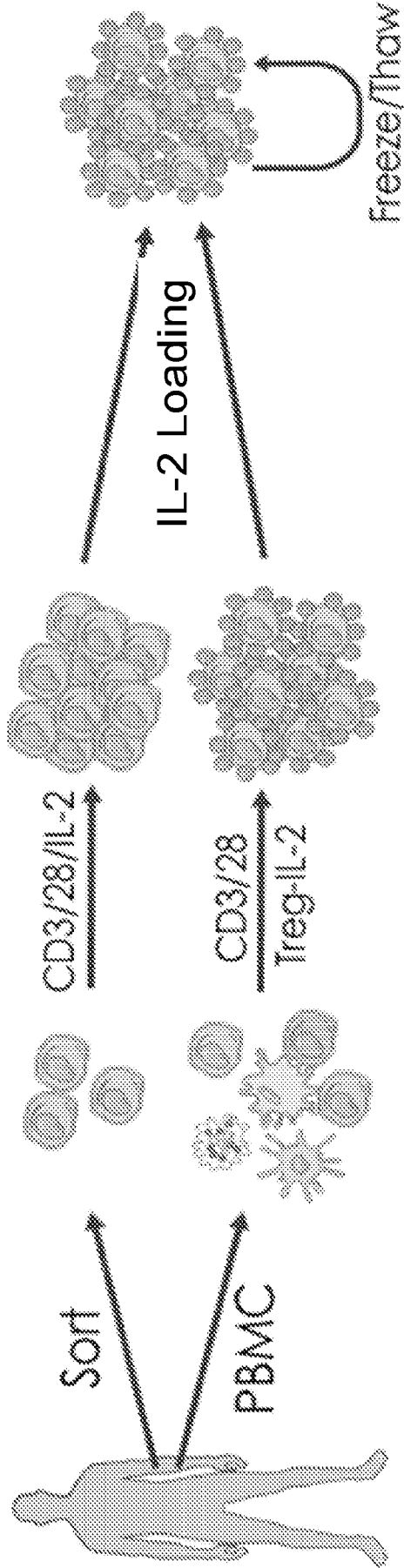


FIG. 1C

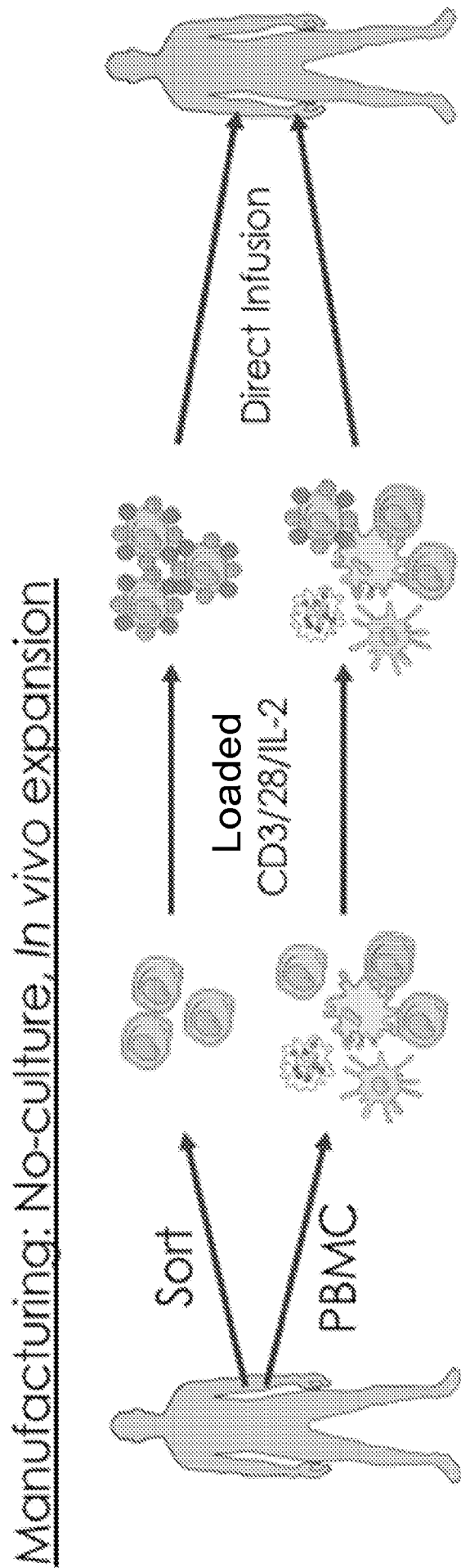


FIG. 1D

IL-2 Loading-Driven, Expansion, Survival and Engraftment

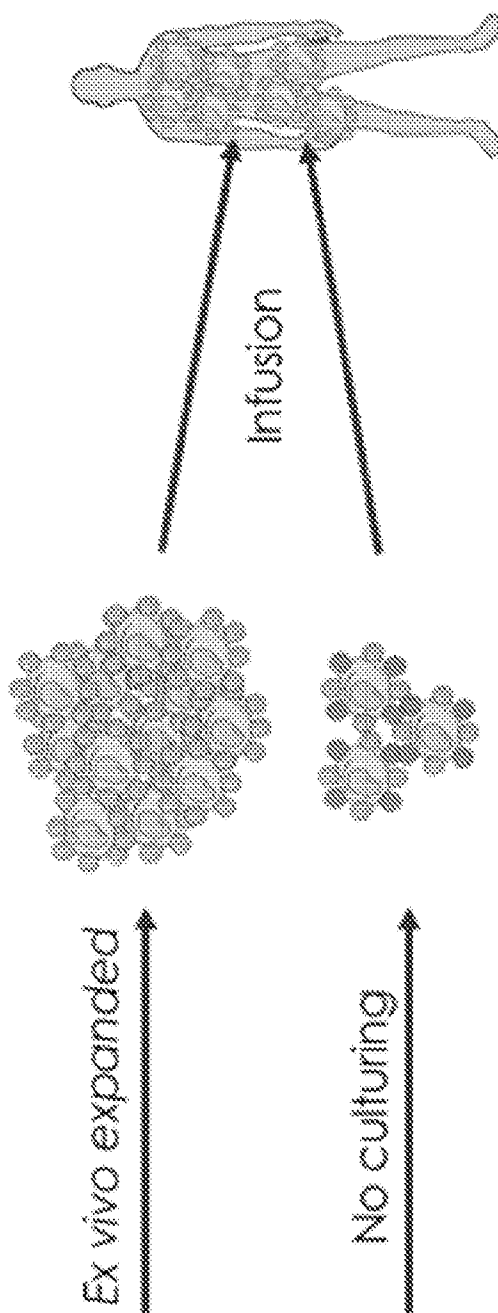


FIG. 1E

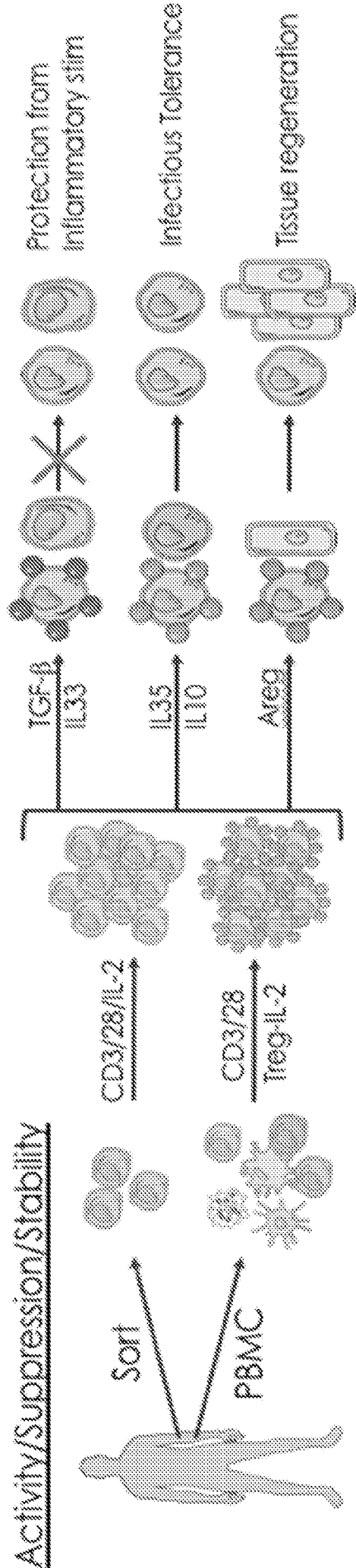


FIG. 1F

Treg-targeting of Tethered Fusions

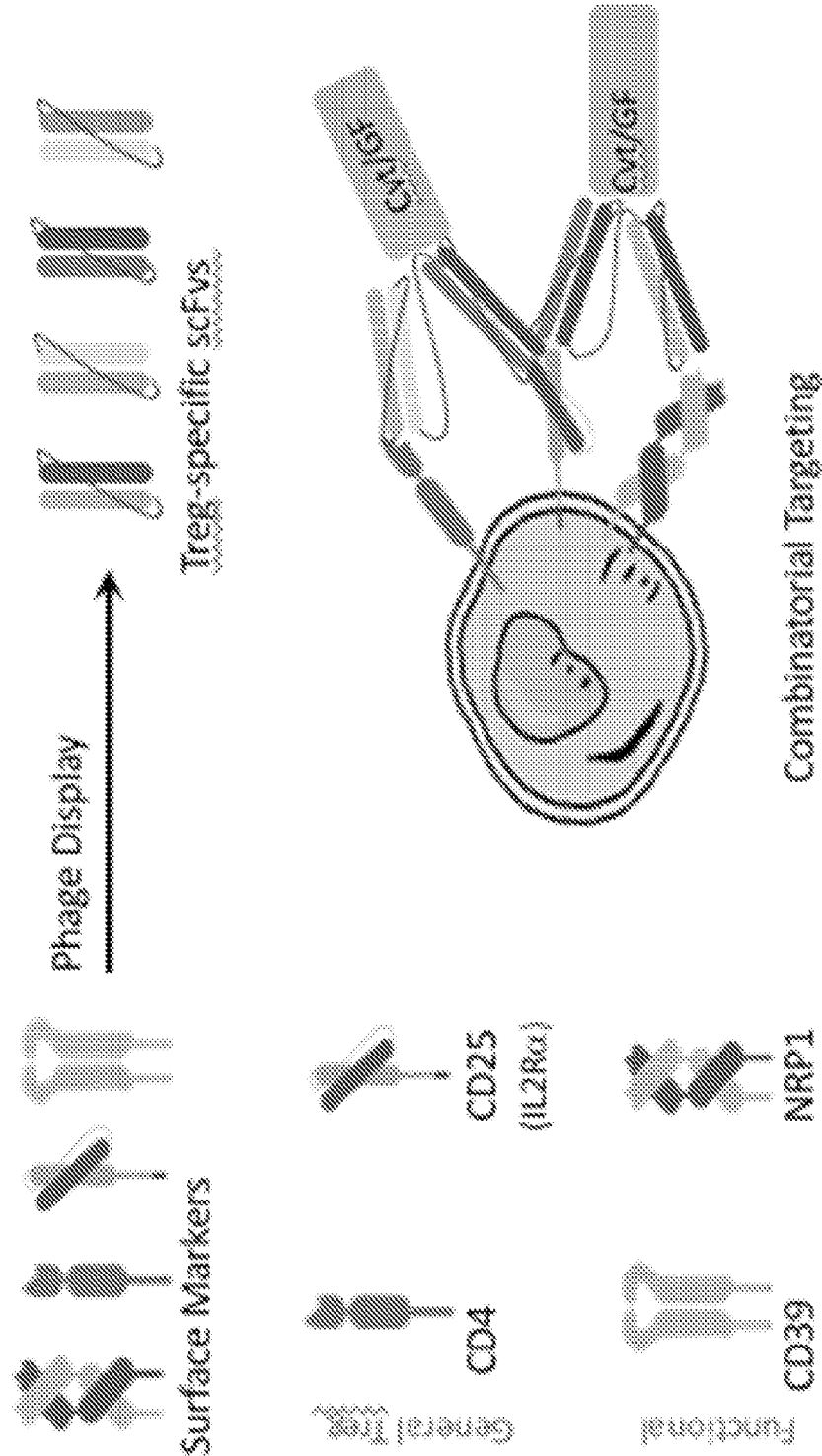


FIG. 1G

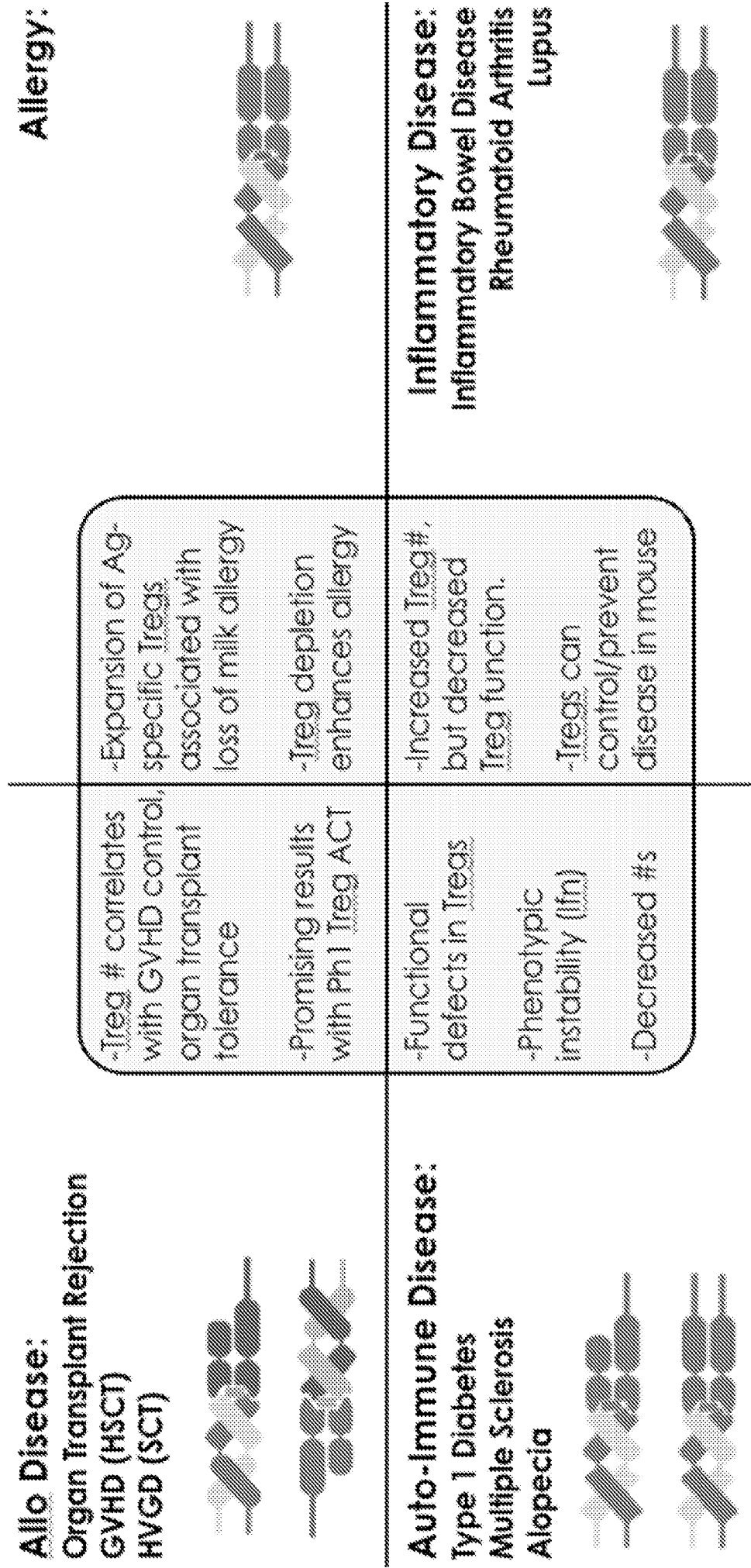


FIG. 1H

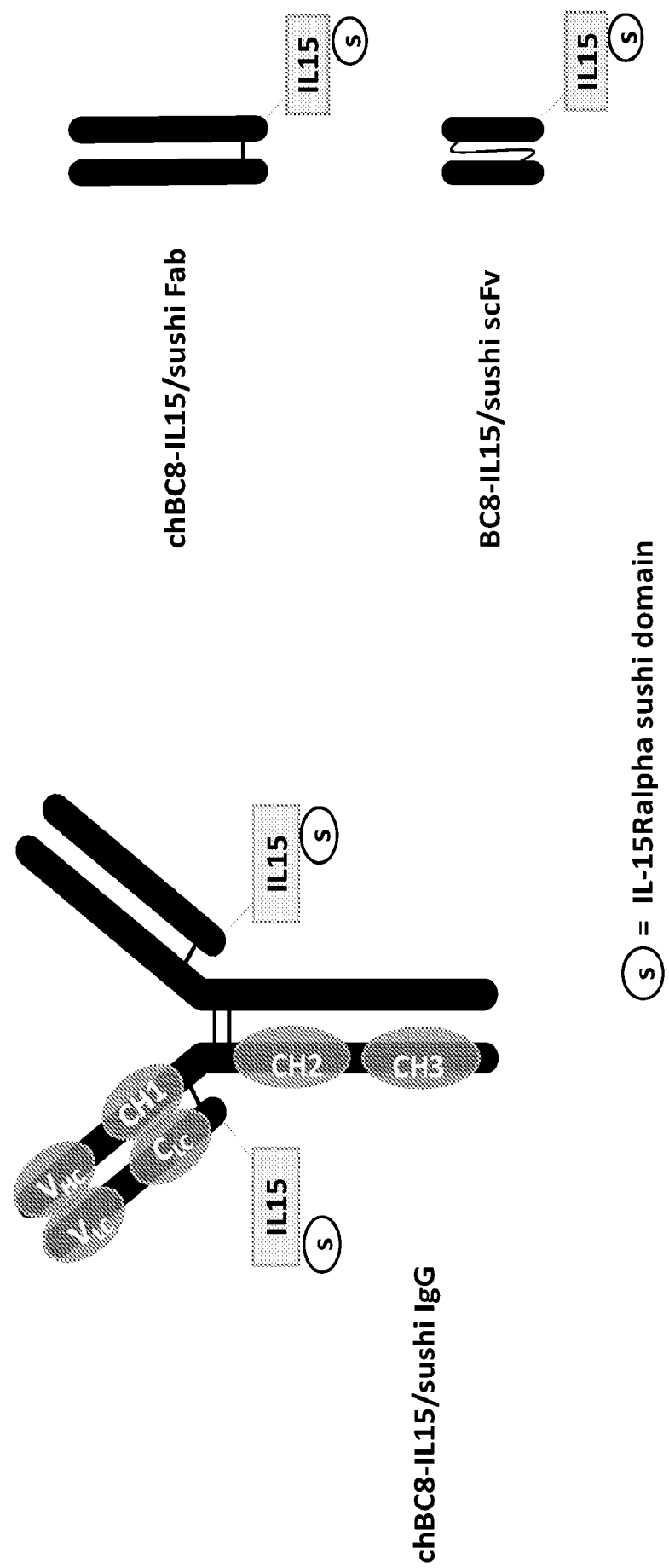


FIG. 2A

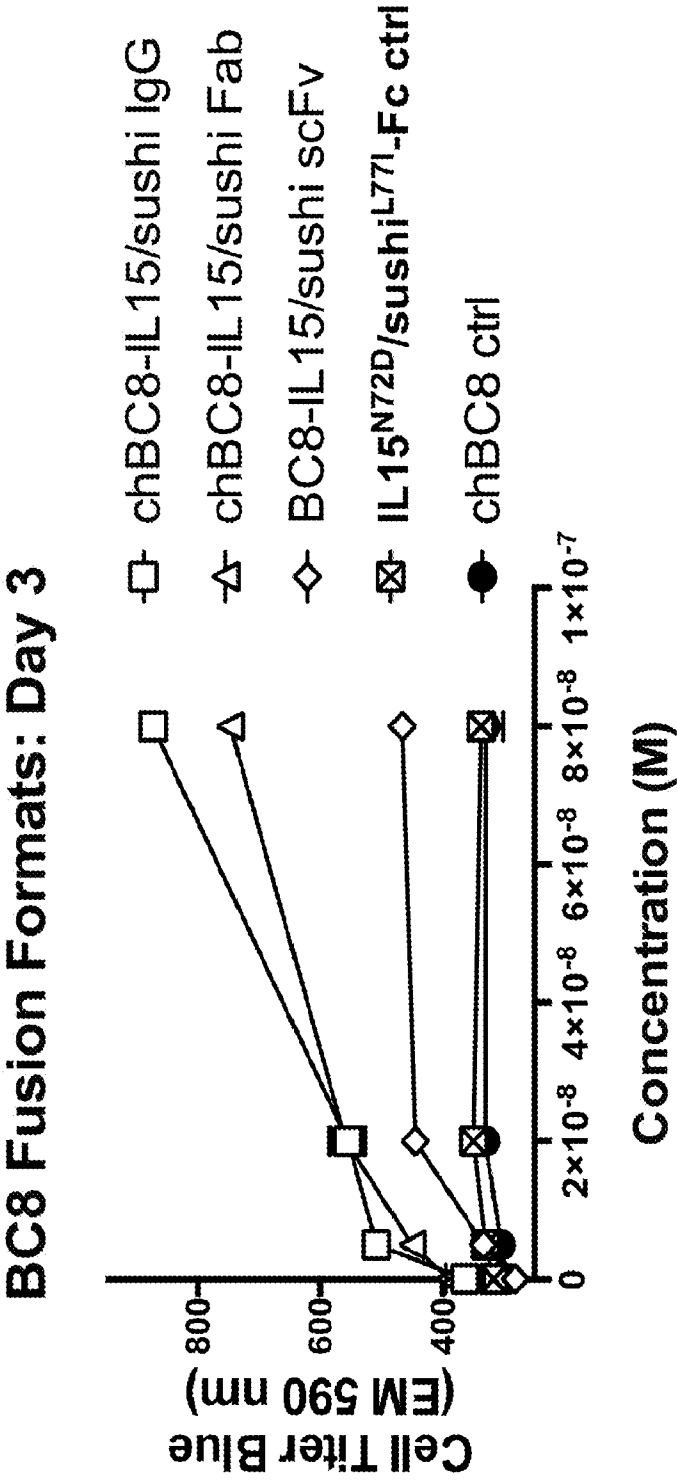


FIG. 2B

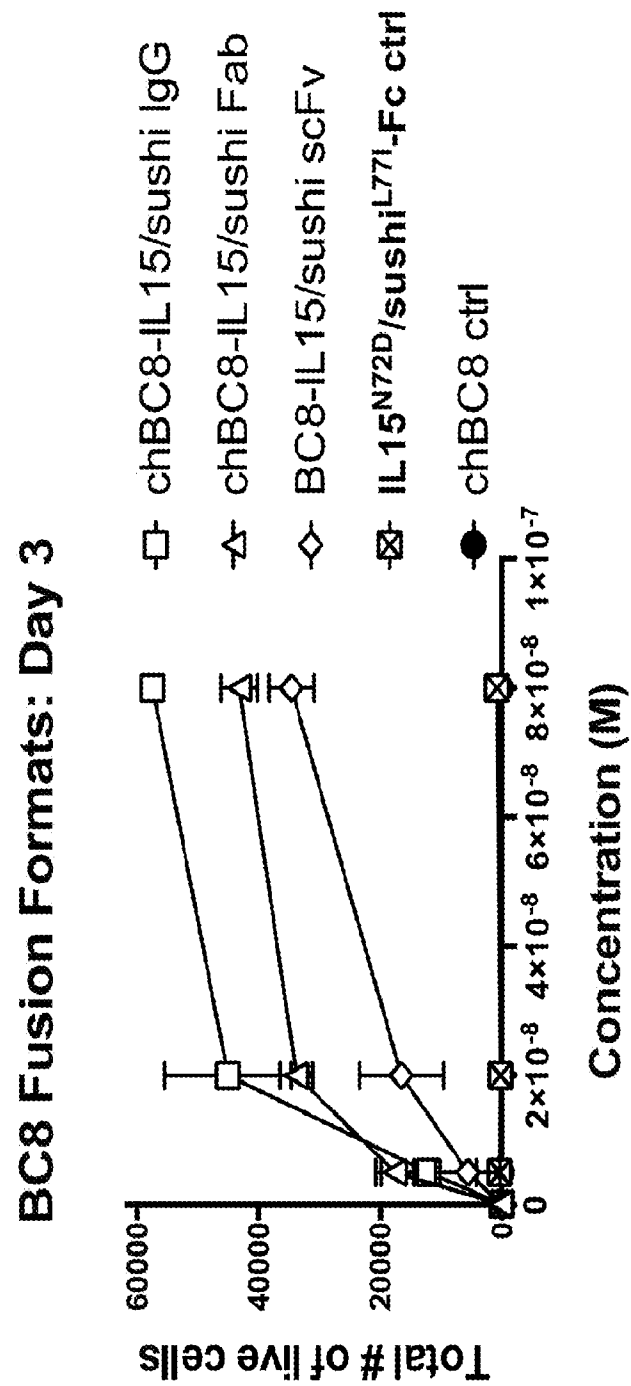


FIG. 2C

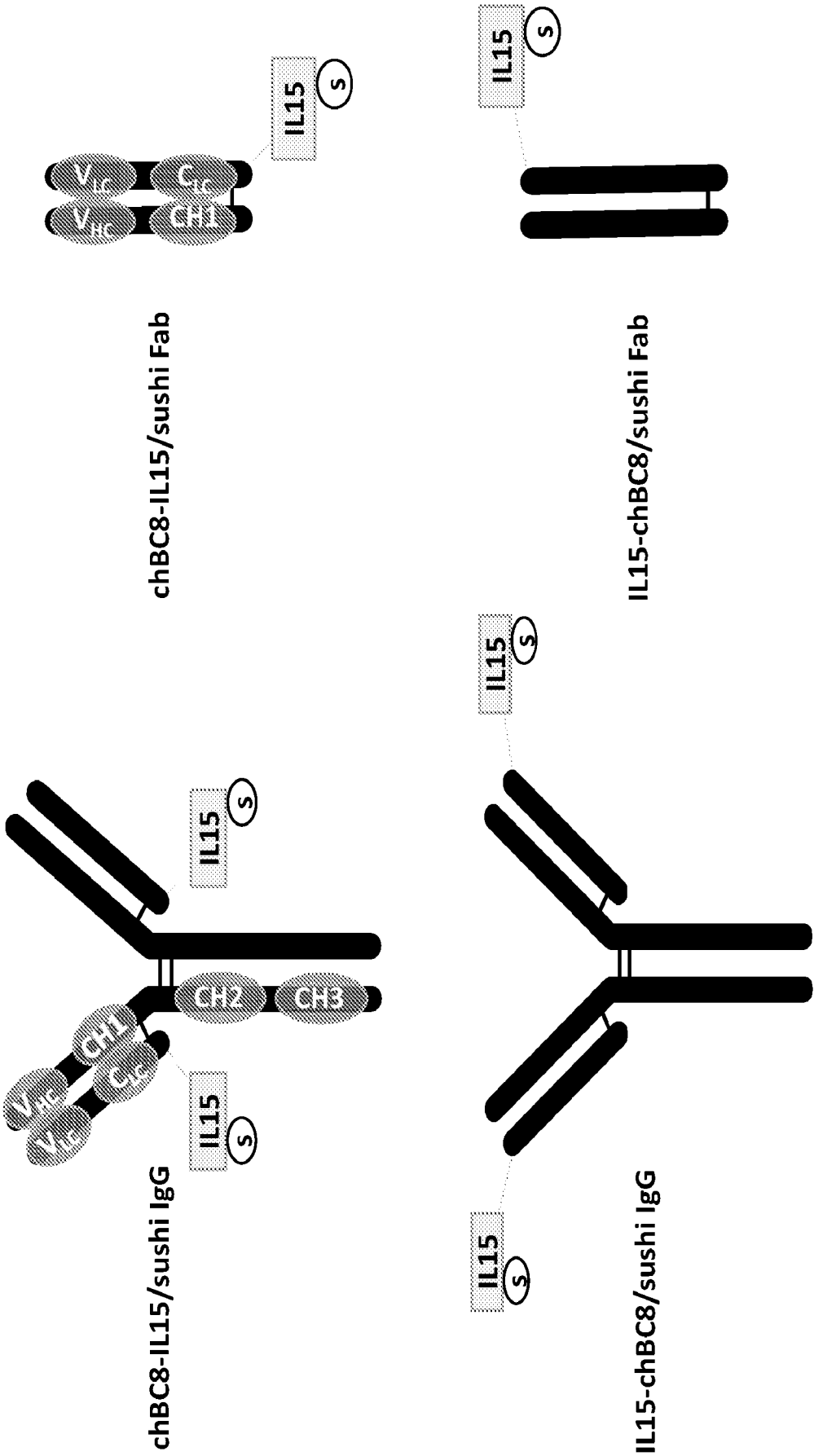


