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(54) Title: ANTI-CHONDROITIN SULFATE PROTEOGLYCAN 4 ANTIBODIES AND USES THEREOF

(57) Abstract: Described herein are antibodies that bind chondroitin sulfate proteoglycan 4 (CSPG4) and, in particular, chimeric and humanized anti-CSPG4 antibodies and fragments thereof. Also provided herein are methods of using individual humanized antibodies or compositions thereof for the detection, prevention, and/or therapeutic treatment of CSPG4-related diseases, in particular, melanoma.



FIG. 1



(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,

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ANTI-CHONDROITIN SULFATE PROTEOGLYCAN 4 ANTIBODIES AND USES THEREOF

BACKGROUND

[001] Antibody-based therapeutics offer significant promise, particularly in cancer treatment. A variety of formats, including monoclonal, murine, chimeric, humanized, human, full-length, Fab, pegylated, radiolabeled, drug-conjugated, multi-specific, etc. are being developed. A 2012 review article, reported that 34 therapeutic antibody agents had received marketing approval in the United States or Europe (see Reichert, mAbs 4:3, 413, May/June 2012, incorporated herein by reference). Still, development of particular effective antibody agents remains a challenge.

SUMMARY OF INVENTION

[002] The present invention provides, among other things, improved humanized antibodies that bind chondroitin sulfate proteoglycan 4 (CSPG4) and contain one or more structural features (e.g., one or more CDRs) of murine antibody 763.74 (referred to herein as 763). In some embodiments, provided antibody agents demonstrate high affinity and unusually slow k_{off} rates as compared to parental murine antibody 763.74. In some embodiments, provided antibody agents have a high affinity to CSPG4 such that said antibody agents do not demonstrate affinity barrier issues.

[003] The present invention also provides, improved multispecific binding agents that include binding moieties that interact with a particular target. In many embodiments, such binding moieties are or comprise antibody components. In some embodiments, multispecific binding agents of the present invention comprise binding elements of a humanized 763 antibody. In some embodiments, multispecific binding agents of the present invention comprise a first binding moiety based on a humanized 763 antibody and a second binding moiety that interacts with immune effector cell (e.g., a T cell). Such provided agents have improved functional characteristics as compared to parental binding agents that lack components described herein.

[004] In some embodiments, the present invention provides a humanized or chimeric antibody or fragment thereof that binds CSPG4, wherein the humanized or chimeric antibody or fragment thereof comprises at least one, at least two, or three of the complementarity determining regions (CDRs) found in the light chain variable region of murine antibody 763 and/or at least one, at least two, or three of the CDRs found in the heavy chain variable region of murine antibody 763. In

some embodiments, humanized or chimeric antibodies of the present invention comprise the three CDRs found in the light chain variable region of murine antibody 763 and the three CDRs found in the heavy chain variable region of murine antibody 763.

[005] In some embodiments, an antibody of the present invention is humanized. In some certain embodiments, humanized antibodies of the present invention comprise a light chain variable region sequence of SEQ ID NO: 12 or SEQ ID NO: 14. In some certain embodiments, humanized antibodies of the present invention comprises a heavy chain variable region of SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 16 or SEQ ID NO: 18.

[006] In some embodiments, a humanized antibody of the present invention comprises a heavy chain variable region of SEQ ID NO: 8 and a light chain variable region of SEQ ID NO: 12. In some embodiments, a humanized antibody of the present invention comprises a heavy chain variable region of SEQ ID NO: 10 and a light chain variable region of SEQ ID NO: 14. In some embodiments, a humanized antibody of the present invention comprises a heavy chain variable region of SEQ ID NO: 16 and a light chain variable region of SEQ ID NO: 12. In some embodiments, a humanized antibody of the present invention comprises a heavy chain variable region of SEQ ID NO: 18 and a light chain variable region of SEQ ID NO: 14.