FIG. 3A

FIG. 3B

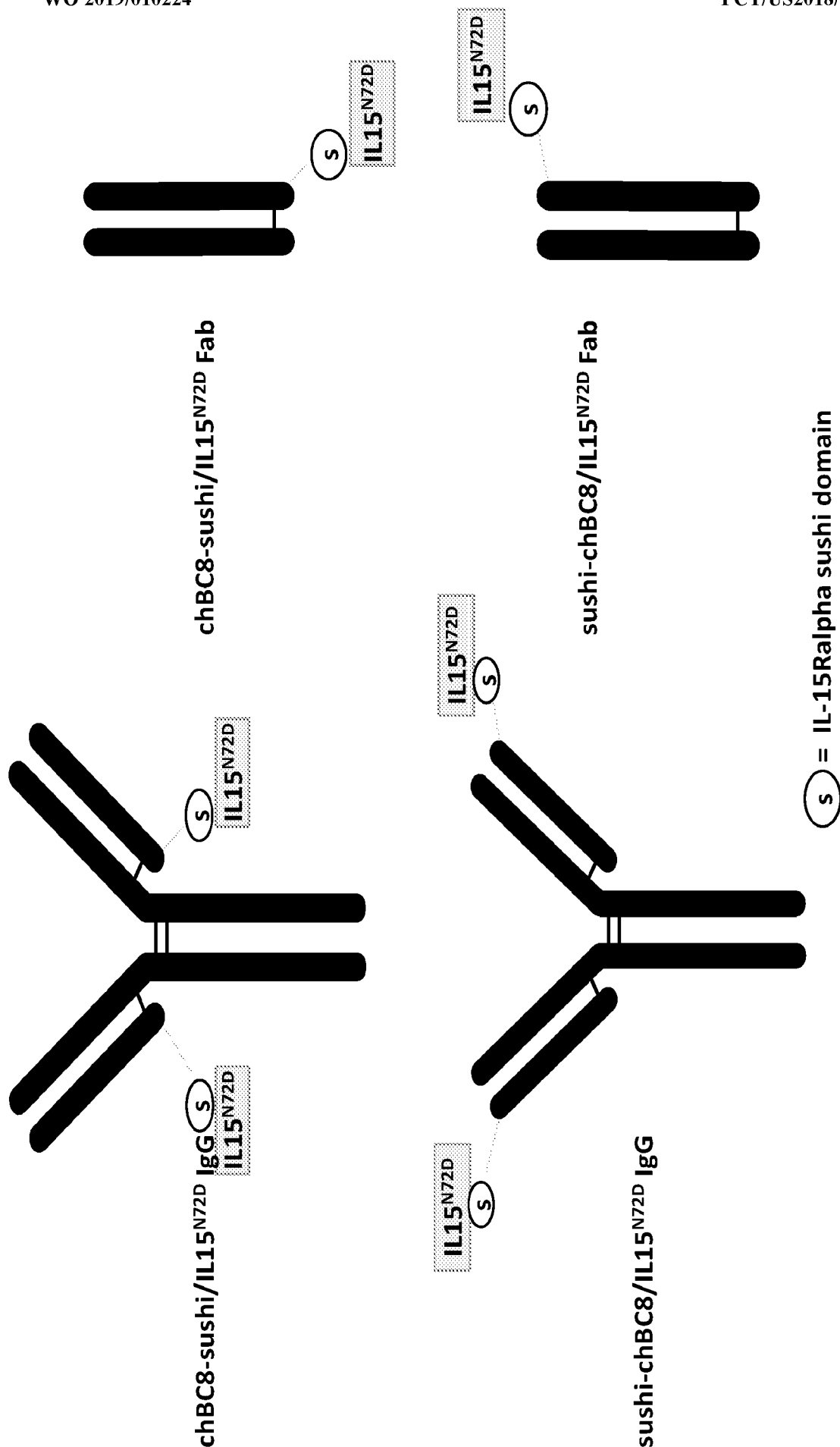


FIG. 3D

FIG. 3C

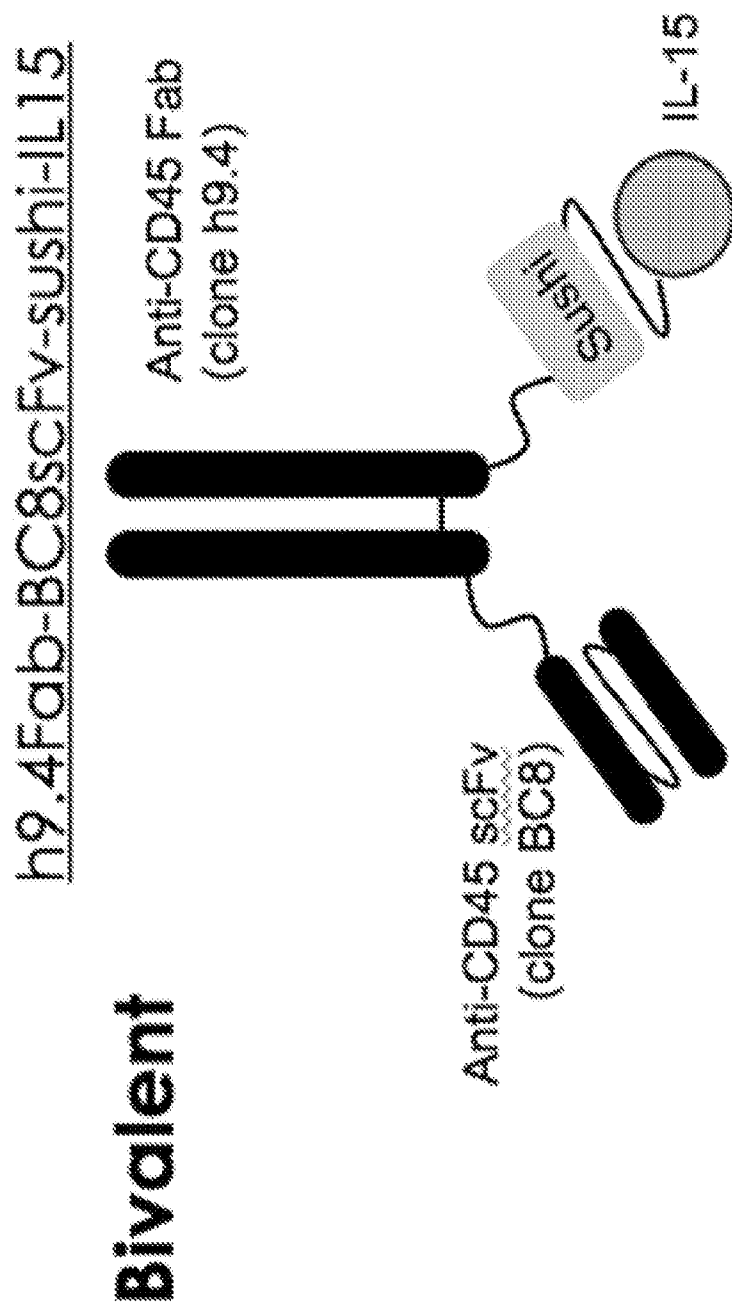


FIG. 3E

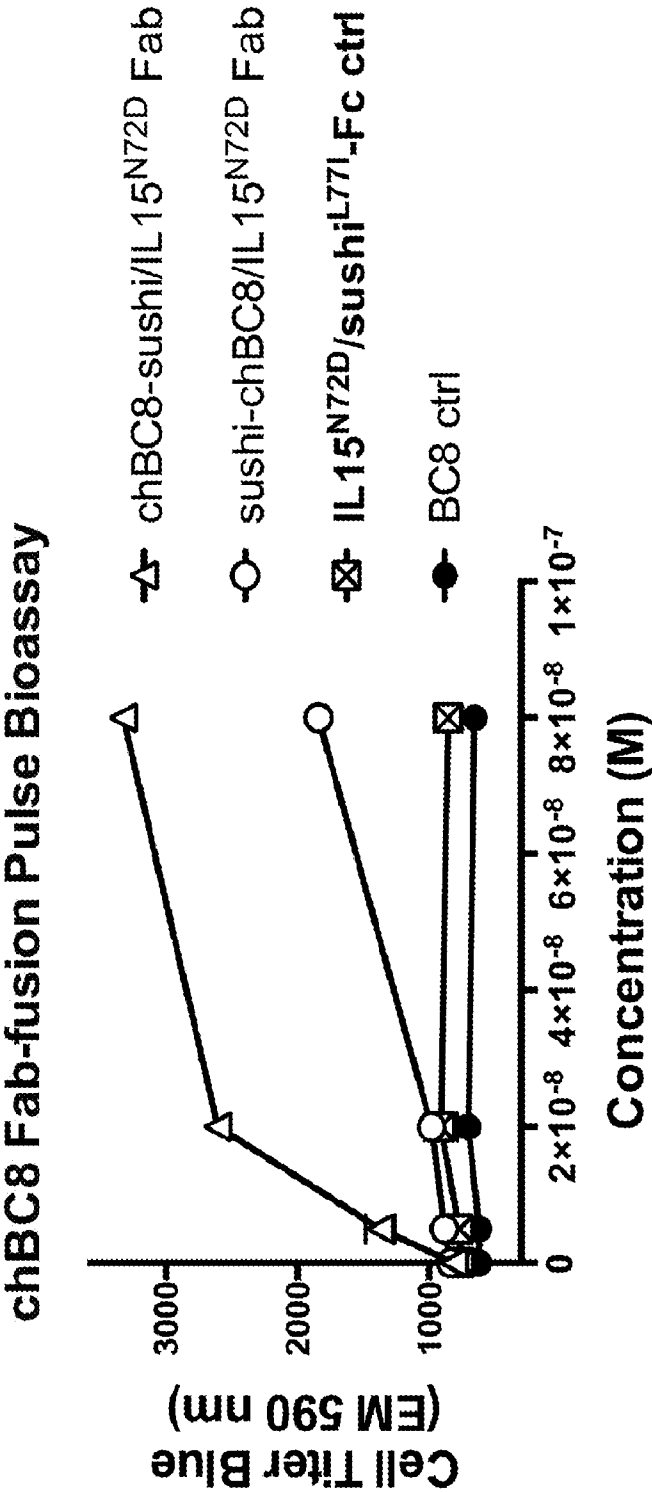


FIG. 4A

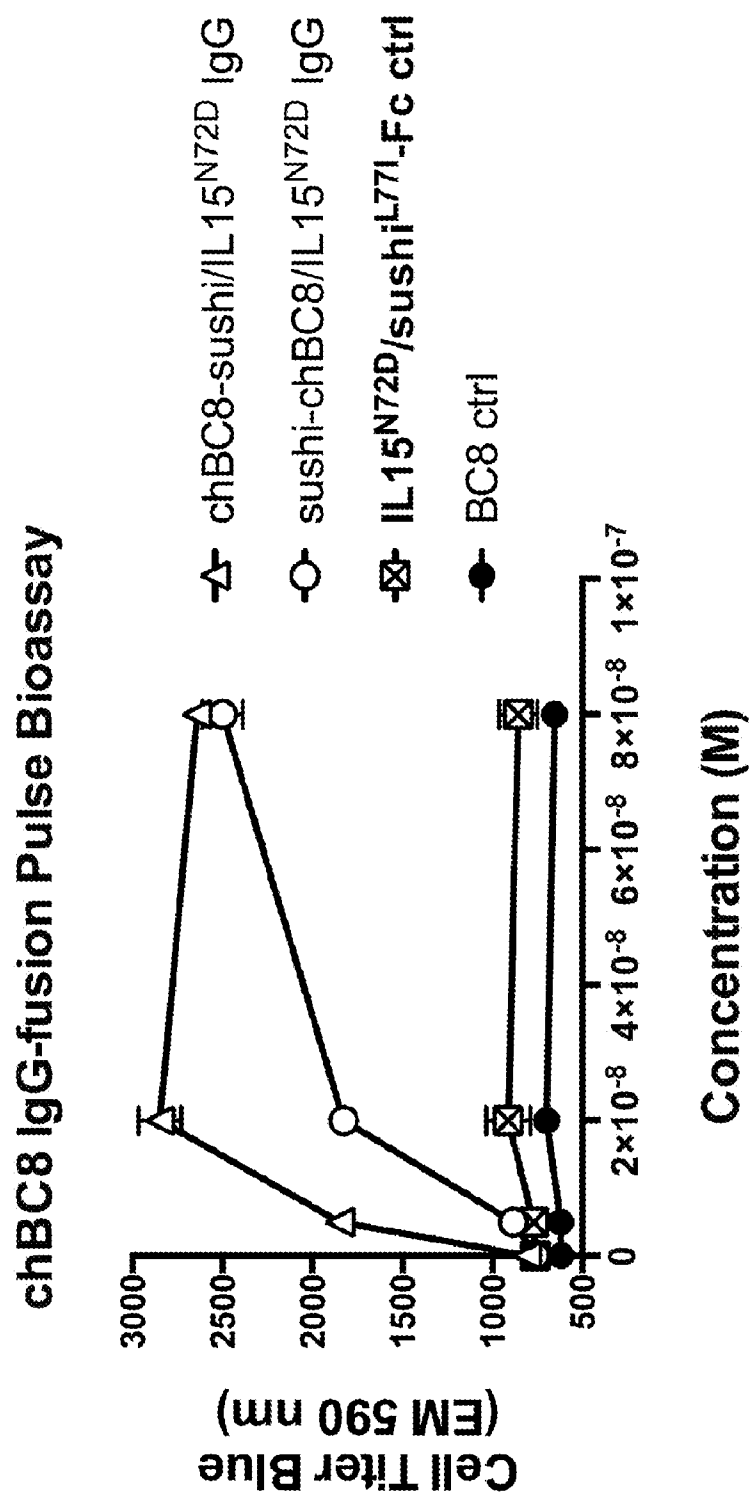


FIG. 4B

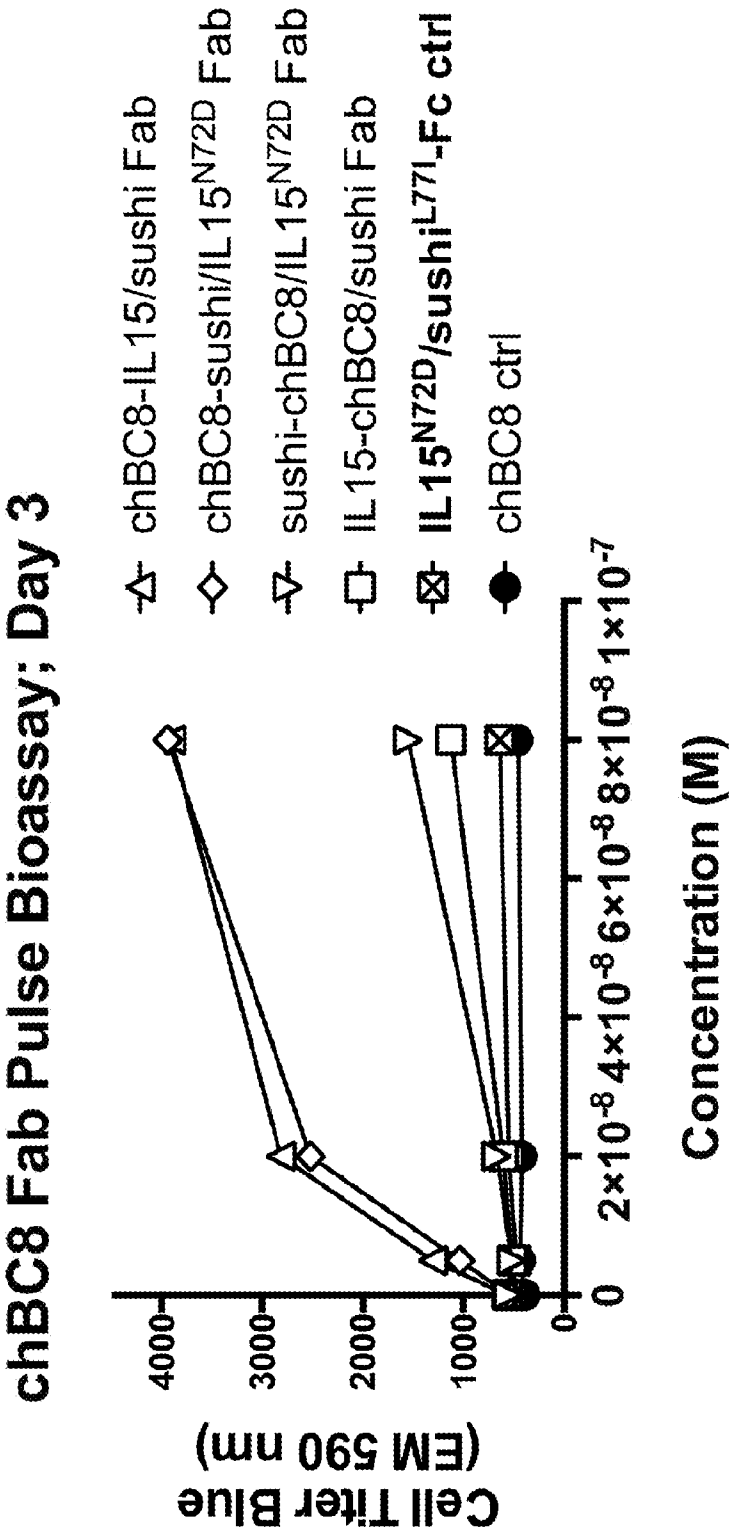


FIG. 5A

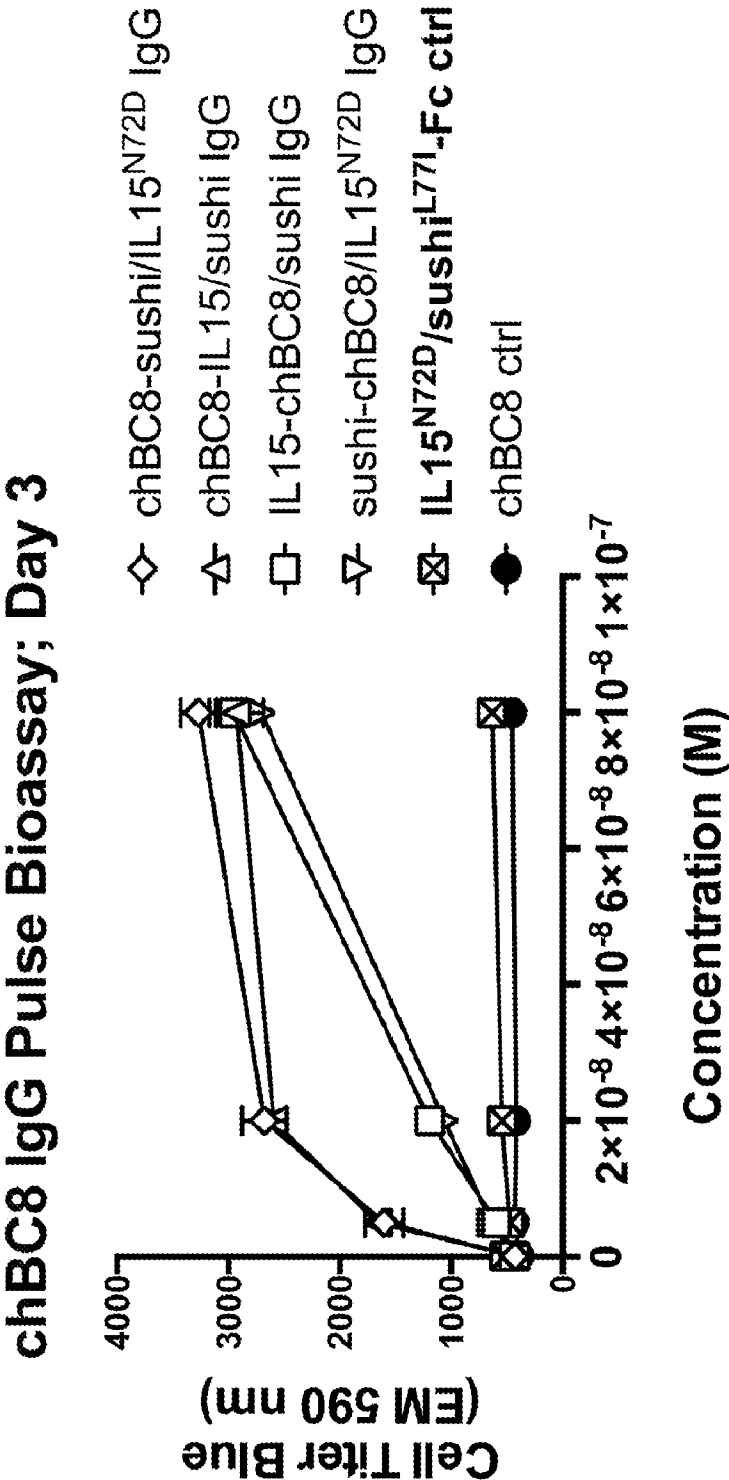


FIG. 5B

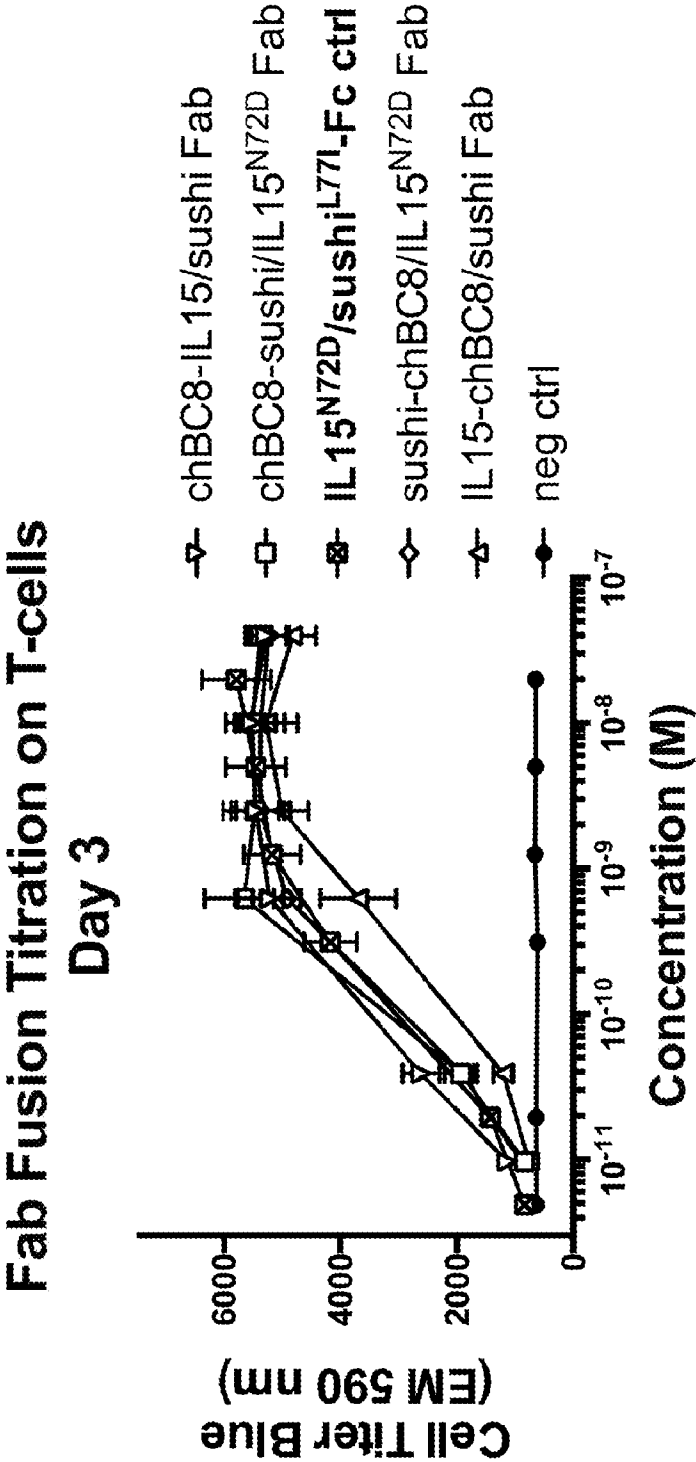


FIG. 5C

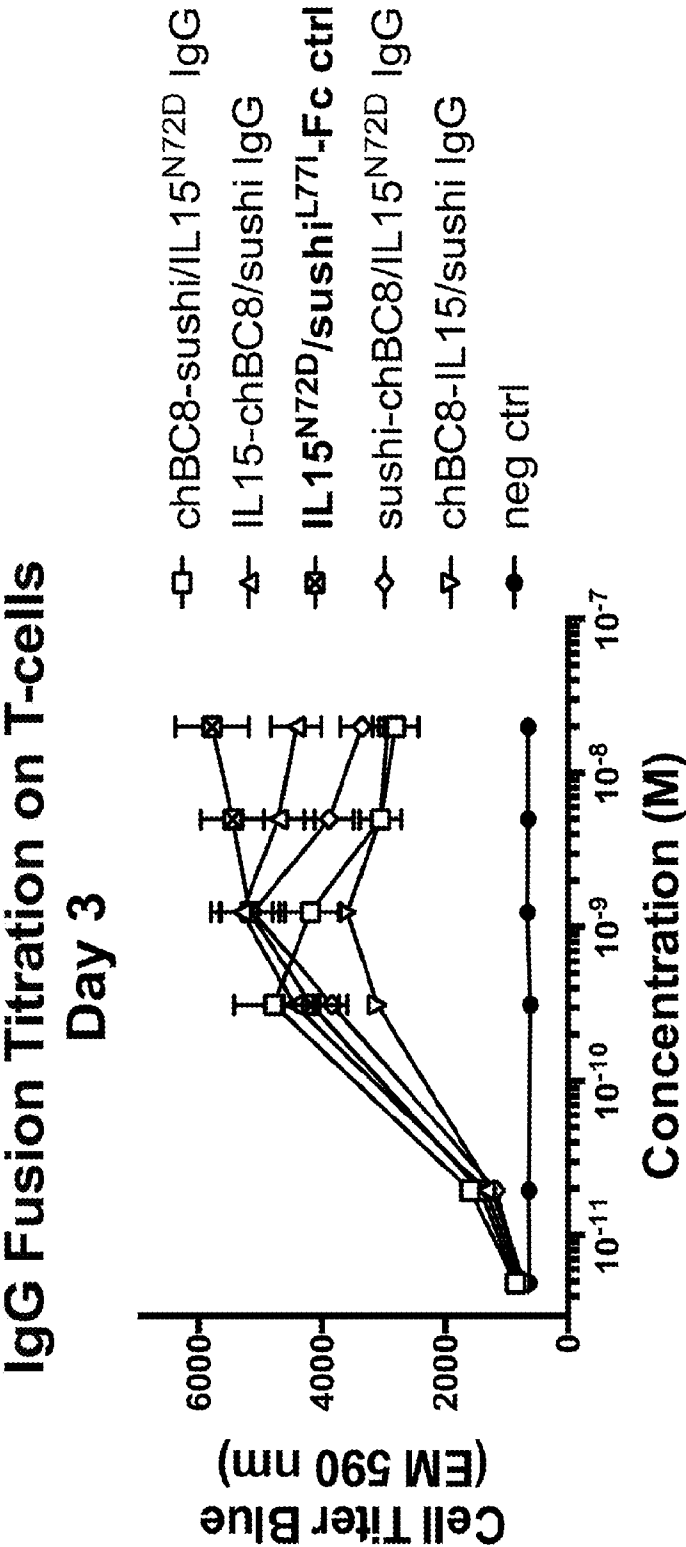


FIG. 5D

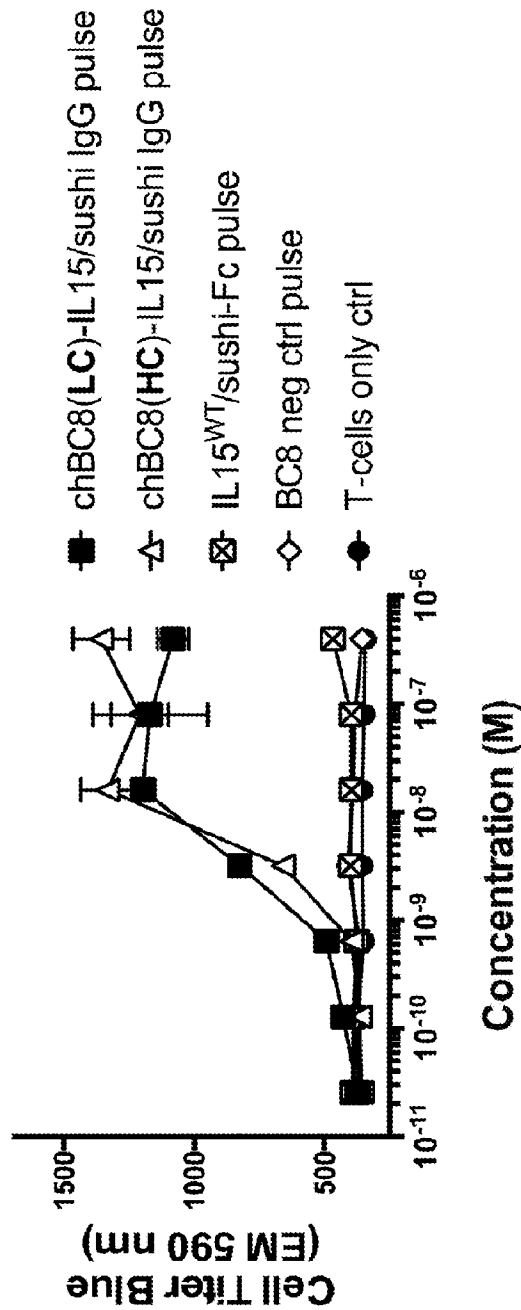
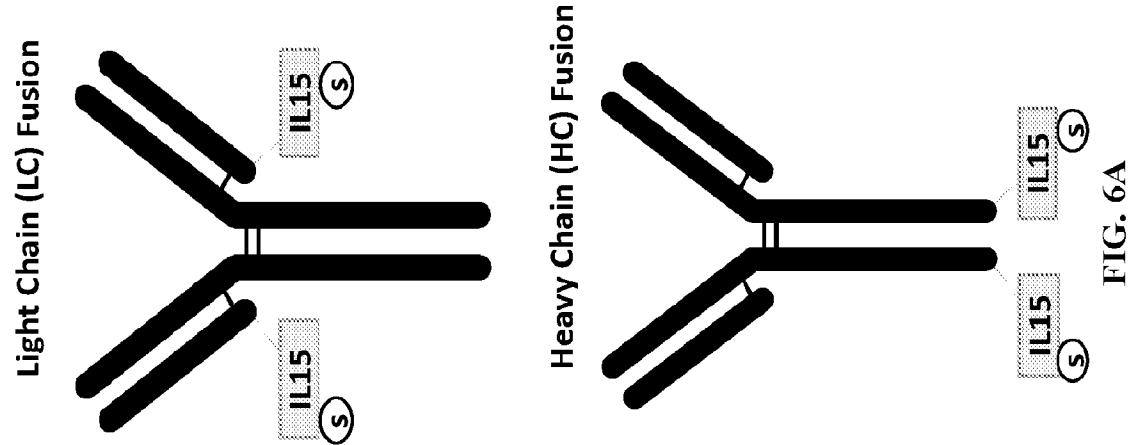


FIG. 6B

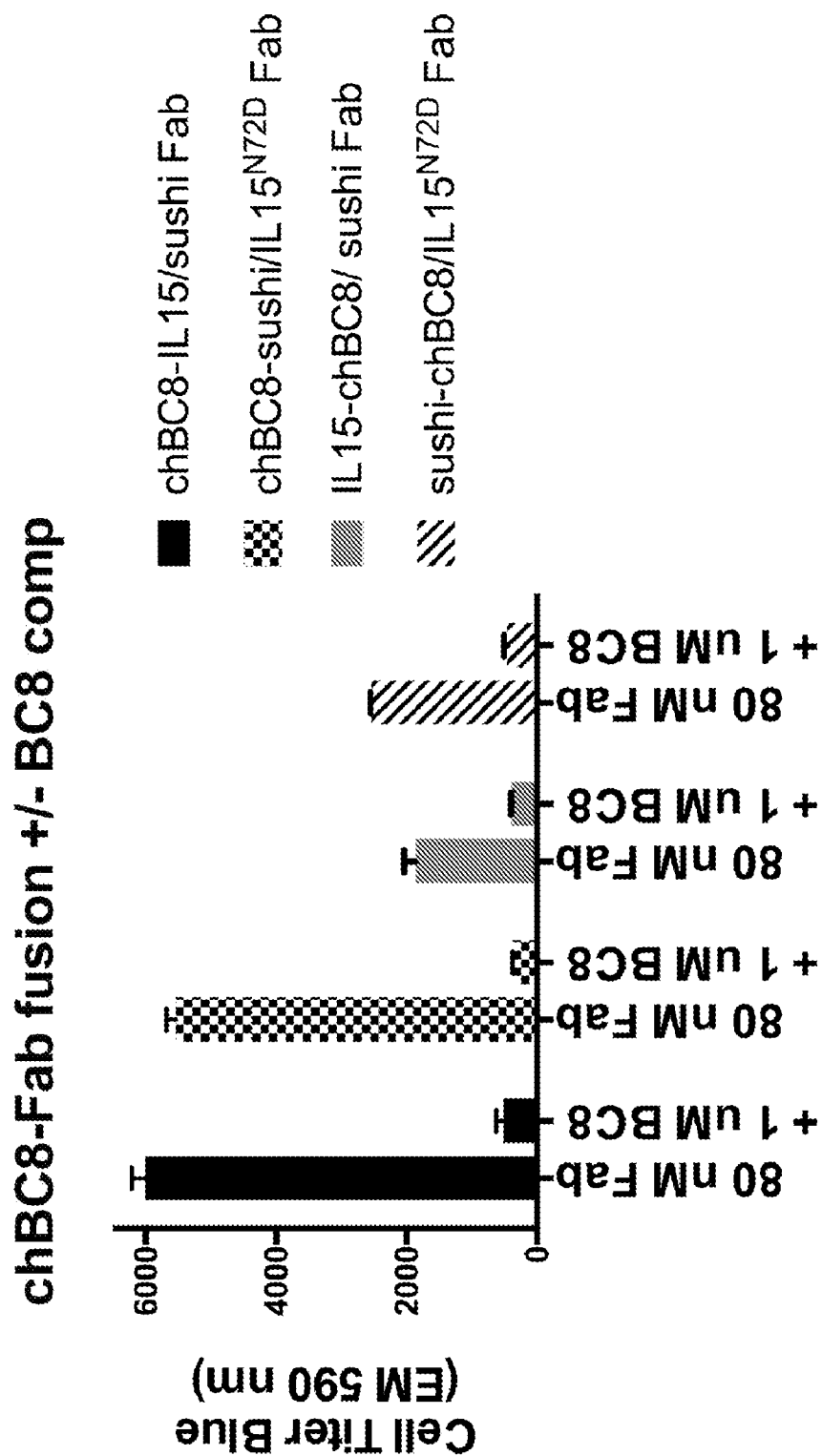


FIG. 7A

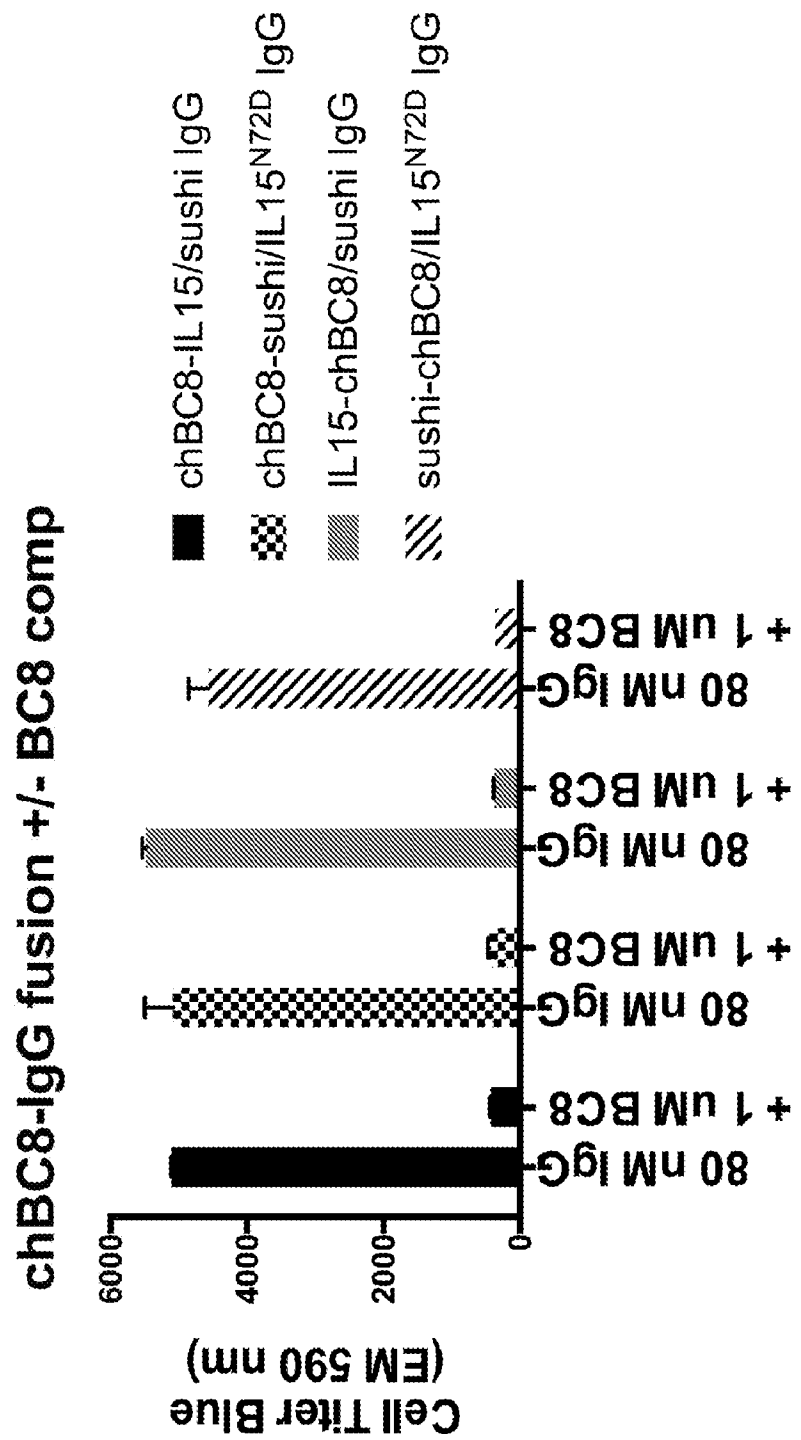


FIG. 7B

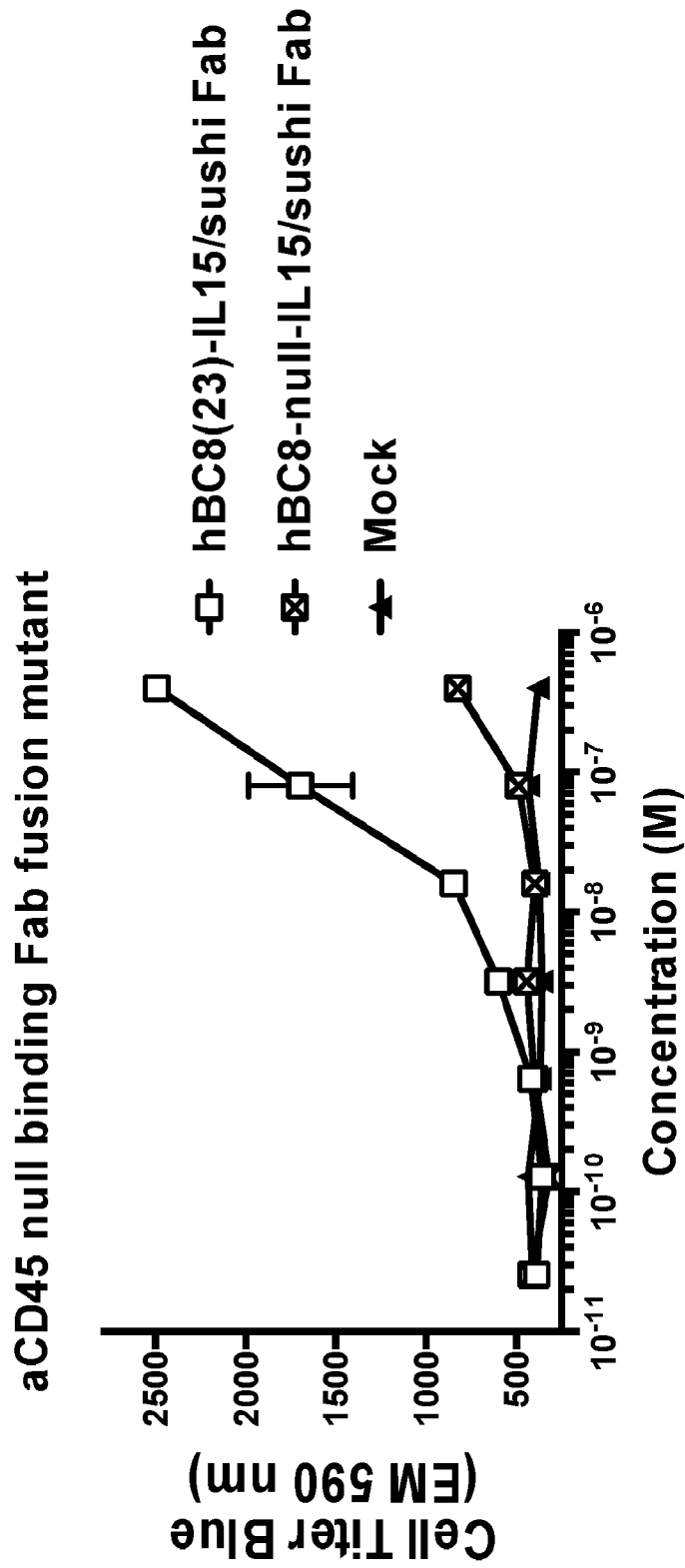


FIG. 7C

Initial cell surface levels

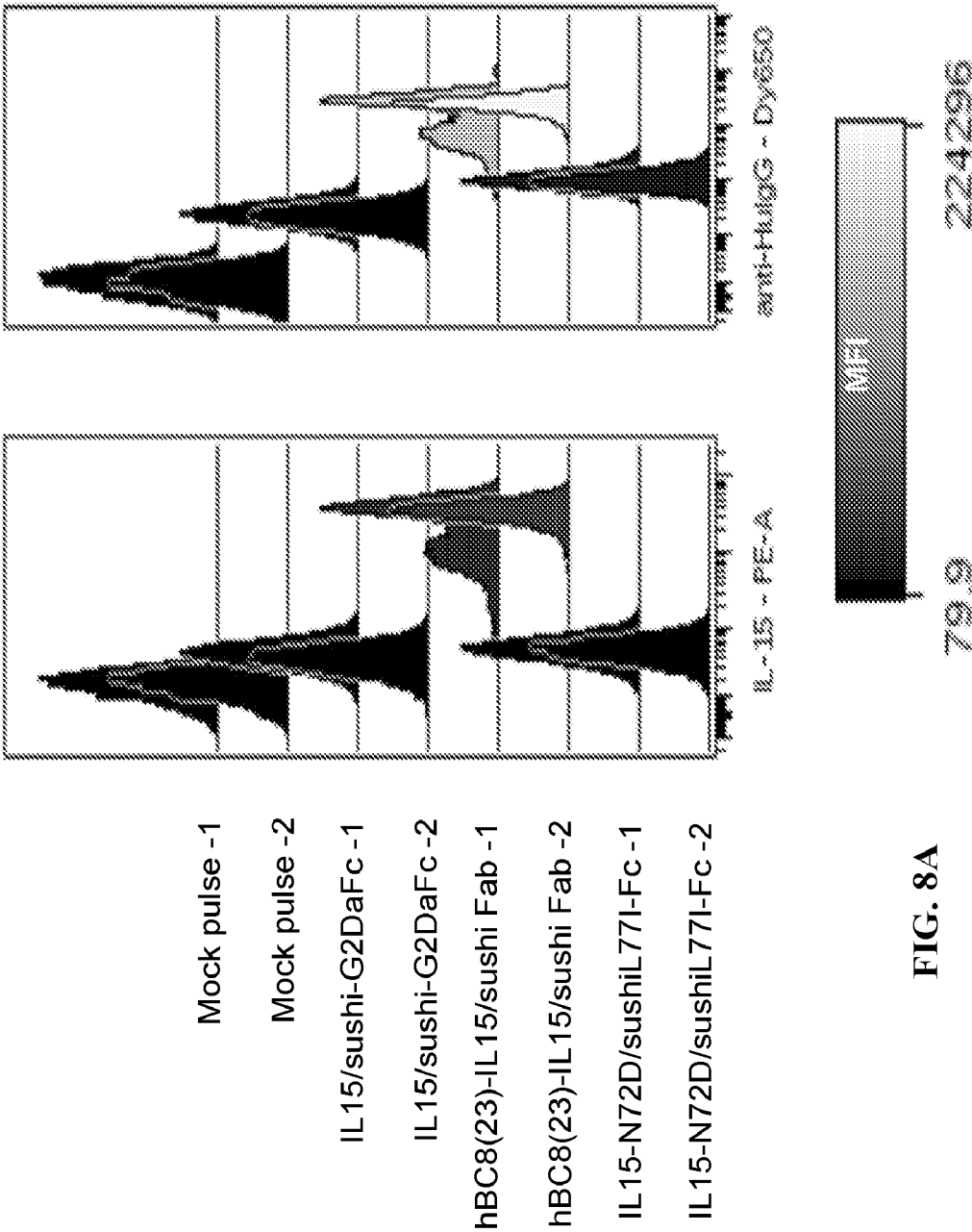


FIG. 8A

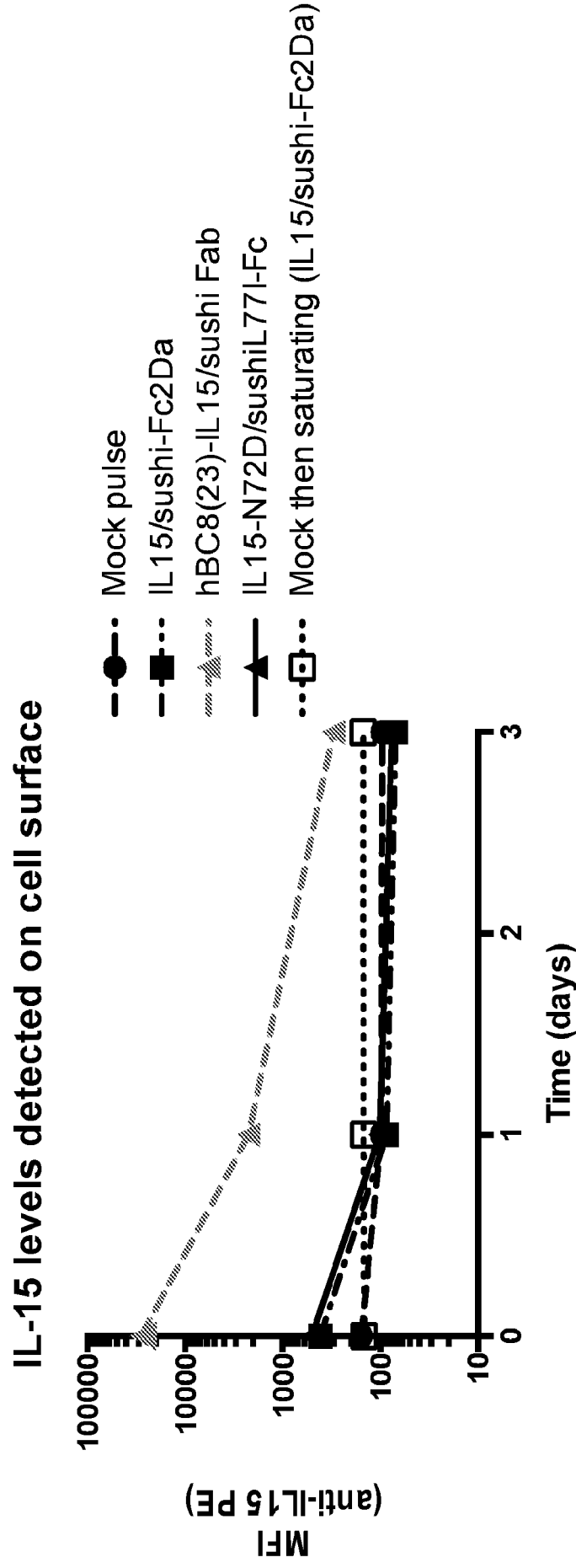


FIG. 8B

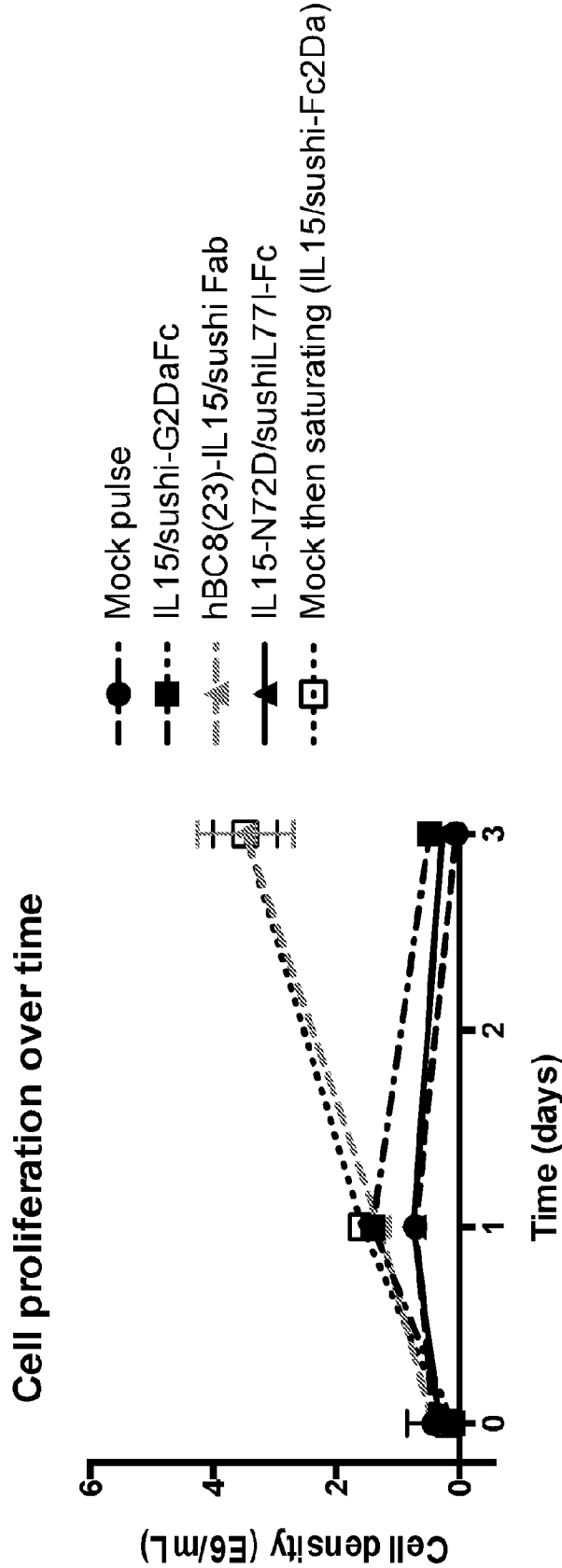


FIG. 8C

Construct	Linker Design amino acid sequence	Linker Length # of amino acids
chBC8-L1-IL15/sushi Fab	(GlyGlyGlySer) x3 GGGGSGGGGGGG GGG	15 GGGS (SEQ ID NO: 125)
chBC8-L2-IL15/sushi Fab	(GlyGlyGlySer) x2 GGGGGGGG	8 GGGS (SEQ ID NO: 124)
chBC8-L3-IL15/sushi Fab	Amino acids from extended IgG1 hinge: DKTHTSPSPAP	12 (SEQ ID NO: 38)