[007] In some embodiments, a humanized antibody of the present invention comprises a heavy chain variable region of SEQ ID NO: 8 and a light chain variable region of SEQ ID NO: 14. In some embodiments, a humanized antibody of the present invention comprises a heavy chain variable region of SEQ ID NO: 10 and a light chain variable region of SEQ ID NO: 12. In some embodiments, a humanized antibody of the present invention comprises a heavy chain variable region of SEQ ID NO: 16 and a light chain variable region of SEQ ID NO: 14. In some embodiments, a humanized antibody of the present invention comprises a heavy chain variable region of SEQ ID NO: 18 and a light chain variable region of SEQ ID NO: 12.

[008] In some embodiments, a humanized antibody of the present invention comprises the heavy chain of SEQ ID NO: 8 and the light chain of SEQ ID NO: 12. In some embodiments, a humanized antibody of the present invention comprises the heavy chain of SEQ ID NO: 10 and a light chain of SEQ ID NO: 14. In some embodiments, a humanized antibody of the present invention comprises the heavy chain of SEQ ID NO: 16 and the light chain of SEQ ID NO: 12. In some embodiments, a humanized antibody of the present invention comprises the heavy chain of SEQ ID NO: 18 and a light chain of SEQ ID NO: 14.

[0009] In some embodiments, a humanized antibody of the present invention comprises the heavy chain of SEQ ID NO: 8 and a light chain of SEQ ID NO: 14. In some embodiments, a humanized antibody of the present invention comprises the heavy chain of SEQ ID NO: 10 and a light chain of SEQ ID NO: 12. In some embodiments, a humanized antibody of the present invention comprises the heavy chain of SEQ ID NO: 16 and a light chain of SEQ ID NO: 14. In some embodiments, a humanized antibody of the present invention comprises the heavy chain of SEQ ID NO: 18 and a light chain of SEQ ID NO: 12.

[0010] In some embodiments, an antibody of the present invention is chimeric. In some certain embodiments, a chimeric antibody of the present invention comprises a light chain variable region sequence of SEQ ID NO: 6. In some certain embodiments, a chimeric antibody of the present invention comprises a heavy chain variable region of SEQ ID NO: 4. In some certain embodiments, a chimeric antibody of the present invention comprises the heavy chain of SEQ ID NO: 4 and the light chain of SEQ ID NO: 6.

[0011] In various embodiments, a humanized or chimeric antibody of the present invention is characterized in that it inhibits tumor uptake of SKMEI-28 xenographs by about 50% as compared to a reference antibody.

[0012] In various embodiments, a humanized or chimeric antibody of the present invention is glycosylated with terminal mannose, N-acetylglucose or glucose, but no fucose.

[0013] In various embodiments, a humanized or chimeric antibody of the present invention is or comprises a human IgG1 or a human IgG4.

[0014] In various embodiments, a humanized or chimeric antibody of the present invention is or comprises a human IgG1 that has a variant glycosylation. In some certain embodiments, variant glycosylation results from an amino acid substitution at residue 297 of the human IgG1 Fc. In some certain embodiments, variant glycosylation results from expression in a engineered cell line. In some embodiments, engineered cell lines include CHO cells; in some certain embodiments, engineered cell lines include GnT1-deficient CHO cells.

[0015] In some embodiments, the present invention provides an isolated nucleic acid molecule that encodes an amino acid sequence described herein. In some embodiments, isolated nucleic acid sequences of the present invention are codon-optimized. In some certain embodiments, isolated nucleic acid sequences are or comprise any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19 and SEQ ID NO: 21.

[0016] In some embodiments, the present invention provides a recombinant or expression vector comprising a nucleic acid molecule as described herein.

[0017] In some embodiments, the present invention provides a host cell comprising a recombinant or expression vector as described herein.

[0018] In some embodiments, the present invention provides compositions comprising a humanized or chimeric antibody or fragment thereof as described herein.

[0019] In some embodiments, humanized or chimeric antibodies of the present invention are conjugated to a cytotoxic agent.

[0020] In some embodiments, the present invention provides a pharmaceutical composition comprising a humanized or chimeric antibody or fragment thereof as described herein or a composition as described herein and further comprise a pharmaceutically acceptable carrier or diluent.

[0021] In some embodiments, the present invention provides a method for producing a humanized or chimeric antibody or fragment thereof as described herein comprising a step of culturing a host cell as described herein in a culture medium under conditions allowing the expression of the humanized or chimeric antibody or fragment thereof and separating the humanized or chimeric antibody or fragment thereof from the culture medium.