FIG. 9A

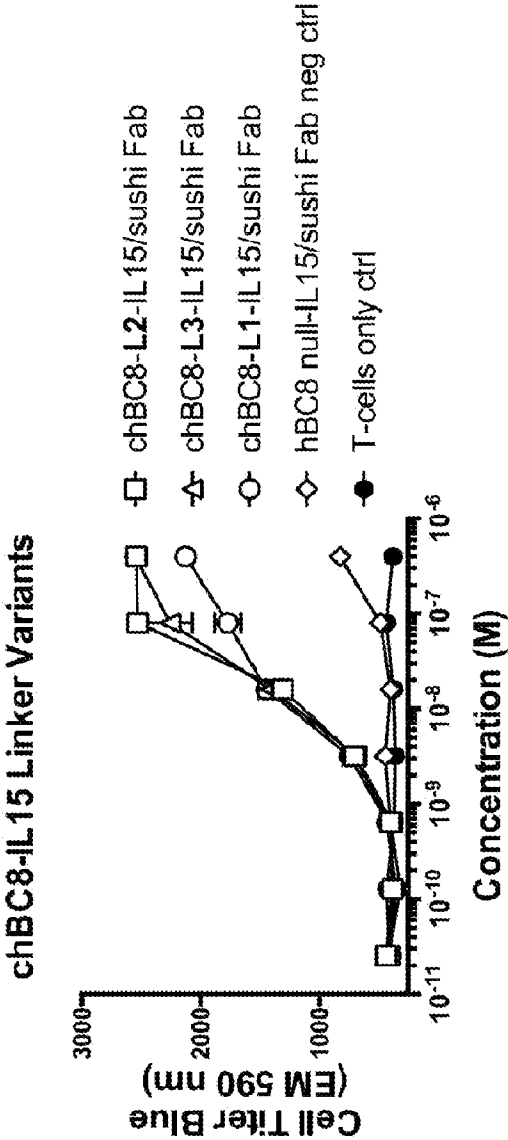


FIG. 9B

Antibody Titration on Human
CD8⁺ T-cells

CD45 mAb	T-Cell binding Apparent K _d (nM)
Humanized BC8(23)	7.8
Chimeric BC8	6.68
Chimeric 4B2	0.44
Chimeric 9.4	0.84
Human BC8 null binding mutant	>500

FIG. 10A

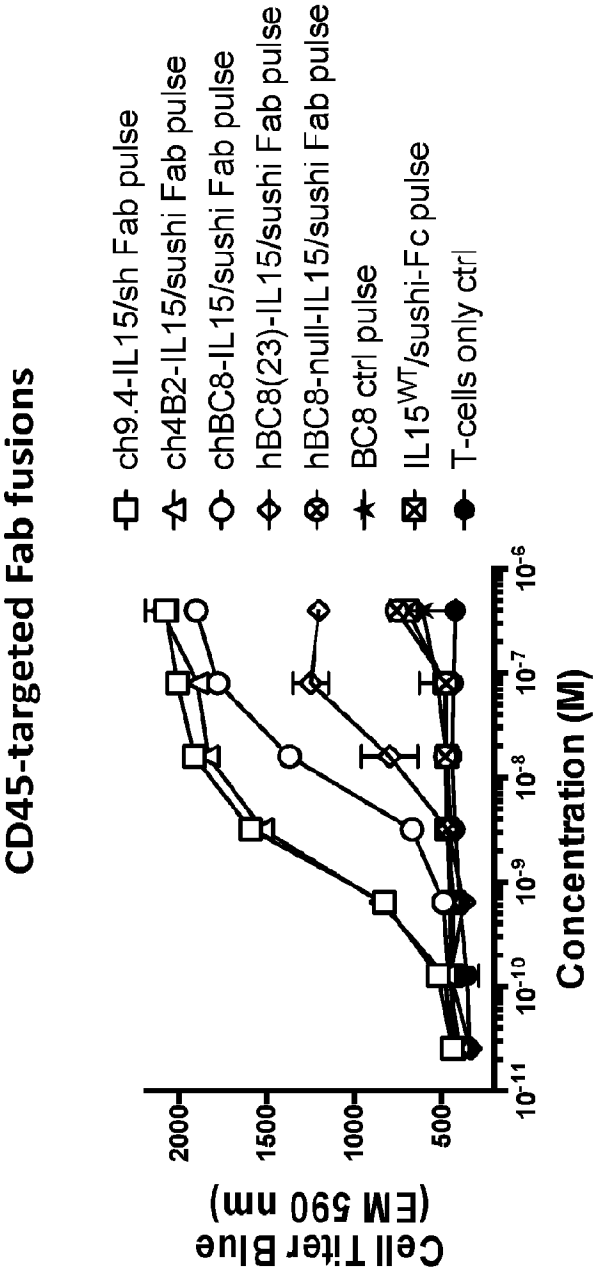
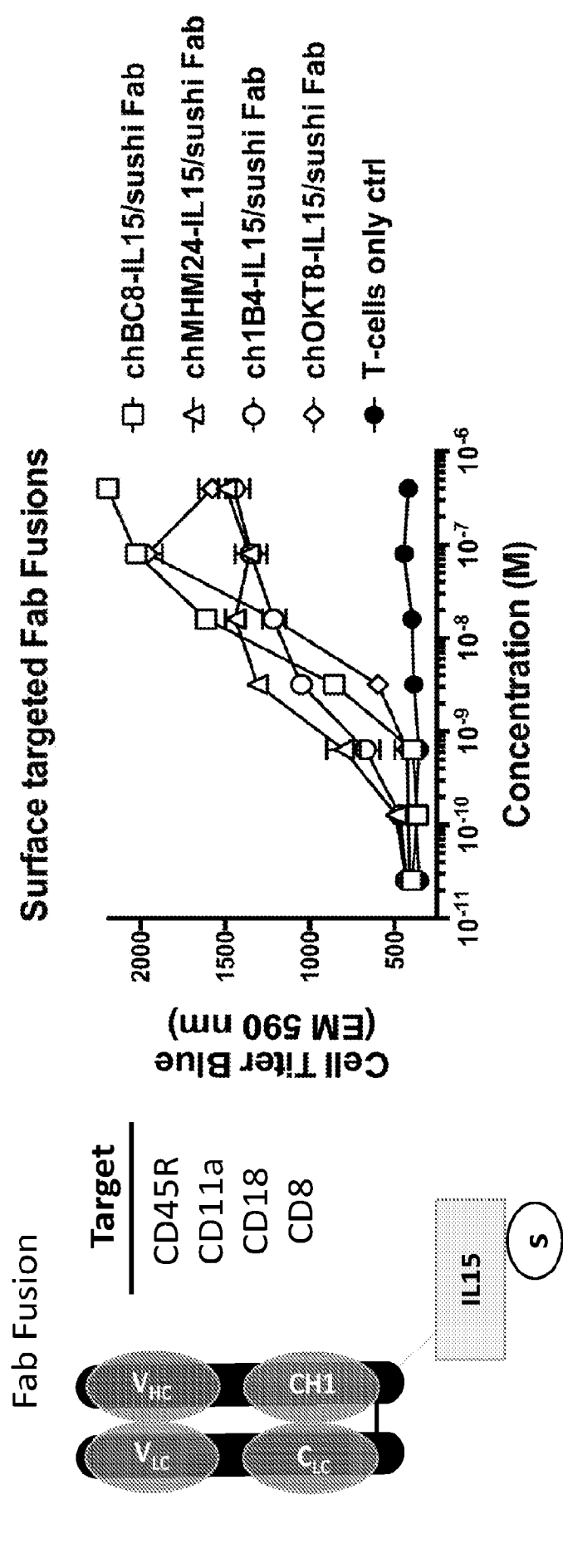