[0022] In some embodiments, the present invention provides a method of treating or preventing a medical condition in a subject, wherein the medical condition characterized by CSPG4 expression, the method comprising administering a therapeutically effective amount of an antibody or fragment thereof as described herein to said subject. In various embodiments, medical conditions include CSPG4-positive tumors. In various embodiments, medical conditions include melanoma, breast cancer, osteosarcoma, head and neck cancers, glioblastomas multiforme, sarcoma and/or mesothelioma.

[0023] In some embodiments, the present invention also provides a bispecific binding agent (e.g., a bispecific antibody) that comprises first and second antigen-binding sites. In many embodiments, first antigen-binding sites are or comprises antibody components derived from a humanized 763 antibody as described herein. In many embodiments, second antigen-binding sites are or comprise antibody components that bind to immune effector cells.

[0024] In some embodiments, the present invention provides a bispecific antibody comprising a first antigen-binding site derived from a humanized 763 antibody and a second antigen-binding site.

In many embodiments, humanized 763 antibodies are or are based on humanized 763 antibodies described herein.

[0025] In some embodiments, first and second antigen-binding sites are or comprise single chain variable fragments (scFvs). In some embodiments, a first antigen-binding site is composed of an immunoglobulin molecule and a second antigen-binding site is composed of an scFv, scFab, Fab or Fv. In some certain embodiments, a second antigen-binding site is an scFv. In some certain embodiments, a first antigen binding site is composed of an immunoglobulin molecule and a second antigen-binding site is an scFv, wherein the scFv is linked to the C-terminal end of the heavy chain of the immunoglobulin. In some certain embodiments, a first antigen binding site is composed of an immunoglobulin molecule and a second antigen-binding site is an scFv, wherein the scFv is linked to the C-terminal end of the light chain of the immunoglobulin.

[0026] In various embodiments, a second antigen-binding site binds an immune cell selected from the group consisting of a T cell, NK cell, B cell, dendritic cell, monocyte, macrophage, neutrophil, mesenchymal stem cell and neural stem cell. In various embodiments, a second antigen binding site binds CD3.

[0027] In various embodiments, a bispecific antibody of the present invention comprise the sequence of SEQ ID NO: 20 or SEQ ID NO: 22. In some embodiments, the present invention provides an isolated nucleic acid comprising a coding sequence for part or all of a polypeptide chain of a bispecific antibody as described herein. In some certain embodiments, coding sequences are codon-optimized.

[0028] In some embodiments, the present invention provides a composition comprising a bispecific antibody as described herein.

[0029] In some embodiments, the present invention provides a pharmaceutical composition comprising a composition comprising a bispecific antibody as described herein or bispecific antibody as described herein.

[0030] In some embodiments, the present invention provides a chimeric antigen receptor comprising an antigen-binding domain of a humanized 763 antibody. In many embodiments, humanized 763 antibodies include such antibodies as described herein. In some embodiments, antigen-binding sites include scFvs.

[0031] In some embodiments, the present invention provides an immune effector cell that expresses a chimeric antigen receptor as described herein.

[0032] In some embodiments, the present invention provides use of a chimeric antigen receptor as described herein for the treatment or detection of a condition related to CSPG4 expression.

[0033] In some embodiments, the present invention provides a bispecific T-cell engaging monoclonal antibody comprising an antigen-binding site based on a humanized 763 antibody. In many embodiments, humanized 763 antibodies include such antibodies as described herein.

[0034] In some embodiments, the present invention provides a method of killing tumor cells, the method comprising the steps of contacting the tumor cells with a bispecific antibody, which bispecific antibody is composed of a first antigen-binding site based on a humanized 763 antibody and a second antigen-binding site that binds CD3, the contacting being performed under conditions and for a time sufficient that T cells to which the bispecific antibody has bound mediate killing of the tumor cells.

[0035] In some embodiments, the present invention provides a method of inhibiting tumor growth, the method comprising the steps of contacting a tumor with a bispecific antibody, which bispecific antibody is composed of a first antigen-binding site based on a humanized 763 antibody and a second antigen-binding site that binds CD3 on T cells, the contacting being performed under conditions and for a time sufficient that T cells to which the bispecific antibody has bound inhibit growth of a tumor.

[0036] In various embodiments, first and second antigen-binding sites are scFvs.

[0037] In various embodiments, a first antigen-binding site is composed of an immunoglobulin molecule and a second antigen-binding site is composed of an scFv. In some certain embodiments, an scFv is linked to the immunoglobulin molecule at the C-terminal end of the heavy chain. In some certain embodiments, an scFv is linked to the immunoglobulin molecule at the C-terminal end of the light chain.

[0038] In some embodiments, the present invention provides a bispecific antibody comprised of an immunoglobulin molecule that binds CSPG4 and an scFv that binds to CD3 on T cells, wherein the bispecific antibody is characterized by an increased efficiency to mediate T cell killing of tumor cells as compared to a reference bispecific antibody. In various embodiments, a bispecific antibody of the present invention is characterized by a high potency to kill tumor cells and a very low EC₅₀. In various embodiments, a bispecific antibody of the present invention is characterized by enhanced tumor CSPG4⁺ tumor targeting as compared to a reference bispecific antibody. In various embodiments, a bispecific antibody of the present invention is characterized by no or substantially no aggregation as compared to a reference bispecific antibody. In various embodiments, a

bispecific antibody of the present invention is characterized a greater binding avidity as compared to a reference bispecific antibody.

[0039] In some embodiments, an immunoglobulin molecule of a bispecific antibody of the present invention is based on murine 763 antibody.

[0040] In some embodiments, an scFv of a bispecific antibody of the present invention is based on a humanized OKT3 antibody. In some certain embodiments, an scFv is linked to the immunoglobulin molecule at the C-terminal end of the heavy or light chain.

[0041] In various embodiments, bispecific antibodies of the present invention comprise SEQ ID NO: 20 and SEQ ID NO: 14. In various embodiments, bispecific antibodies of the present invention comprise SEQ ID NO: 20 and/or SEQ ID NO: 12.

[0042] In various embodiments, bispecific antibodies of the present invention comprises SEQ ID NO: 22 and the heavy chain variable region of SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 16 or SEQ ID NO: 18. In various embodiments, bispecific antibodies of the present invention comprise SEQ ID NO: 22 and one of SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 16 or SEQ ID NO: 18.

[0043] In some embodiments, the present invention provides a kit comprising a humanized 763 antibody or a bispecific antibody described herein.

In some embodiments, the present invention provides use of a humanized 763 antibody or bispecific antibody described herein in the manufacture of a medicament for use in medicine. In some embodiments, the present invention provides use of a humanized 763 antibody or bispecific antibody described herein in the manufacture of a medicament for use in a diagnostic test or assay. In some embodiments, the present invention provides use of a humanized 763 antibody or bispecific antibody described herein in the manufacture of a medicament for the diagnosis of cancer. In some embodiments, the present invention provides use of a humanized 763 antibody or bispecific antibody described herein in the manufacture of a medicament for the treatment of cancer. In some embodiments, the present invention provides use of a humanized 763 antibody or bispecific antibody described herein in the manufacture of a medicament for the treatment of melanoma, breast cancer, osteosarcoma, head and neck cancers, glioblastoma multiforme, sarcoma or mesothelioma.

BREIF DESCRIPTION OF THE DRAWING

[0044] The Drawing included herein, which is composed of the following Figures, is for illustration purposes only not for limitation.

[0045] **Figure 1** shows exemplary mean fluorescence intensity for anti-CSPG4 antibody binding to CSPG4⁺ M14 tumor cells after multiple washes with a wash buffer (PBS with 2 mM EDTA).

[0046] **Figure 2** shows exemplary biochemical analysis of humanized 763 antibodies by reduced SDS-PAGE (A) and SE-HPLC chromatography (B). The major peak (around 16.7 min) in SE-HPLC is the main peak of IgG1 and IgG4. Twenty-five minutes corresponds to the salt buffer peak.