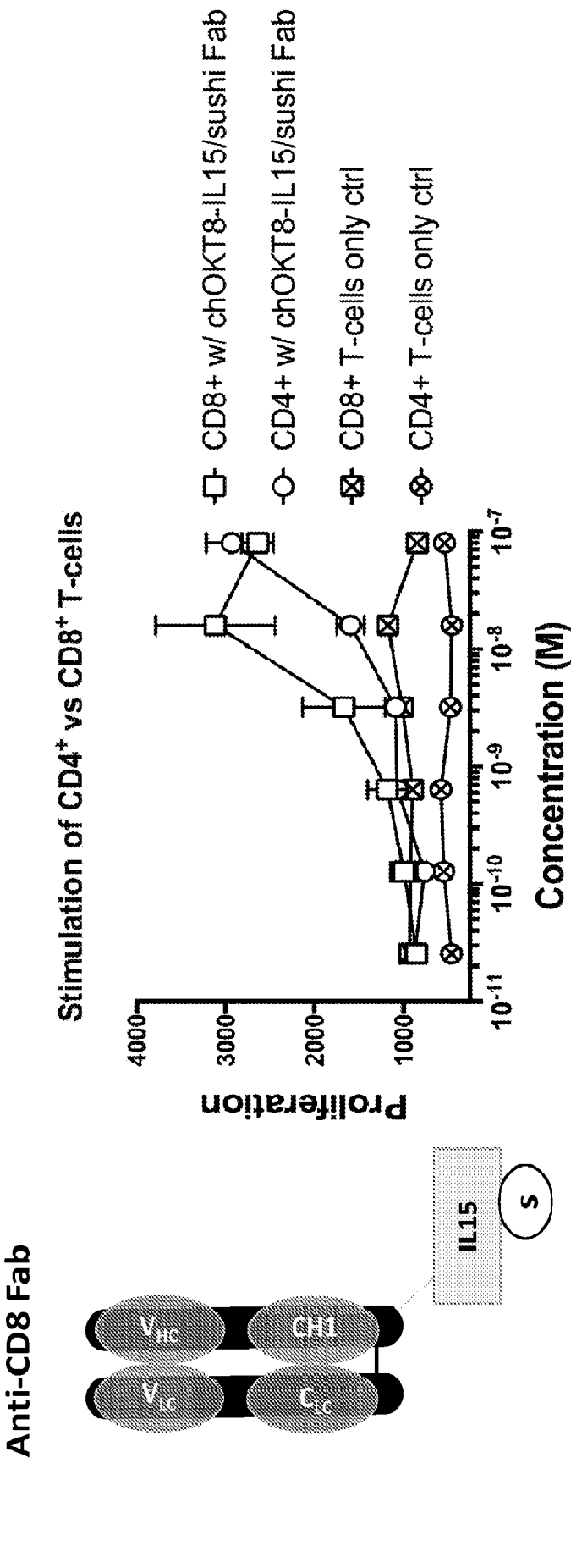
FIG. 10B



(S) = IL15Ralpha sushi domain

FIG. 11A

FIG. 11B



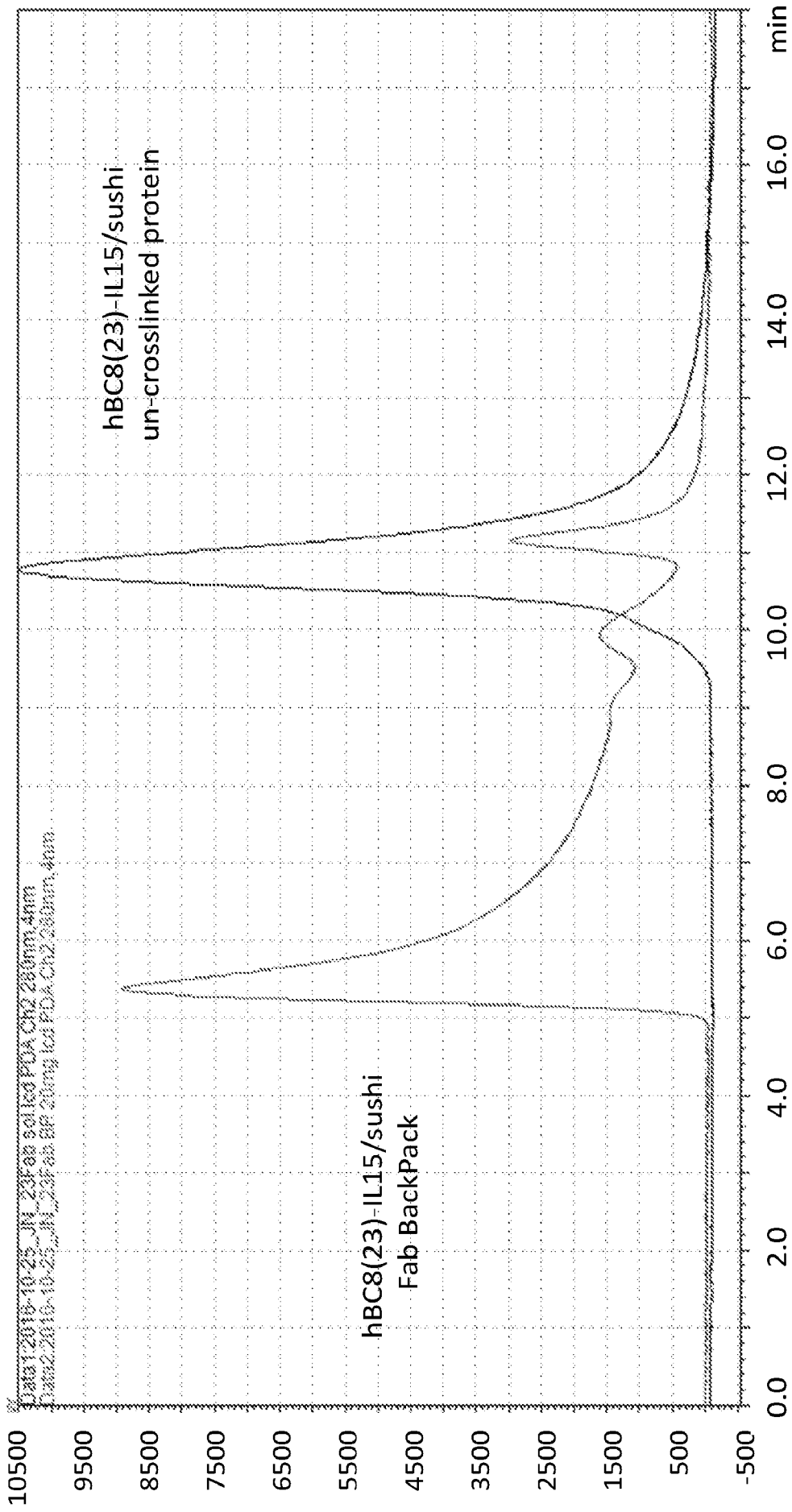


FIG. 13A

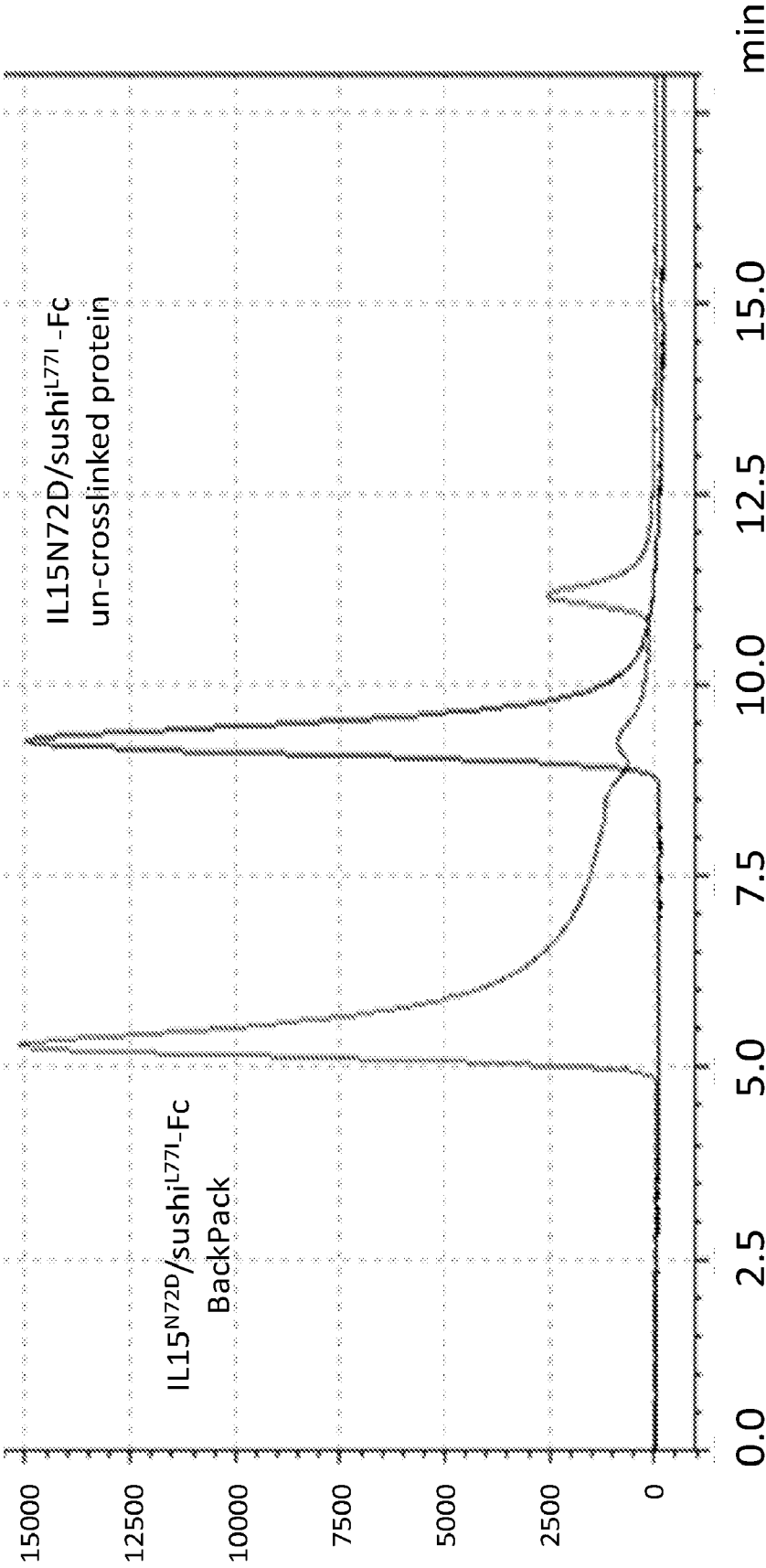


FIG. 13B

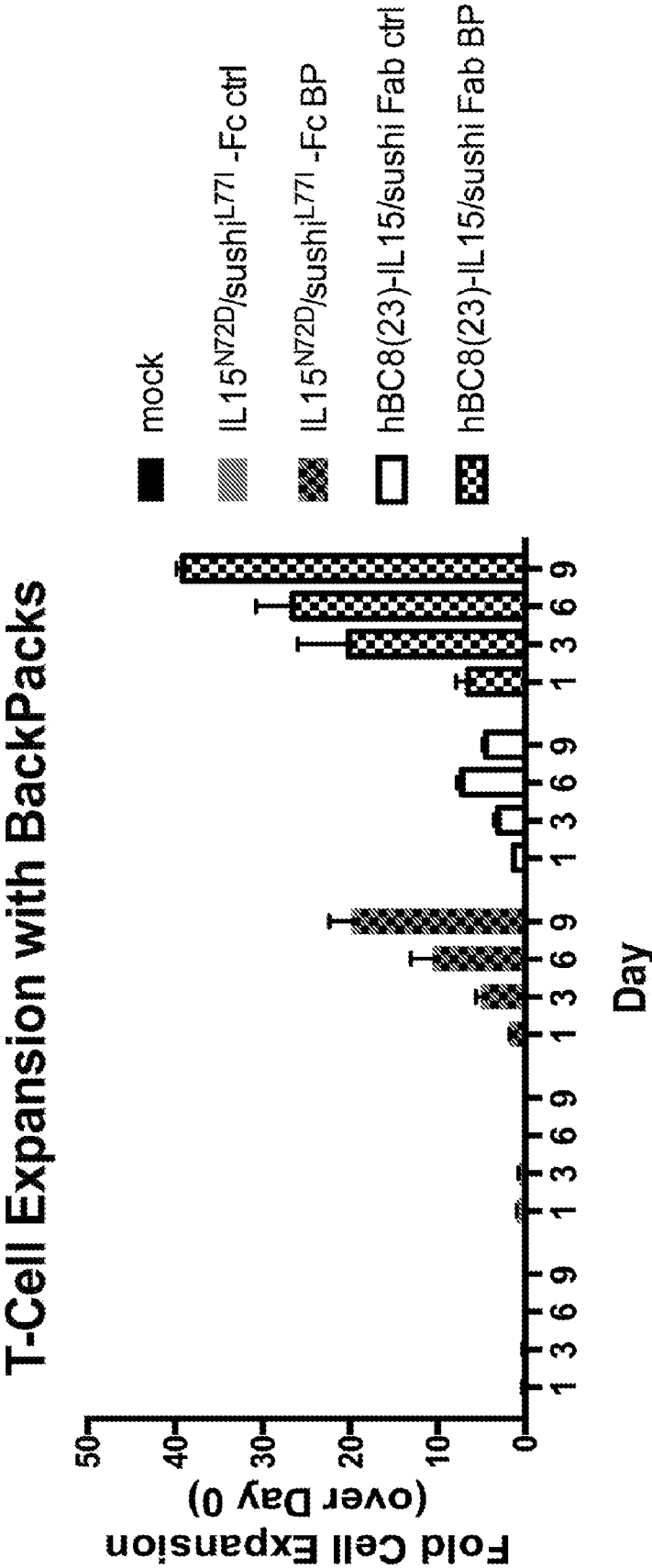


FIG. 13C

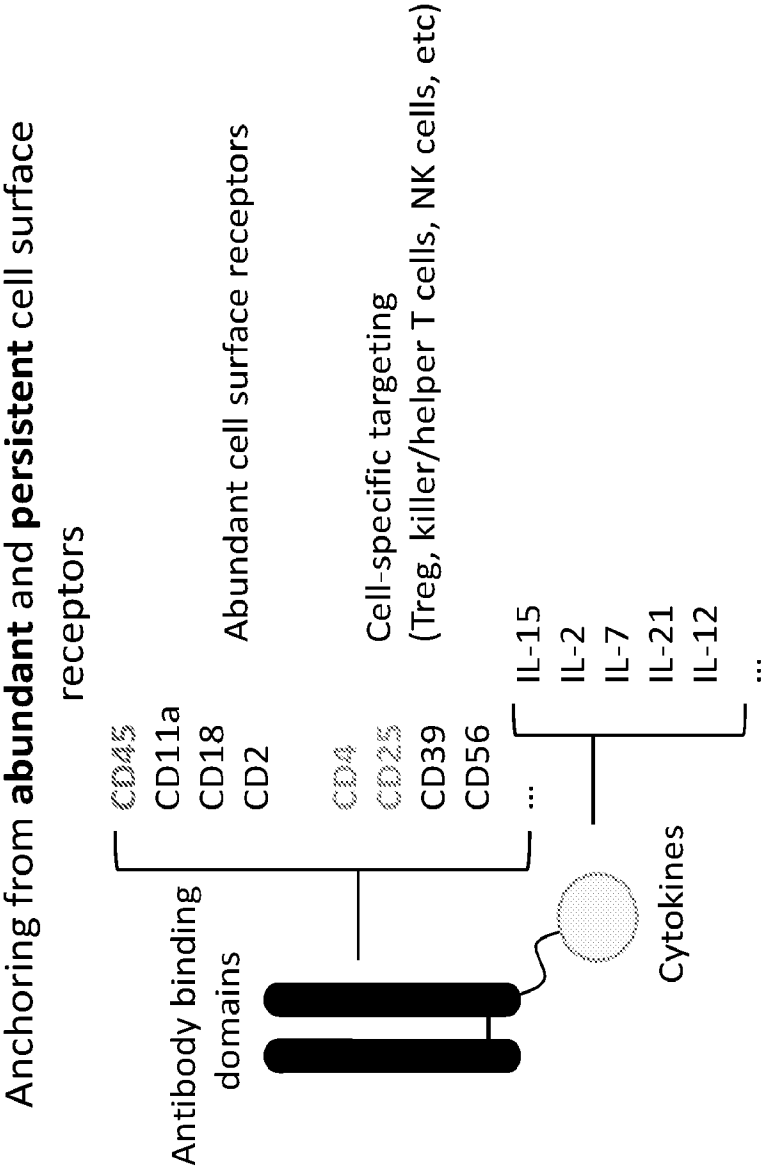


FIG. 14

Example gating of CD4 and CD8 populations

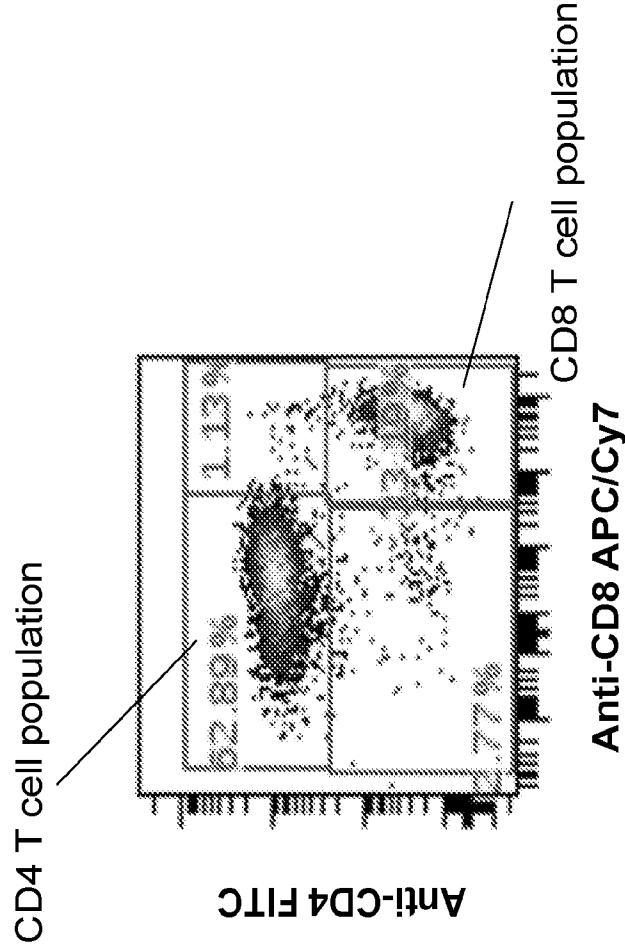


FIG. 15A

Cell-specific targeting

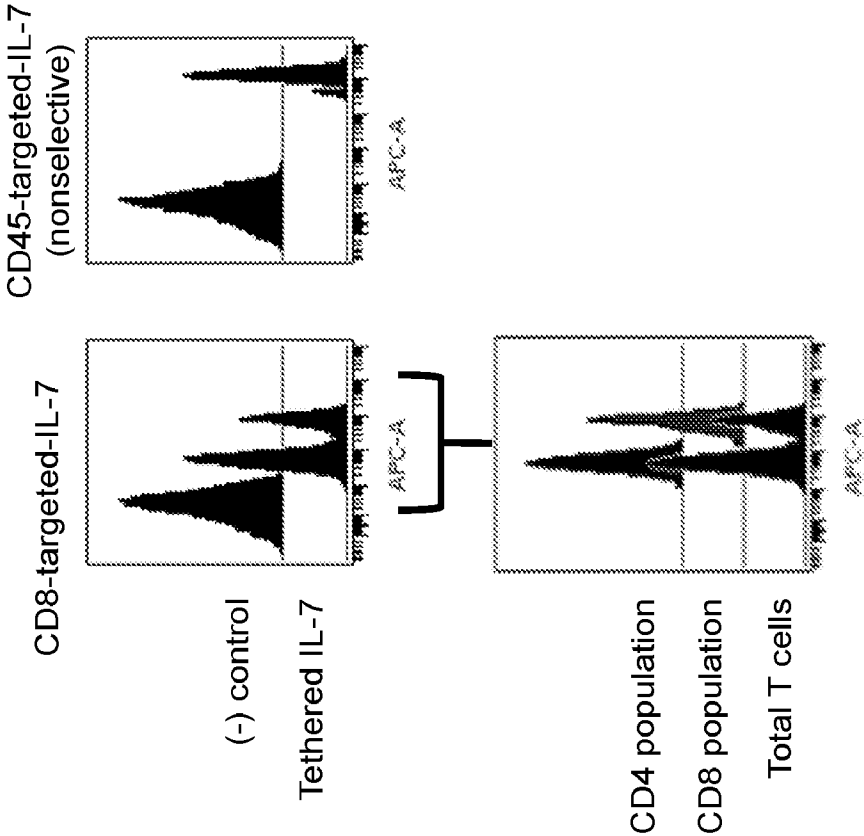
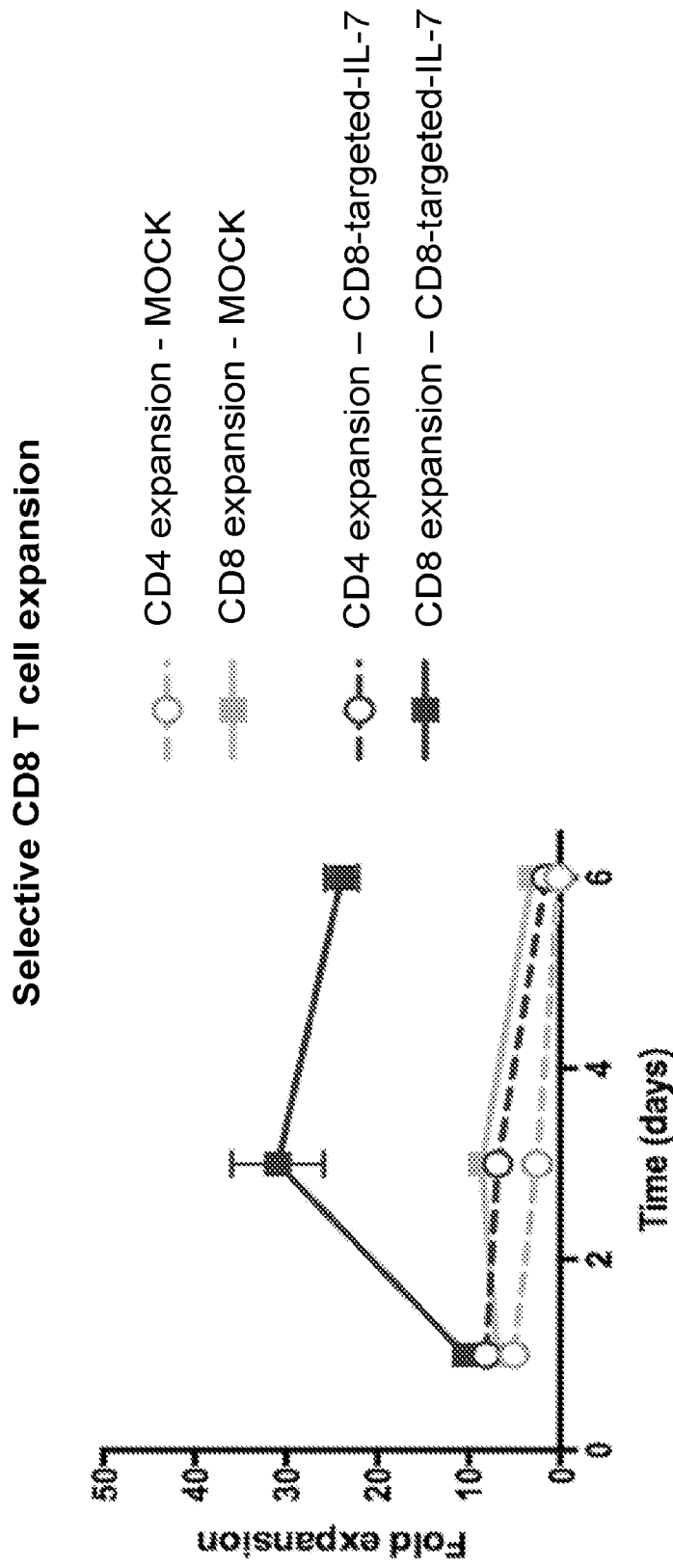


FIG. 15B



Expansion of CD4 vs CD8 T cells in cultures of activated total CD3 human T cells by pulse incubation with CD8-targeted IL-7