[0047] **Figure 3** shows exemplary antigen binding of chimeric and humanized 763 antibodies. A-C, Composite sensograms showing the binding of chimeric and humanized 763 antibodies on antigen D2.8.11-coated chips (A) or on anti-763 idiotype antibody MK2-23 coated chips (B and C), which were measured by surface plasmon resonance (Biacore T-100). D-E, Tumor antigen binding of humanized 763 antibodies. D, FACS analysis of humanized or mouse 763 antibodies to M14 cells. Hu763-IgG1 was also subjected to five cycles of a freeze and thaw process. For humanized 763 IgG1, binding was detected with a FITC conjugated goat anti-human secondary antibody. For mouse 763, binding was detected with a FITC conjugated goat anti-mouse secondary antibody. Antigen binding was expressed as % of MFI of maximum binding at 5 μ g. E, ELISA analysis of hu763-IgG1 and hu763-IgG1n on M14 cells coated plates. Binding was detected with HRP conjugated goat anti-human secondary antibody. Hu763-IgG1n was subjected to five cycles of a freeze and thaw process.

[0048] **Figure 4** shows exemplary antibody-dependent cell-mediated cytotoxicity (ADCC) of NK92Mi(CD16) in the presence of humanized 763 antibodies. A, Specific lysis of M14 cells. B, Specific lysis of U2OS cells. E:T ratio was 20:1.

[0049] **Figure 5** shows exemplary targeting of ¹²⁴I-labelled humanized 763 antibodies to SKMEL-28 xenografts.

[0050] **Figure 6** shows a schematic illustration, not to scale, of humanized bispecific antibody formats (e.g., IgG-scFv) that employ humanized 763 antibodies described herein.

[0051] **Figure 7** shows exemplary biochemical analysis of humanized 763 bispecific antibodies (hu763-BsAb) by SE-HPLC chromatography (A) and reduced SDS-PAGE (B). Major peaks for the hu763-HC (15.494 min) and hu763-LC (15.912 min) formats are indicated. Twenty-five minutes is the salt buffer peak.

[0052] **Figure 8** shows exemplary antigen binding of humanized 763 bispecific antibodies to M14 cells (A) and T cells (B). Control antibody: monospecific antibody that does not bind CSPG4

or T cells. A phycoerythrin conjugated goat anti-human antibody was used as secondary antibody and binding was analyzed by FACS analysis.

[0053] **Figure 9** shows exemplary redirected T cell killing of CSPG4 positive tumor cells by humanized 763 bispecific antibodies. Activated T cells (ATC) were incubated with target cells labeled with ^{51}Cr (E:T at 10:1) in the presence of different concentration of bispecific antibodies. Cytotoxicity was measured by release of ^{51}Cr in the supernatant counted by a γ -counter. Control BsAb: control bispecific antibody that binds CSPG4 and an organic compound.

[0054] **Figure 10** shows exemplary tumor growths in a melanoma xenograft model using humanized 763 bispecific antibodies. BALBRag2-/-IL-2R-rC-KO (DKO) mice were implanted with 1×10^6 M14 (melanoma) cells. Treatment was initiated on day 4 and schedules are indicated (A). Tumor growth was assessed by bioluminescence once a week starting on day 4. A, Bioluminescence quantitation of luciferin for different treatment groups. B, Fluorescent imaging of mice in different treatment groups on day 24.

DEFINITIONS

[0055] This invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention is defined by the claims.

[0056] Unless defined otherwise, all terms and phrases used herein include the meanings that the terms and phrases have attained in the art, unless the contrary is clearly indicated or clearly apparent from the context in which the term or phrase is used. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, particular methods and materials are now described. The contents of all cited references (including non-patent literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

[0057] In order for the present invention to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms are set forth through the specification.

[0058] “*Affinity*”: As is known in the art, “affinity” is a measure of the tightness with a particular ligand binds to its partner. Affinities can be measured in different ways. In some

embodiments, affinity is measured by a quantitative assay. In some such embodiments, binding partner concentration may be fixed to be in excess of ligand concentration so as to mimic physiological conditions. Alternatively or additionally, in some embodiments, binding partner concentration and/or ligand concentration may be varied. In some such embodiments, affinity may be compared to a reference under comparable conditions (e.g., concentrations).

[0059] "*Affinity matured*" (or "*affinity matured antibody*"), as used herein, refers to an antibody with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). In some embodiments, affinity matured antibodies will have nanomolar or even picomolar affinities for a target antigen. Affinity matured antibodies may be produced by any of a variety of procedures known in the art. Marks et al., *BioTechnology* 10:779-783 (1992) describes affinity maturation by V_H and V_L domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. *Proc. Nat. Acad. Sci. U.S.A* 91:3809-3813 (1994); Schier et al., *Gene* 169: 147-155 (1995); Yelton et al., *J. Immunol.* 155: 1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al., *J. Mol. Biol.* 226:889-896 (1992).

[0060] "*Amelioration*", as used herein, refers to the prevention, reduction or palliation of a state, or improvement of the state of a subject. Amelioration includes, but does not require complete recovery or complete prevention of a disease, disorder or condition (e.g., radiation injury).

[0061] "*Animal*", as used herein refers to any member of the animal kingdom. In some embodiments, "*animal*" refers to humans, of either sex and at any stage of development. In some embodiments, "*animal*" refers to non-human animals, at any stage of development. In certain embodiments, the non-human animal is a mammal (e.g., a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, insects, and/or worms. In certain embodiments, the animal is susceptible to infection by DV. In some embodiments, an animal may be a transgenic animal, genetically engineered animal, and/or a clone.

[0062] "*Antibody*", as used herein, has its art understood meaning and refers to an immunoglobulin (Ig) that binds specifically to a particular antigen. As is known by those of ordinary skill in the art, antibodies produced in nature are typically comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains. Each heavy and light chain is comprised of a variable region (abbreviated herein as HCVR or V_H and LCVR or V_L, respectively) and a constant region. The constant region of a heavy chain comprises a C_H1, C_H2 and C_H3 domain (and

optionally a C_H4 domain in the case of IgM and IgE). The constant region of a light chain is comprised of one domain, C_L. The V_H and V_L regions further contain regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, which are termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. Immunoglobulin molecules can be of any type (e.g., IgM, IgD, IgG, IgA and IgE), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass.

[0063] *Antibody agent*: As used herein, the term “antibody agent” refers to an agent that specifically binds to a particular antigen. In some embodiments, the term encompasses any polypeptide with immunoglobulin structural elements sufficient to confer specific binding. In various embodiments, suitable antibody agents may include, but are not limited to, monoclonal antibodies, polyclonal antibodies, humanized antibodies, primatized antibodies, chimeric antibodies, human antibodies, bi-specific or multi-specific antibodies, single domain antibodies (e.g., shark single domain antibodies (e.g., IgNAR or fragments thereof)), conjugated antibodies (i.e., antibodies conjugated or fused to other proteins, radiolabels, cytotoxins), Small Modular ImmunoPharmaceuticals (“SMIPsTM”), single chain antibodies, cameloid antibodies, antibody fragments, etc. In some embodiments, the term can refer to a stapled peptide. In some embodiments, the term can refer to an antibody-like binding peptidomimetic. In some embodiments, the term can refer to an antibody-like binding scaffold protein. In some embodiments, the term can refer to monobodies or adnectins. In many embodiments, an antibody agent is or comprises a polypeptide whose amino acid sequence includes one or more structural elements recognized by those skilled in the art as a complementarity determining region (CDR); in some embodiments an antibody agent is or comprises a polypeptide whose amino acid sequence includes at least one CDR (e.g., at least one heavy chain CDR and/or at least one light chain CDR) that is substantially identical to one found in a reference antibody. In some embodiments an included CDR is substantially identical to a reference CDR in that it is either identical in sequence or contains between 1-5 amino acid substitutions as compared with the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that it shows at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that it shows at least 96%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference CDR. In some embodiments an included CDR is substantially identical

to a reference CDR in that at least one amino acid within the included CDR is deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical with that of the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that 1-5 amino acids within the included CDR are deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical to the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that at least one amino acid within the included CDR is substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical with that of the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that 1-5 amino acids within the included CDR are deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical to the reference CDR. In some embodiments, an antibody agent is or comprises a polypeptide whose amino acid sequence includes structural elements recognized by those skilled in the art as an immunoglobulin variable domain. In some embodiments, an antibody agent is a polypeptide protein having a binding domain, which is homologous or largely homologous to an immunoglobulin-binding domain. In some embodiments, an antibody agent is or comprises a polypeptide that includes all CDRs found in a particular reference antibody chain or chains (e.g., heavy chain and/or light chain).