FIG. 15C

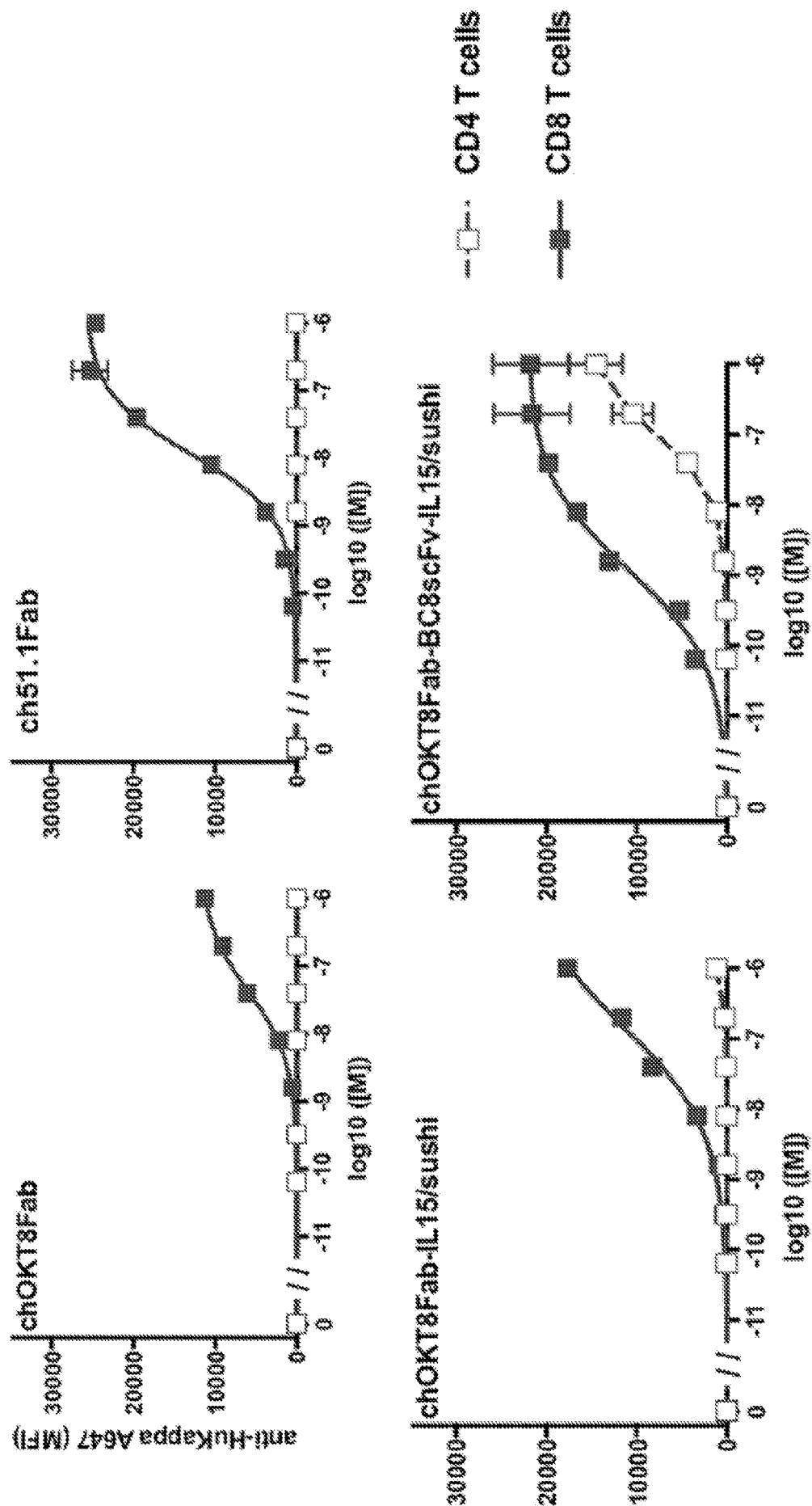


FIG. 15D

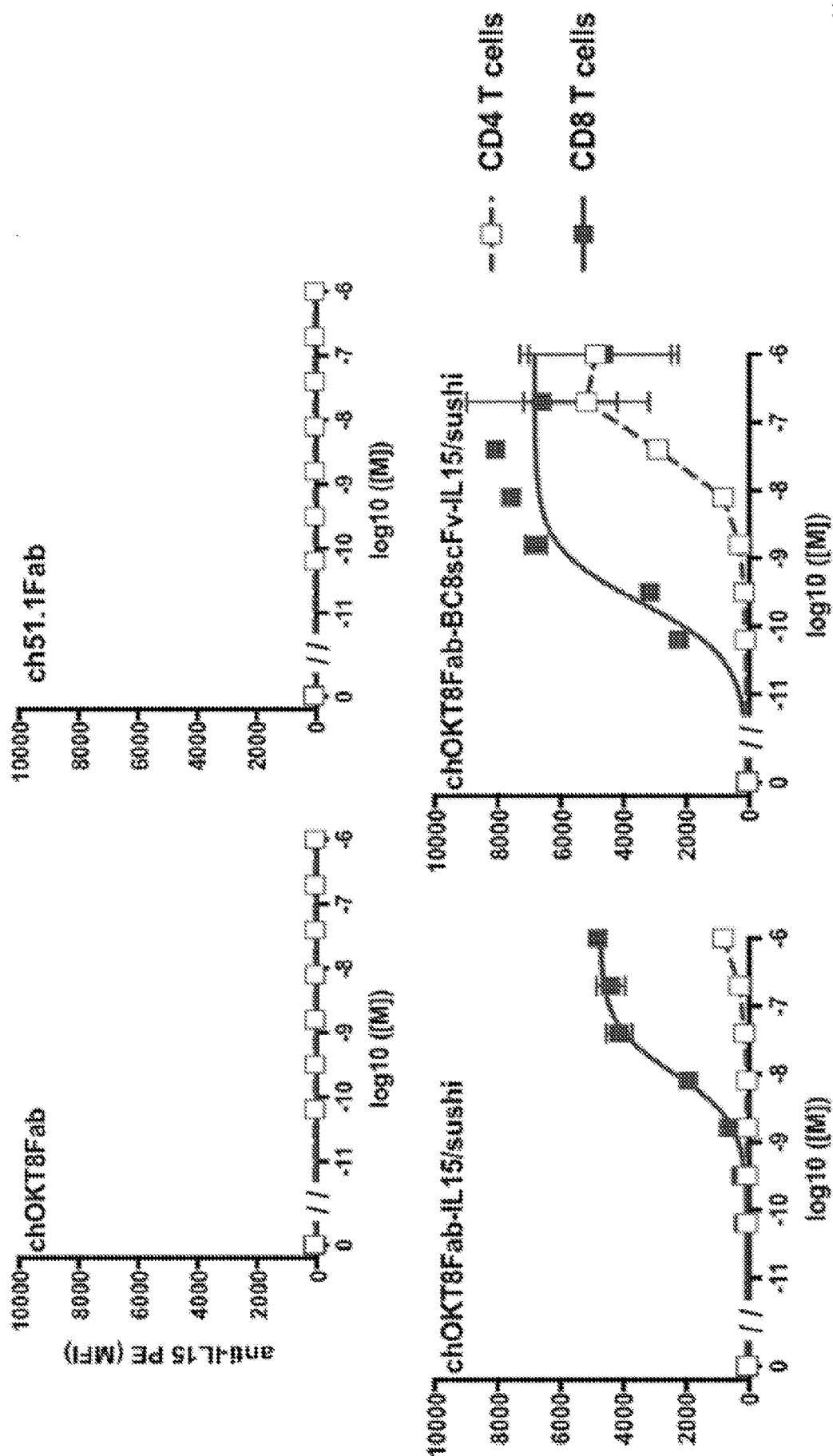


FIG. 15E

Comparison of CD8 T cell binding

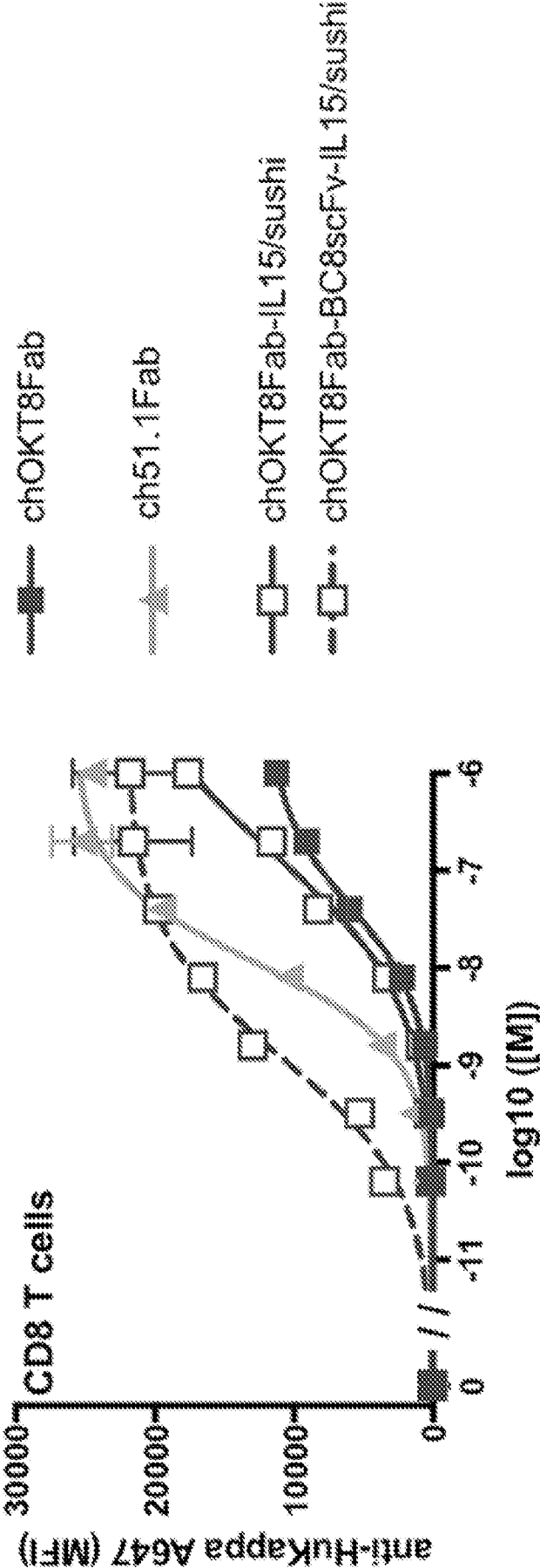


FIG. 15F

T cell binding by BC8 scFv monomer or dimer

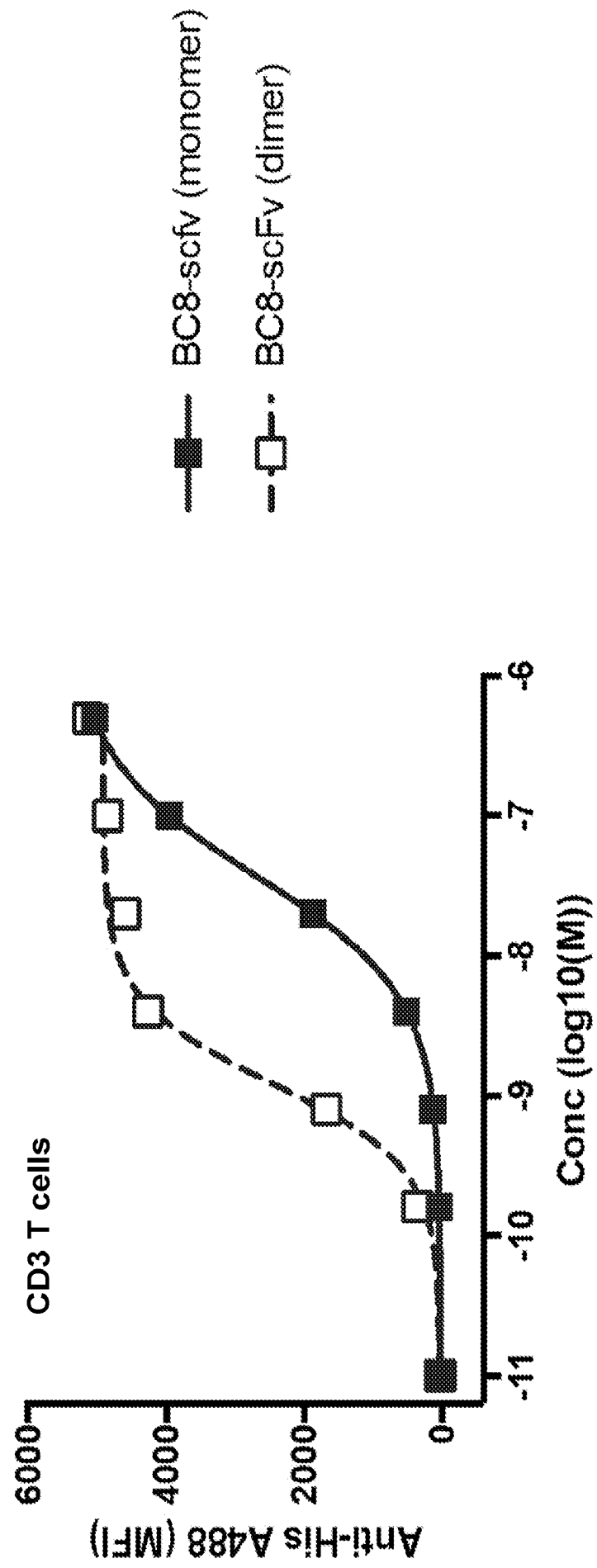


FIG. 15G

Titration of IL-15 variants on human T cells

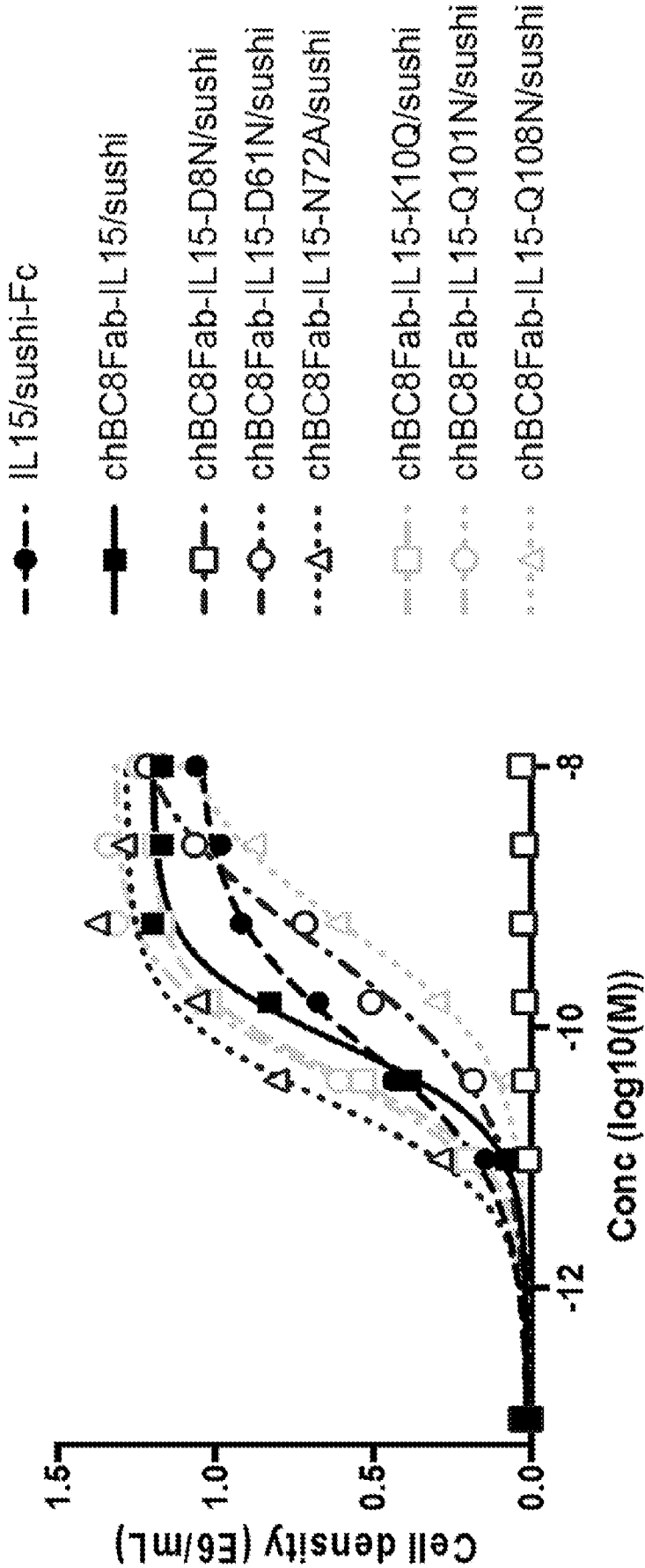


FIG. 16A

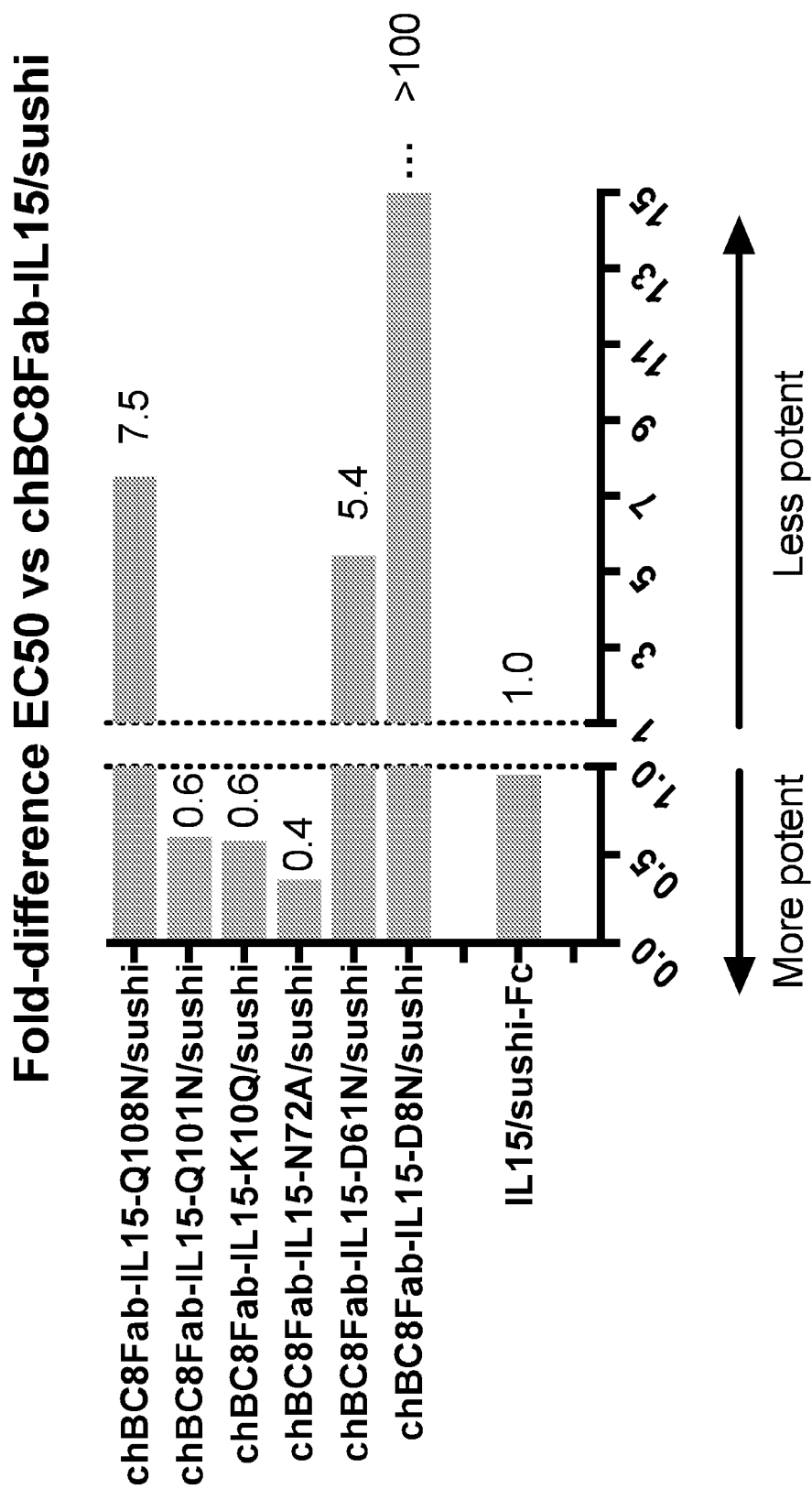


FIG. 16B

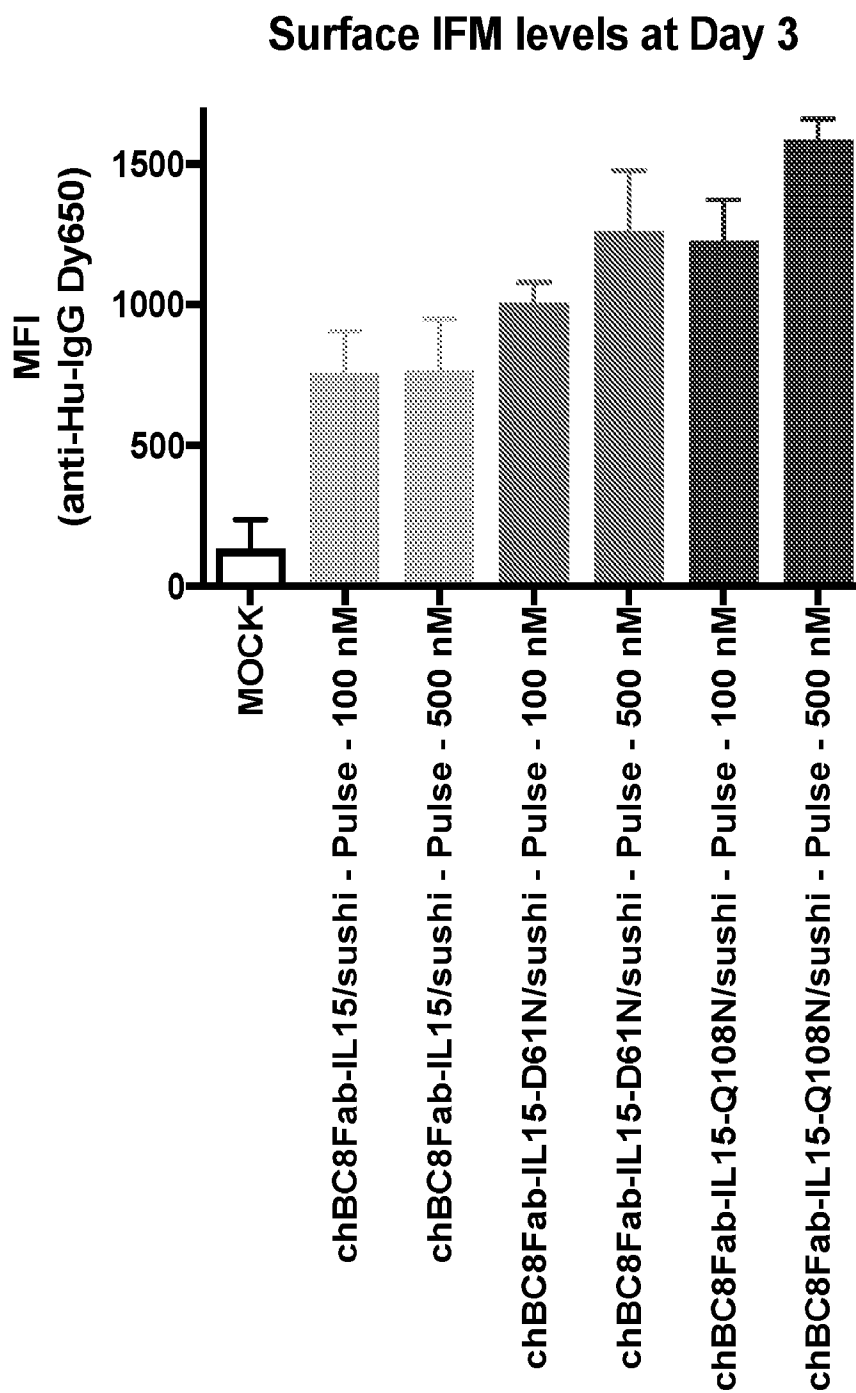


FIG. 16C

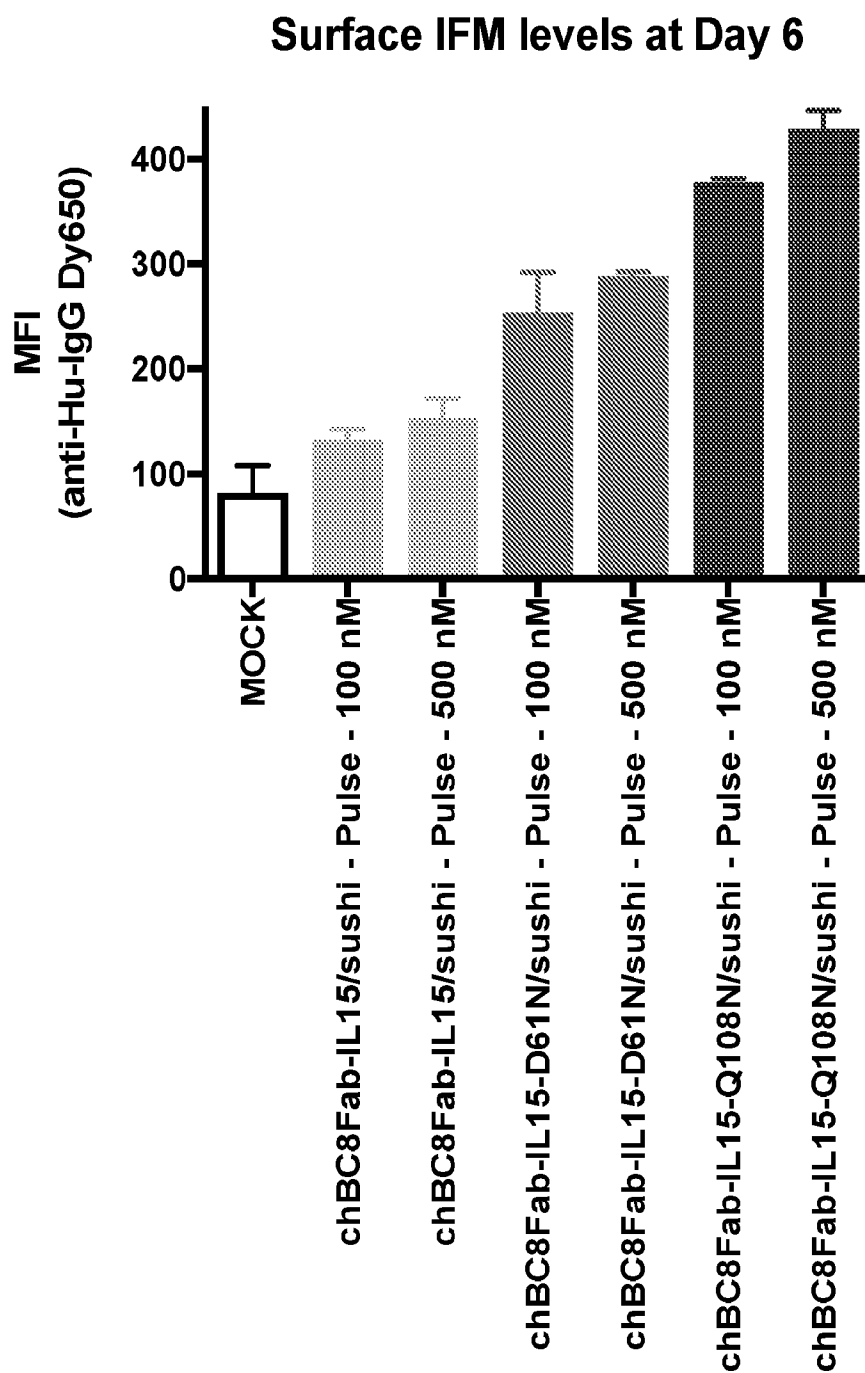


FIG. 16C
(cont.)

CD8-targeted IFM binding to CD4 T cells

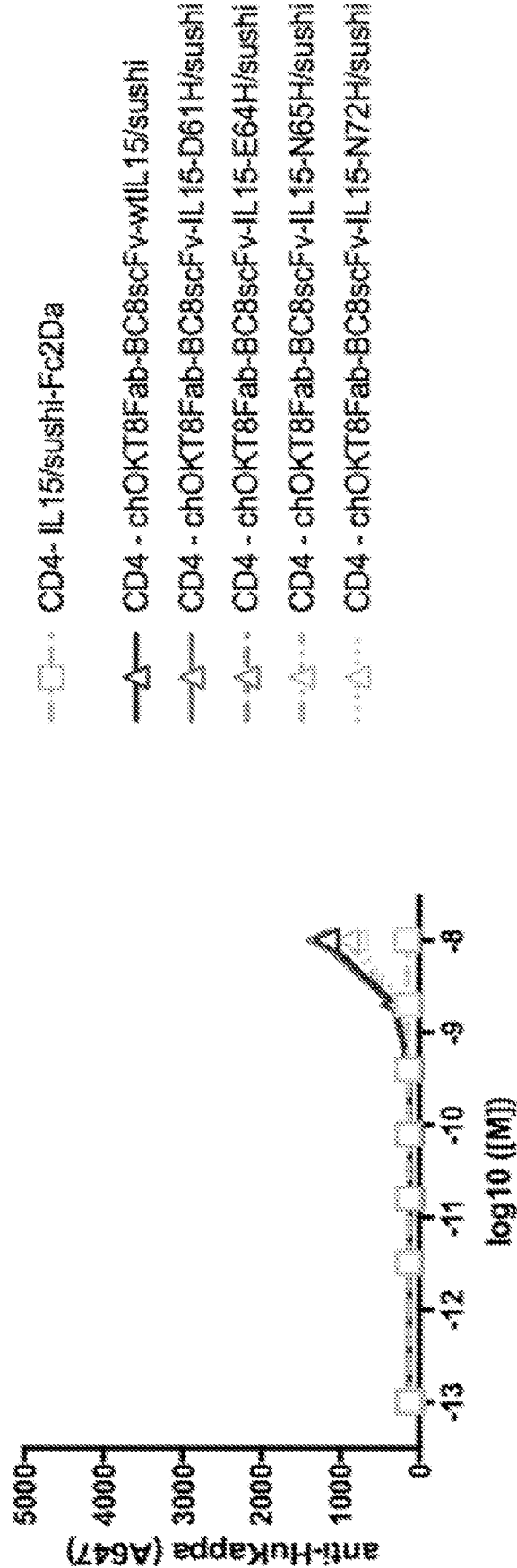


FIG. 16D

CD8-targeted IFM binding to CD8 T cells

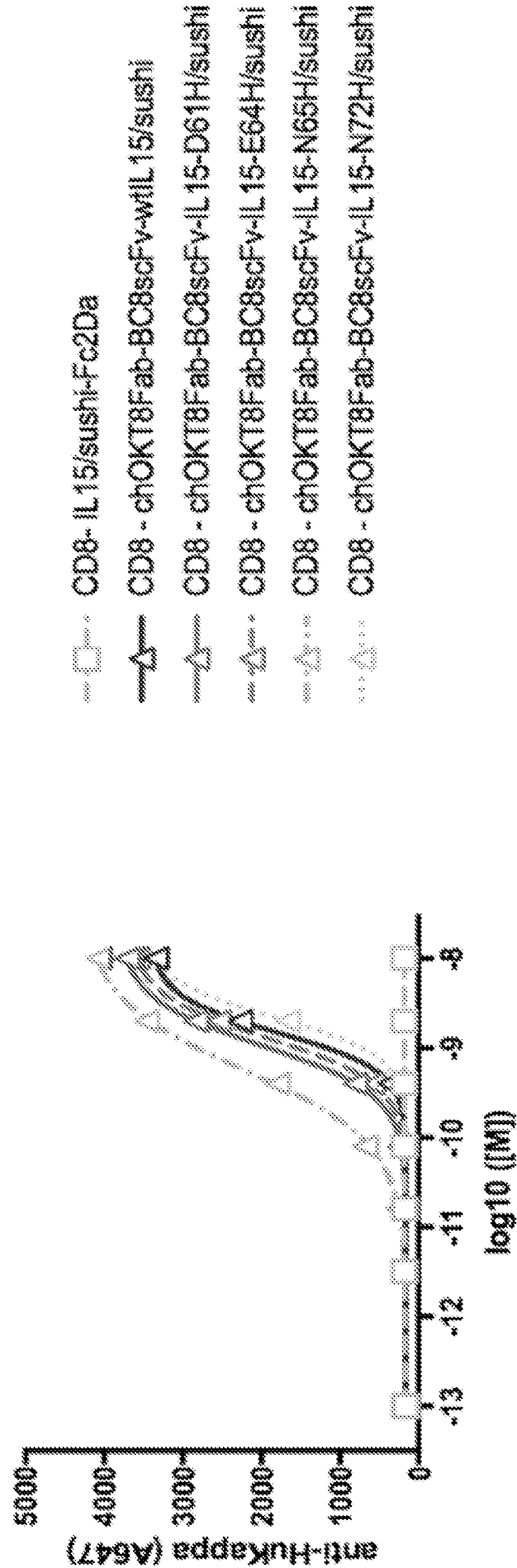


FIG. 16D (cont.)

Cell expansion by IL-15 point mutants

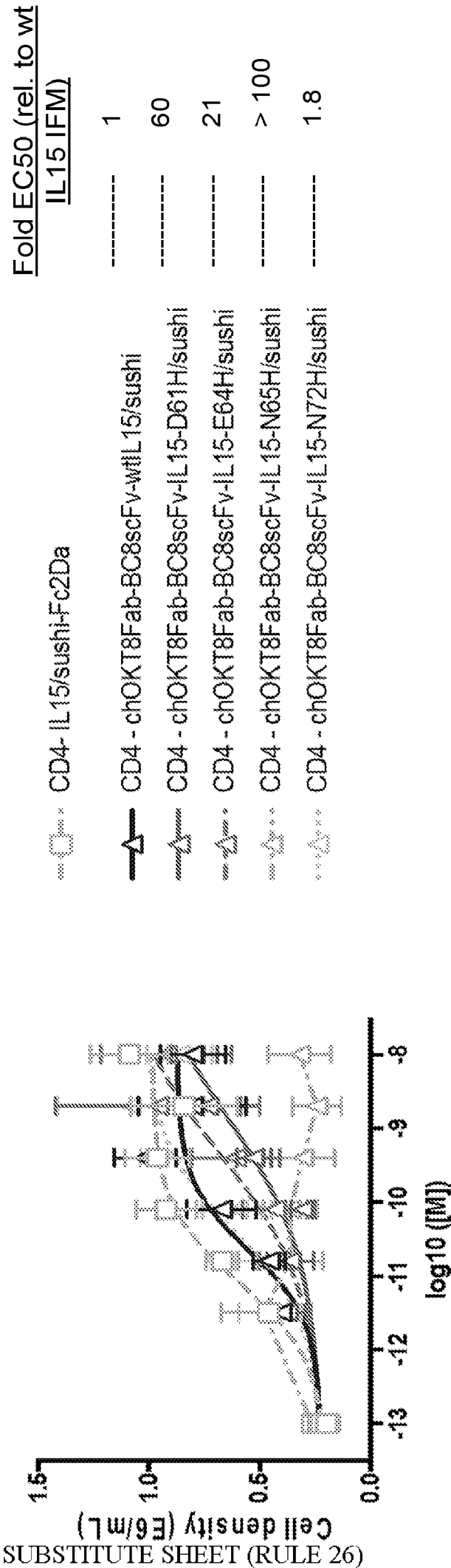


FIG. 16E

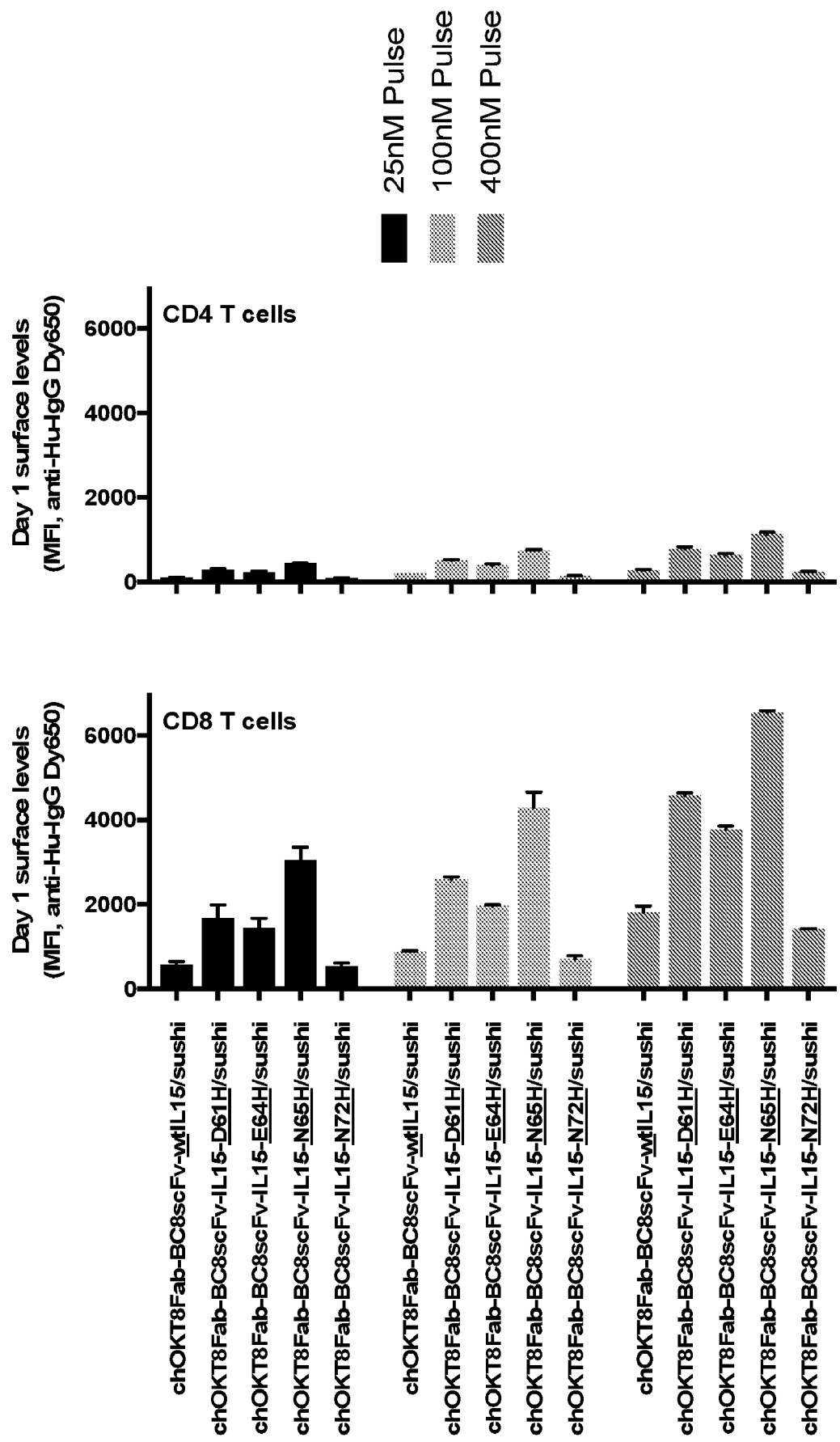


FIG. 17A

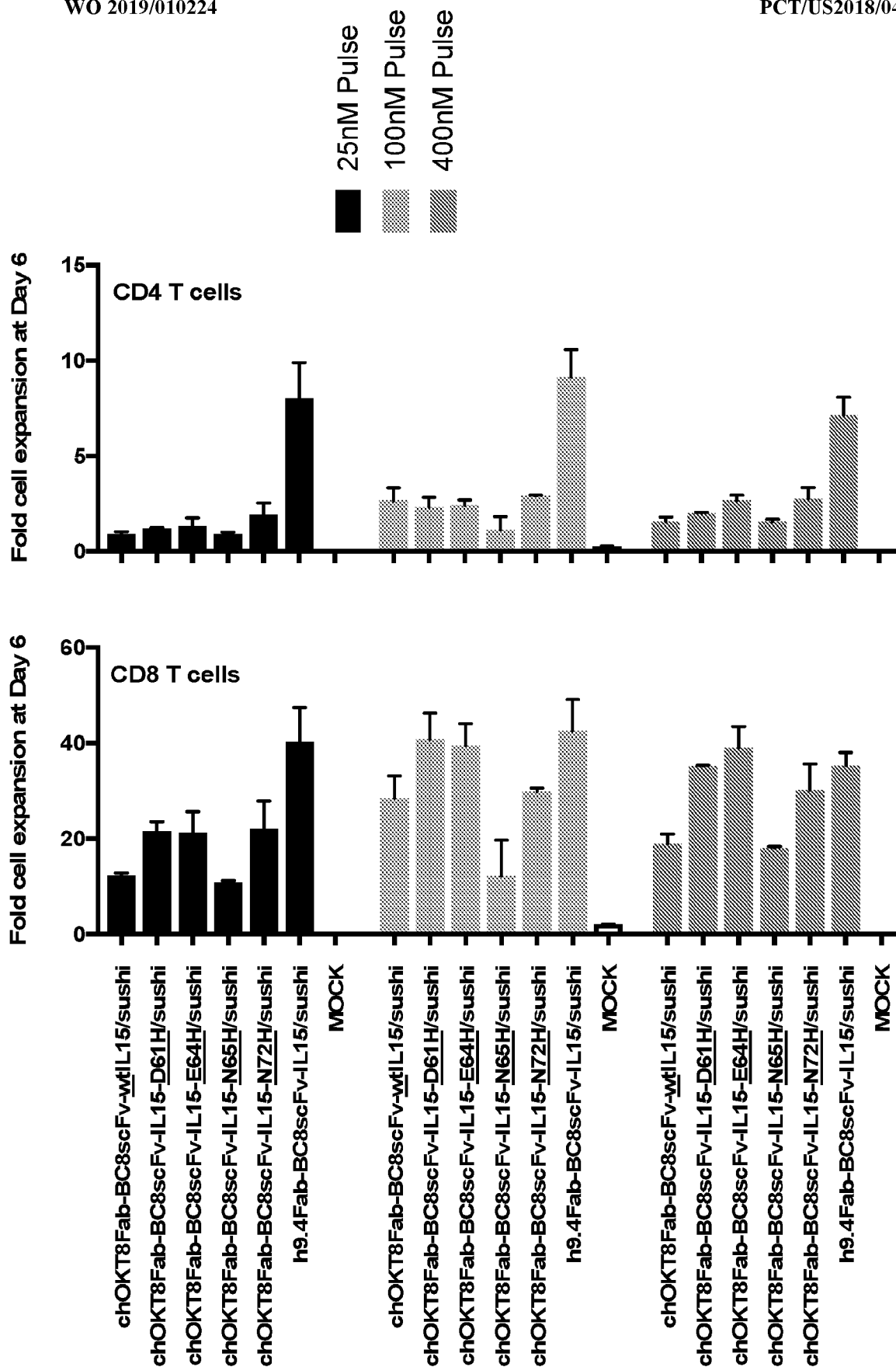


FIG. 17B

Improved CD8 T cell expansion

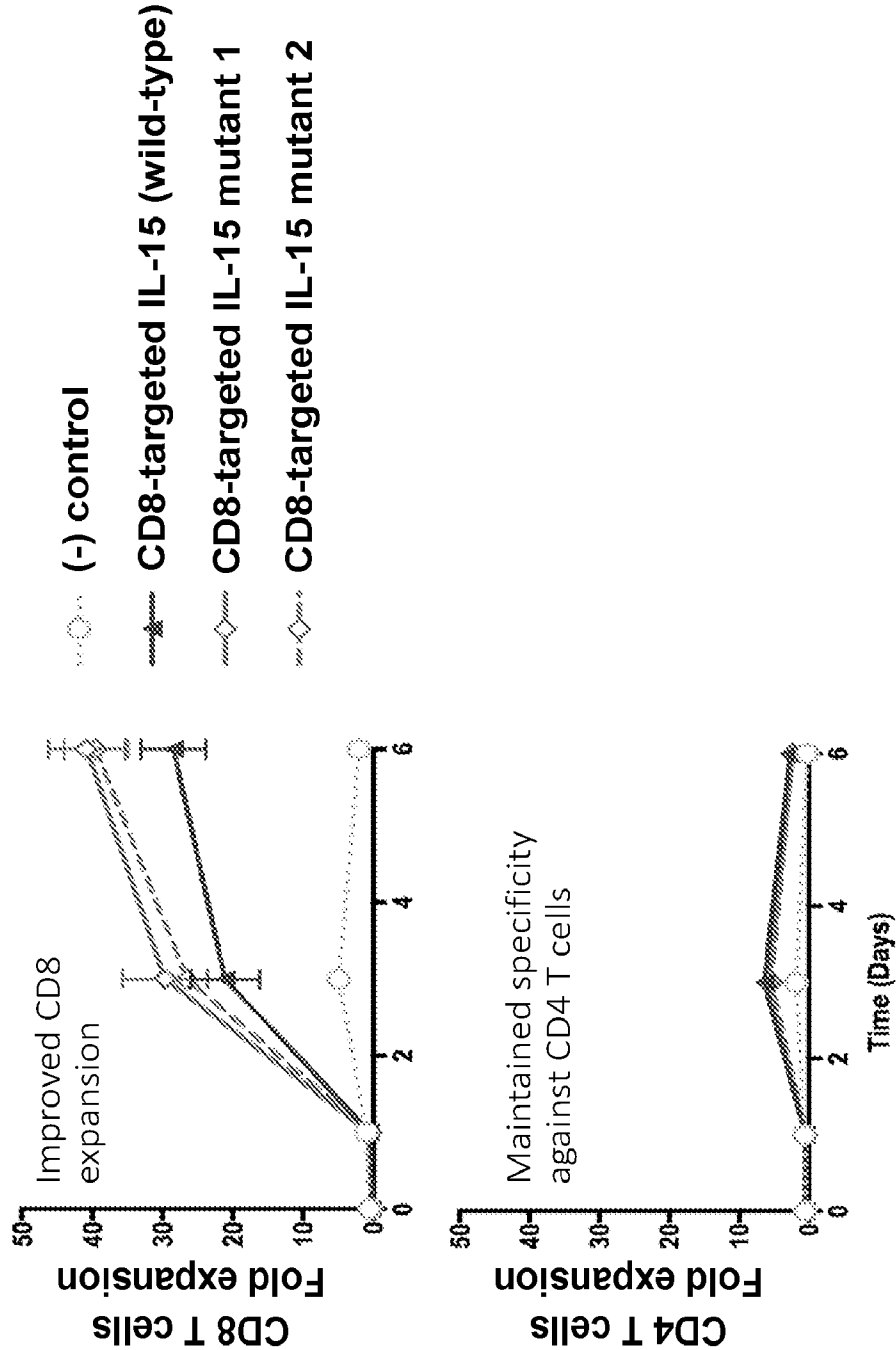


FIG. 17C

Expansion of CD4 vs CD8 T cells in cultures of activated total CD3 human T cells by pulse incubation with CD8-targeted IL-15 variants

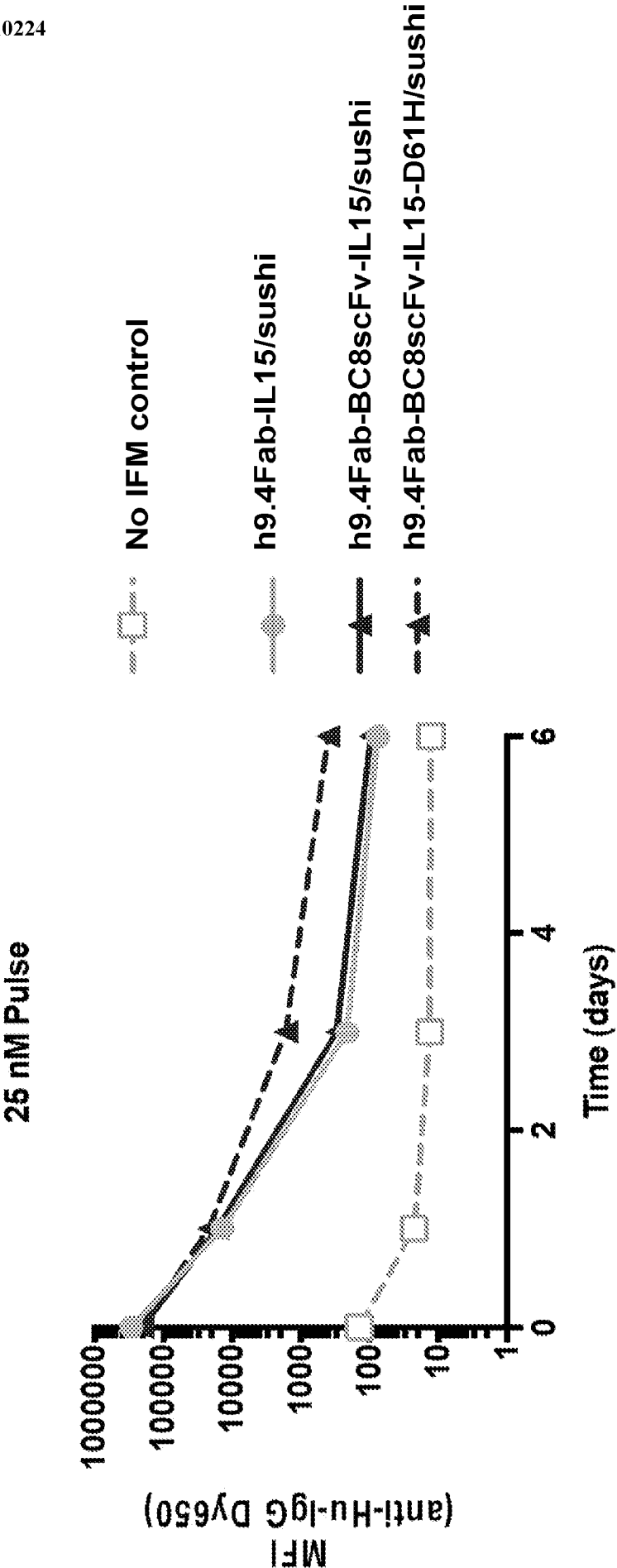


FIG. 17D

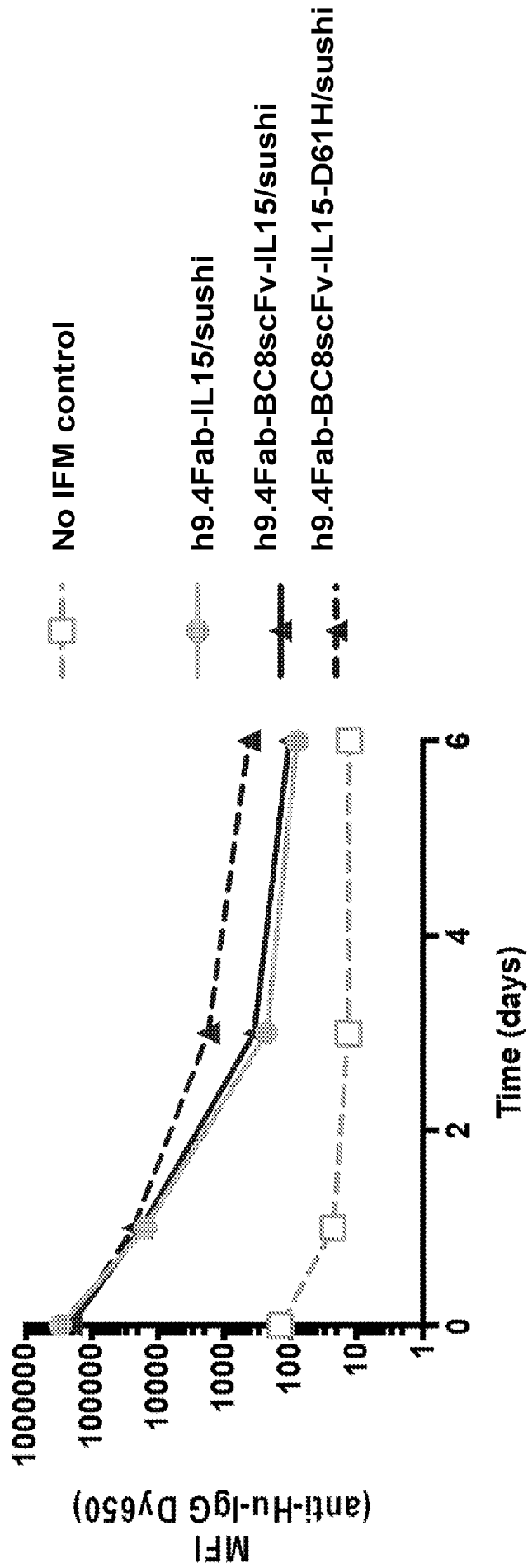


FIG. 17D (cont.)

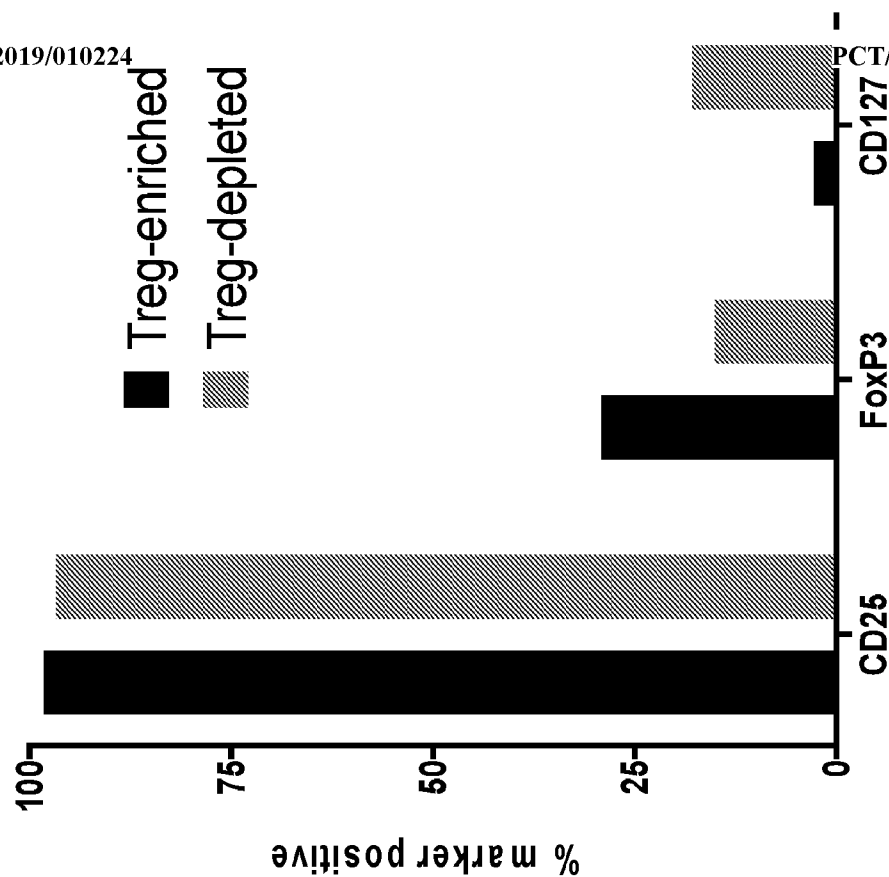
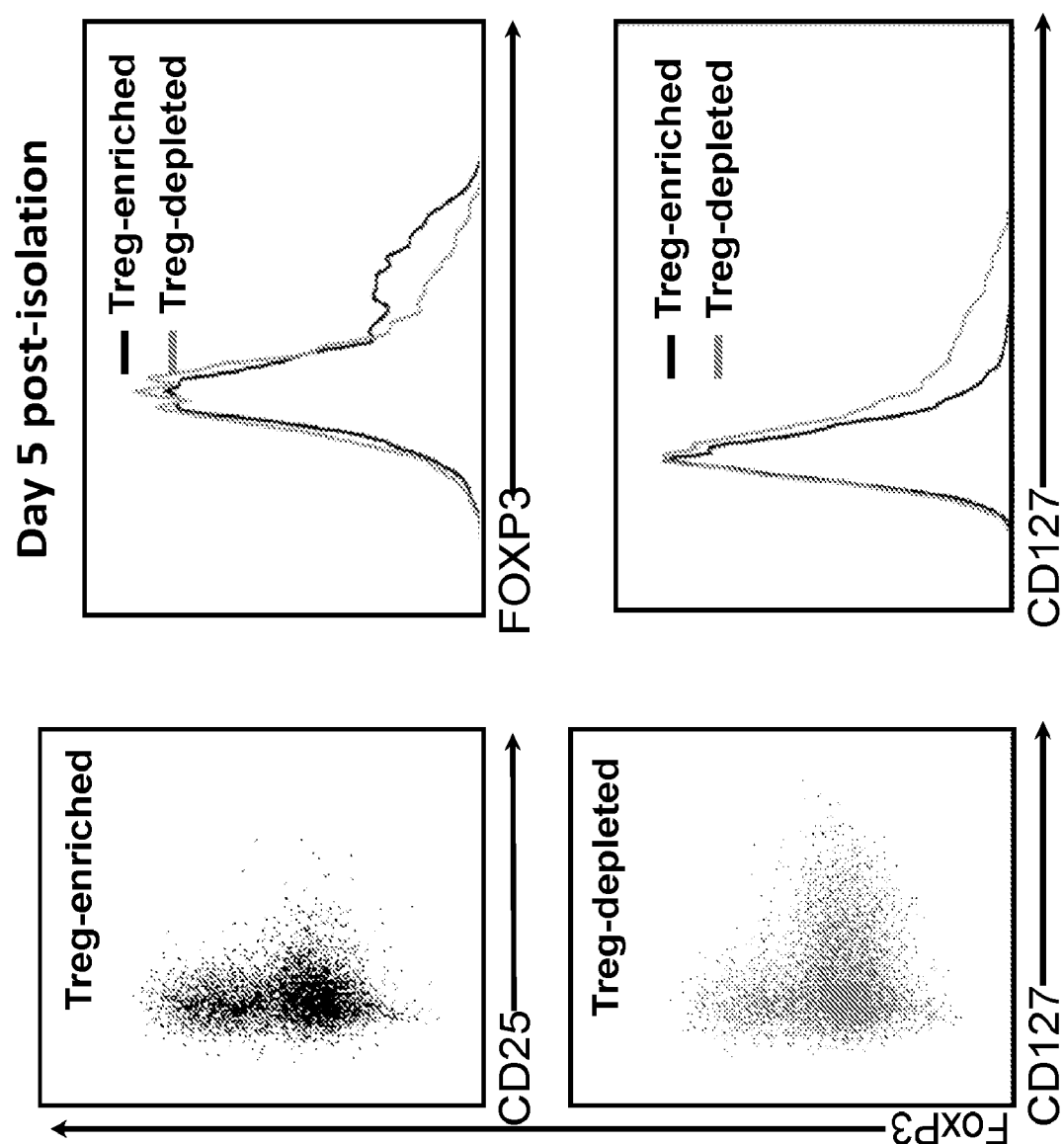


FIG. 18

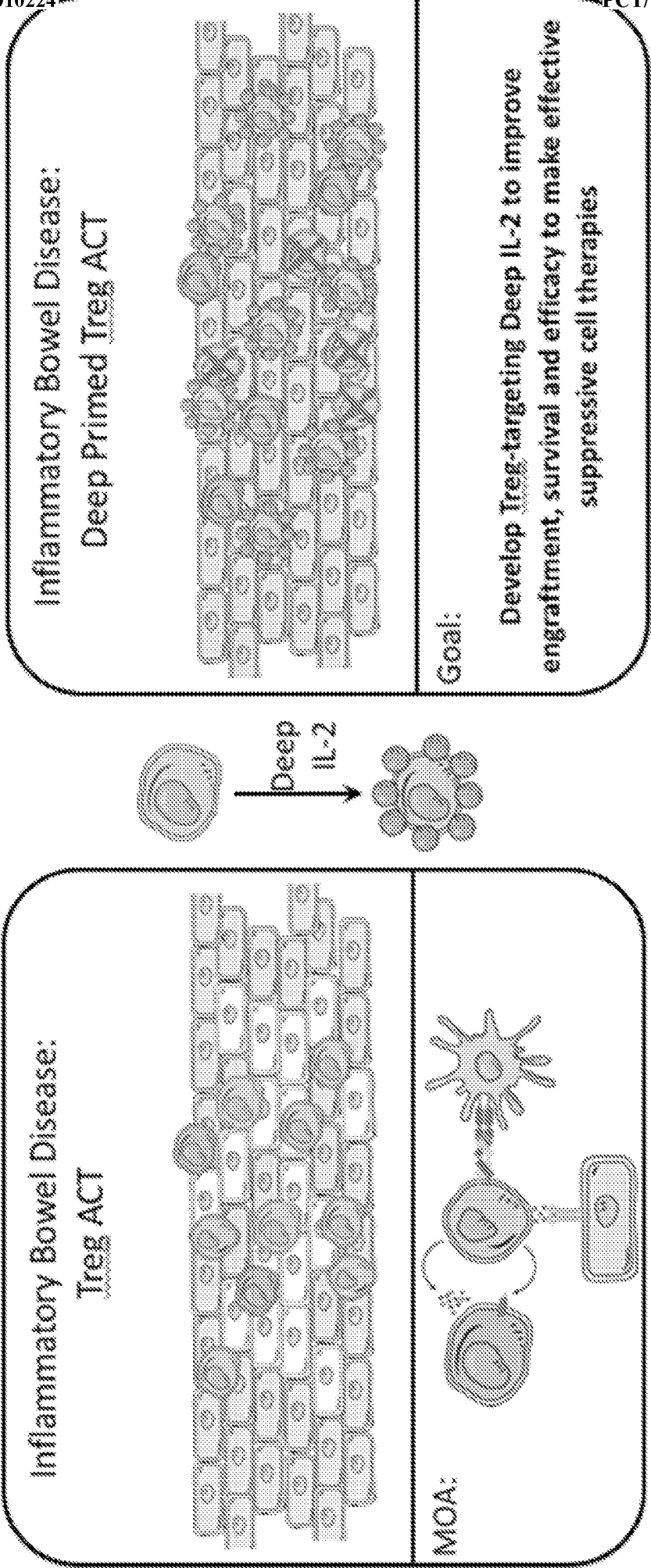
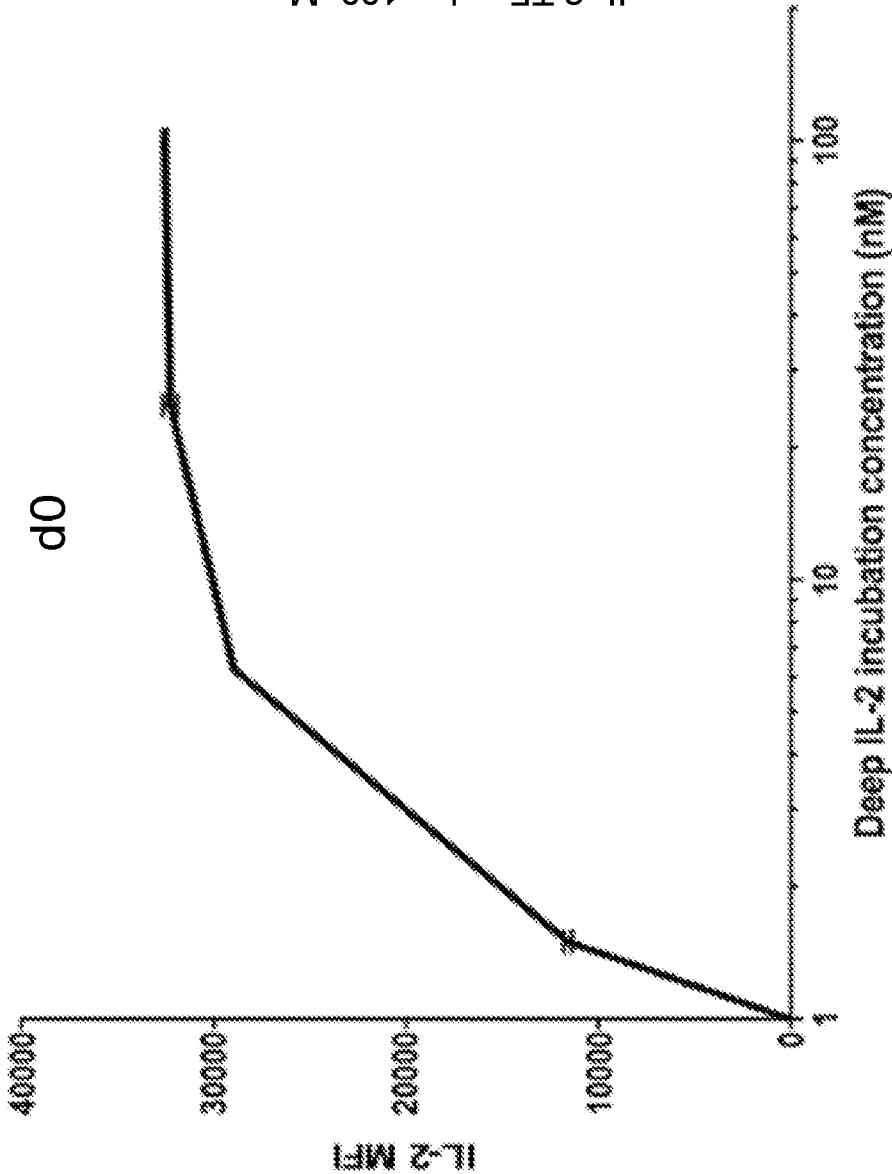


FIG. 19

Surface stain immediately post-Fab-HSA-IL2 loading



d0

Surface stain time course

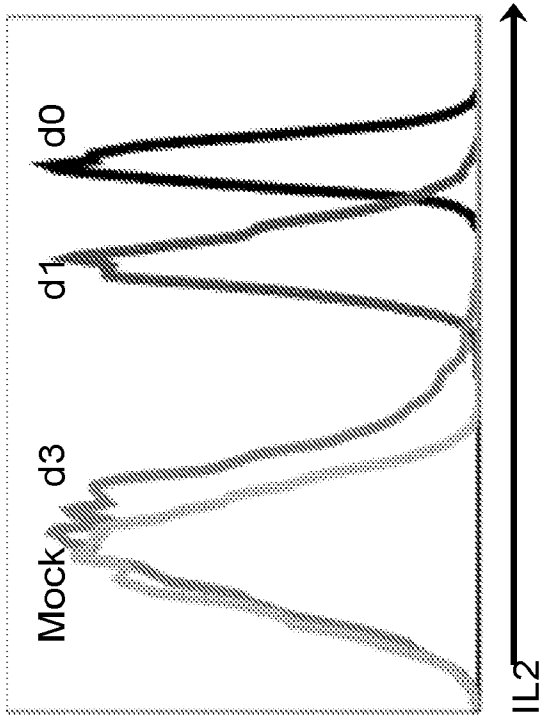


FIG. 20

Expansion and survival 3 Days post-Fab-HSA-IL2 loading

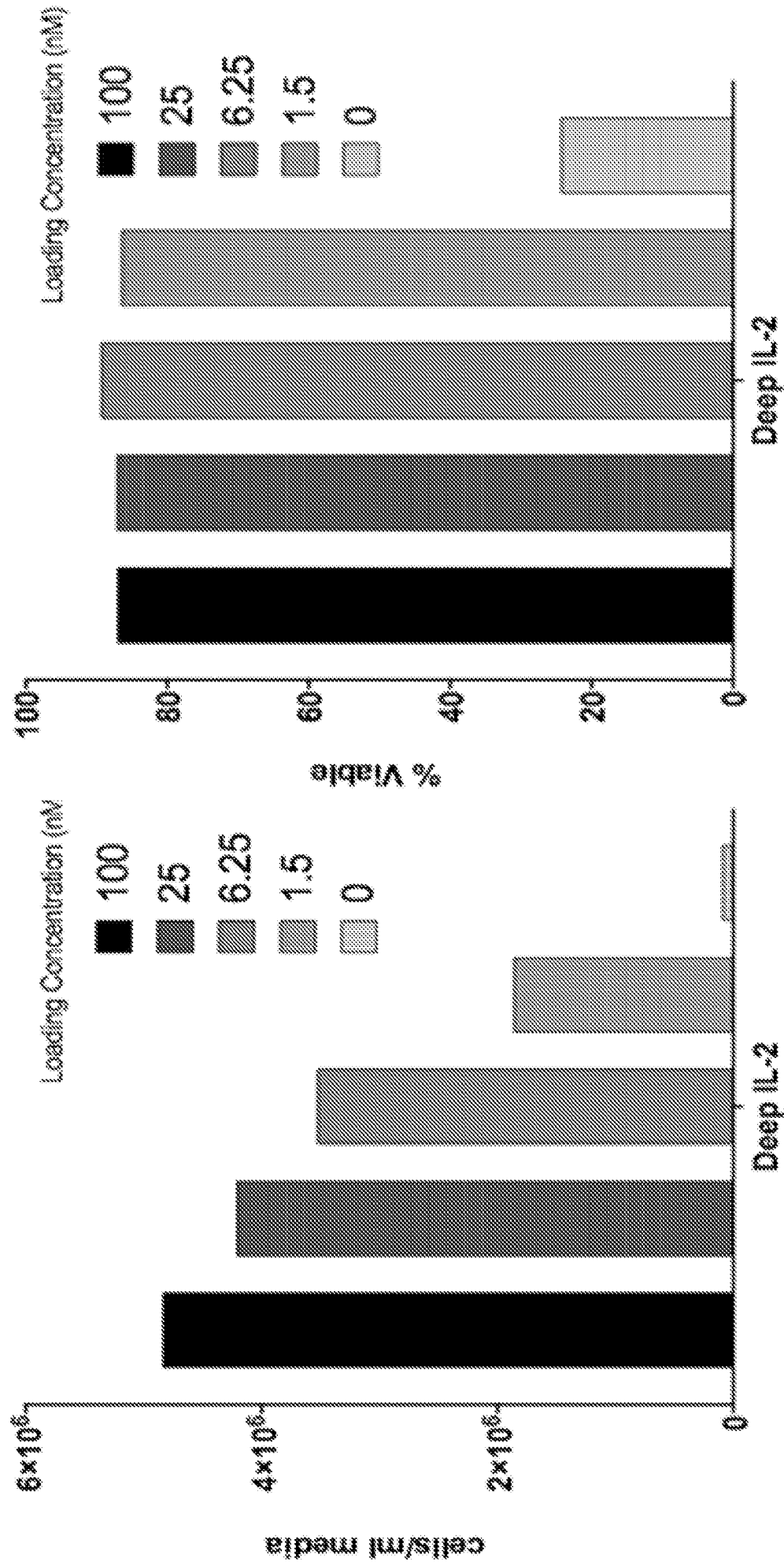


FIG. 20 (cont.)

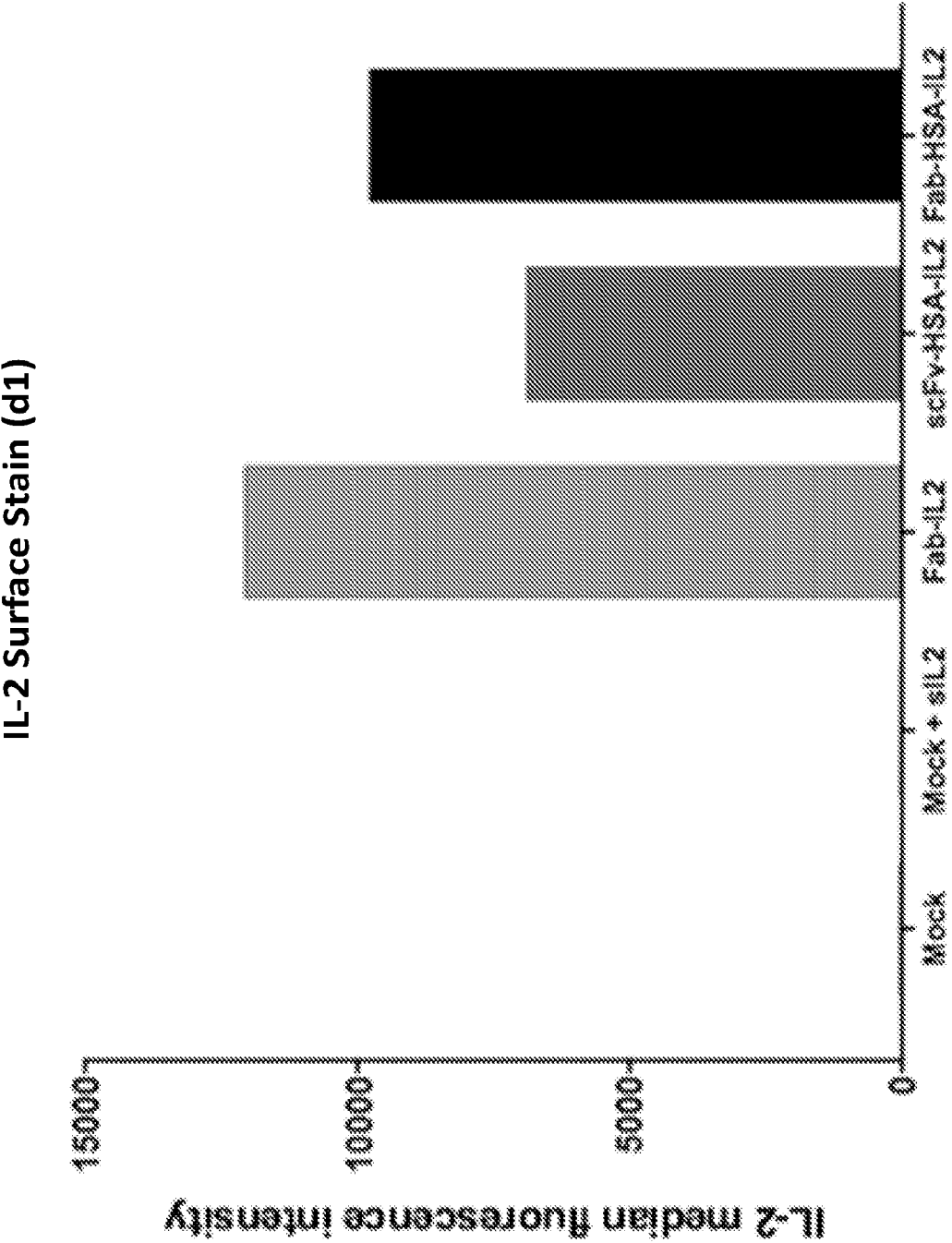


FIG. 21

Cell Number and Viability (d3)

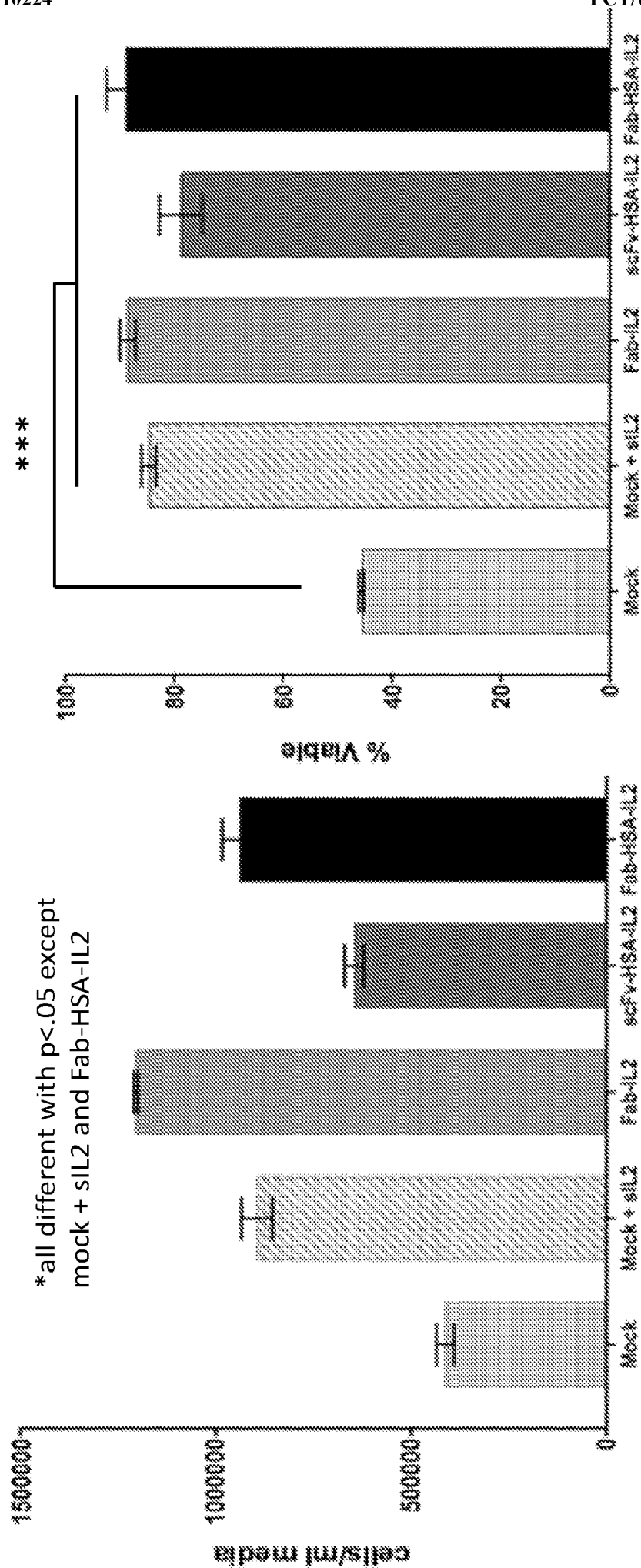


FIG. 21 (cont.)

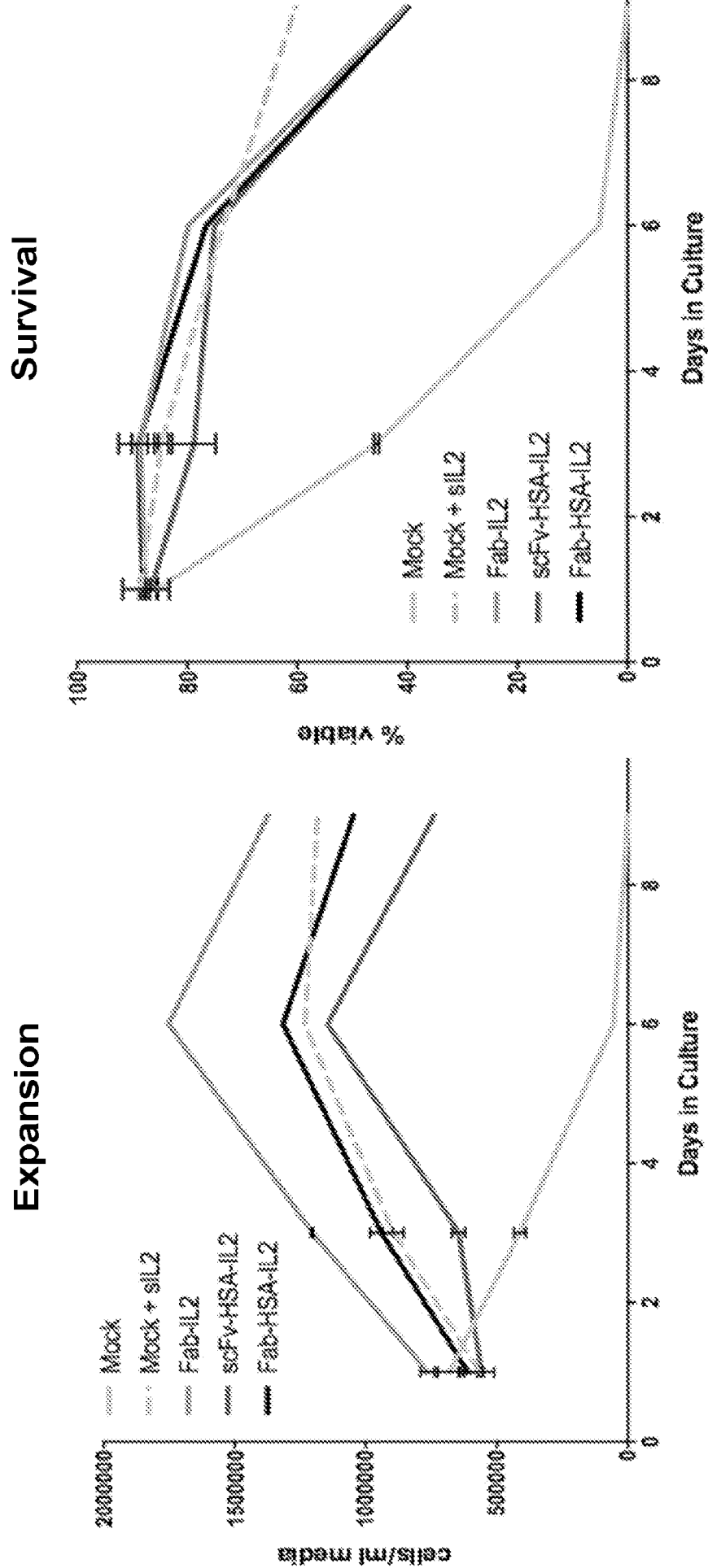


FIG. 22

d10 post-ACT

Deep Primed Cell Expansion Time Course

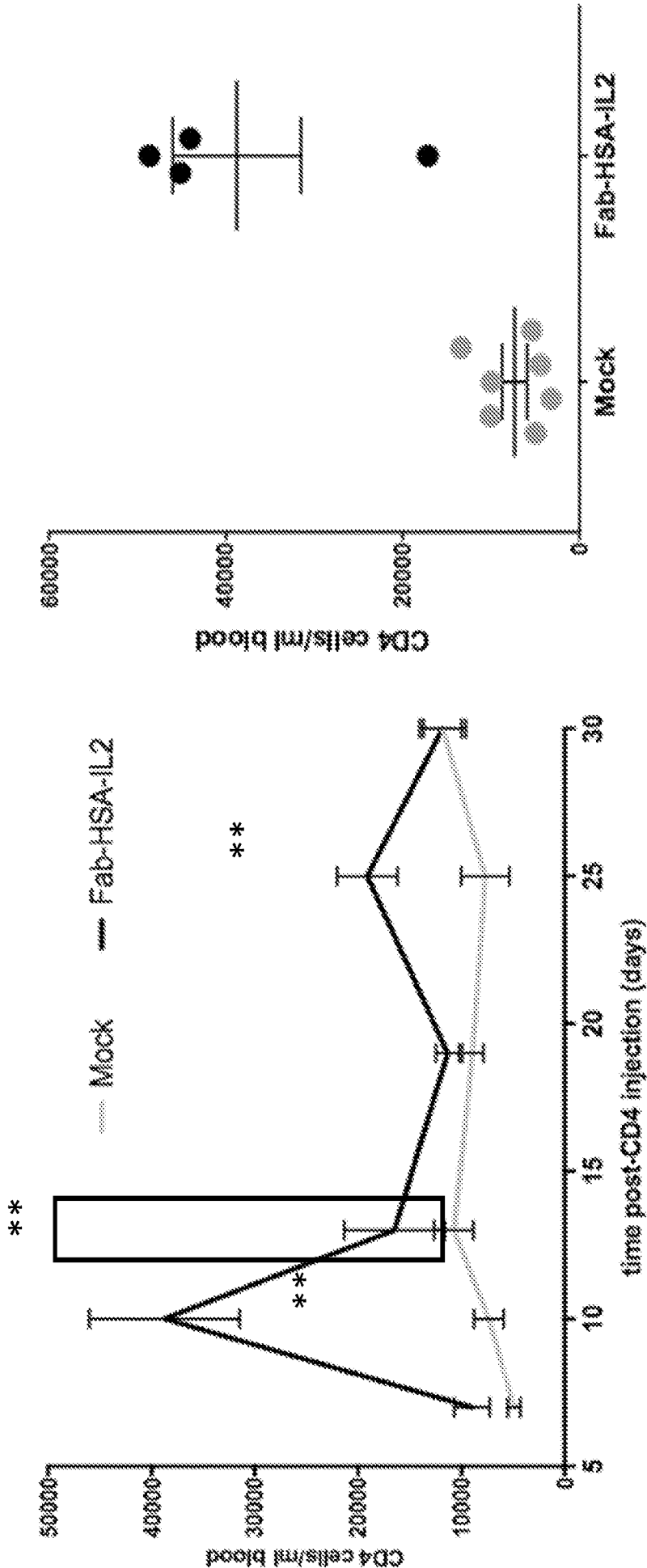


FIG. 23

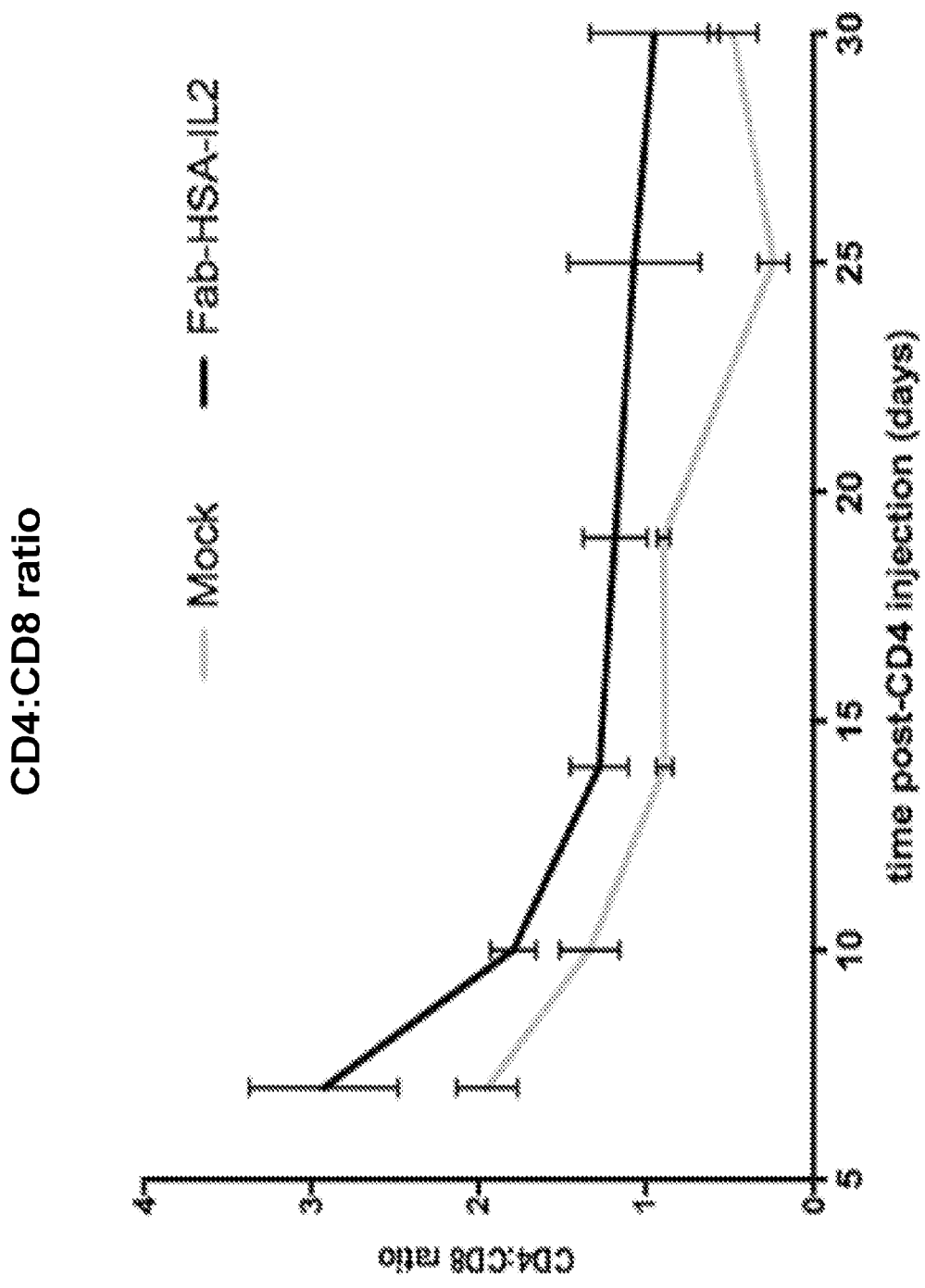


FIG. 24

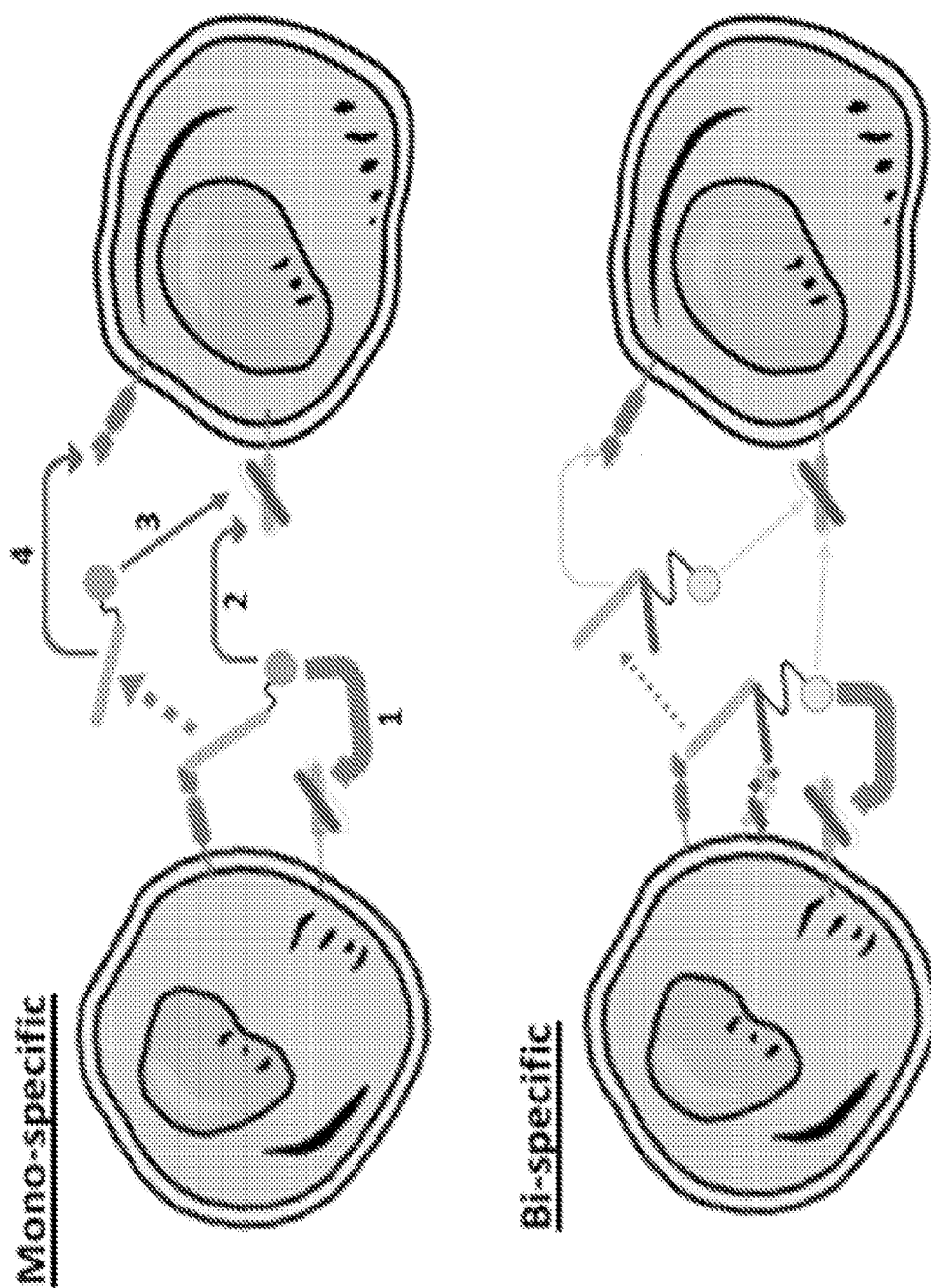


FIG. 25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/040786

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/18; A61K 38/19; A61K 38/20; A61K 39/395; C07K 16/28; C07K 16/46 (2018.01)

CPC - A61K 2039/505; A61K 2039/507; C07K 14/475; C07K 14/52; C07K 16/28; C07K 2317/31; C07K 2317/565; C07K 2319/00 (2018.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/85.2; 424/134.1; 424/136.1; 424/178.1; 435/328; 514/7.6; 530/351; 530/387.3 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/0037131 A1 (BERNETT et al) 09 February 2017 (09.02.2017) entire document	1-8, 14, 28-33, 35a, 37, 47
--		
Y		39
Y	WO 2016/179288 A1 (UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.) 10 November 2016 (10.11.2016) entire document	39
A	US 2016/0145355 A1 (BIOMED VALLEY DISCOVERIES, INC.) 26 May 2016 (26.05.2016) entire document	1-8, 14, 28-33, 35a, 37, 39, 47
A	US 2017/0080104 A1 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 23 March 2017 (23.03.2017) entire document	1-8, 14, 28-33, 35a, 37, 39, 47
A	US 2016/0168269 A1 (MERRIMACK PHARMACEUTICALS, INC.) 16 June 2016 (16.06.2016) entire document	1-8, 14, 28-33, 35a, 37, 39, 47

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 November 2018

Date of mailing of the international search report

10 DEC 2018

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/040786

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☐ forming part of the international application as filed:
☐ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

ISA/225 mailed on 24 July 2018. No approved electronic sequence listing was submitted in response to the ISA/225.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/040786

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 34
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 34 is held unsearchable as a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit, furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

3. ☒ Claims Nos.: 9-13, 15-27, 35b, 36, 38, 40-46
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.