[0064] "*Antibody component*", as used herein, refers to a polypeptide element (that may be a complete polypeptide, or a portion of a larger polypeptide, such as for example a fusion polypeptide as described herein) that specifically binds to an epitope or antigen and includes one or more immunoglobulin structural features. In general, an antibody component is any polypeptide whose amino acid sequence includes elements characteristic of an antibody-binding region (e.g., an antibody light chain or variable region or one or more complementarity determining regions ("*CDRs*") thereof, or an antibody heavy chain or variable region or one more CDRs thereof, optionally in presence of one or more framework regions). In some embodiments, an antibody component is or comprises a full-length antibody. In some embodiments, an antibody component is less than full-length but includes at least one binding site (comprising at least one, and preferably at least two sequences with structure of known antibody "*variable regions*"). In some embodiments, the term "*antibody component*" encompasses any protein having a binding domain, which is homologous or largely homologous to an immunoglobulin-binding domain. In particular embodiments, an included "antibody component" encompasses polypeptides having a binding

domain that shows at least 99% identity with an immunoglobulin binding domain. In some embodiments, an included "antibody component" is any polypeptide having a binding domain that shows at least 70%, 75%, 80%, 85%, 90%, 95% or 98% identity with an immunoglobulin binding domain, for example a reference immunoglobulin binding domain. An included "*antibody component*" may have an amino acid sequence identical to that of an antibody (or a portion thereof, e.g., an antigen-binding portion thereof) that is found in a natural source. An antibody component may be monospecific, bi-specific, or multi-specific. An antibody component may include structural elements characteristic of any immunoglobulin class, including any of the human classes: IgG, IgM, IgA, IgD, and IgE. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Such antibody embodiments may also be bispecific, dual specific, or multi-specific formats specifically binding to two or more different antigens. Examples of binding fragments encompassed within the term "*antigen-binding portion*" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_H , V_L , C_{H1} and C_L 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 V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_H and V_L domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341 :544-546), which comprises a single variable domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_H and V_L , are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_H and V_L regions pair to form monovalent molecules (known as 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). In some embodiments, an "*antibody component*", as described herein, is or comprises such a single chain antibody. In some embodiments, an "antibody component" is or comprises a diabody. Diabodies are bivalent, bispecific antibodies in which V_H and V_L domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see e.g., Holliger, P., et al., (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak, R. J., (1994) Structure 2(12):1121-1123). Such antibody binding portions are known in the art (Kontermann and Dubel eds., Antibody Engineering (2001) Springer-Verlag. New York. 790 pp. (ISBN 3-540-41354-5). In some embodiments, an antibody component is or comprises a single chain "*linear antibody*"

comprising a pair of tandem Fv segments (V_H - C_{H1} - V_H - C_{H1}) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata et al., (1995) Protein Eng. 8(10): 1057-1062; and U.S. Patent No. 5,641,870). In some embodiments, an antibody component may have structural elements characteristic of chimeric or humanized antibodies. In general, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some embodiments, an antibody component may have structural elements characteristic of a human antibody.

[0065] "*Biological activity*", as used herein, refers to an observable biological effect or result achieved by an agent or entity of interest. For example, in some embodiments, a specific binding interaction is a biological activity. In some embodiments, modulation (e.g., induction, enhancement, or inhibition) of a biological pathway or event is a biological activity. In some embodiments, presence or extent of a biological activity is assessed through detection of a direct or indirect product produced by a biological pathway or event of interest.

[0066] "*Bispecific antibody*", as used herein, refers to a bispecific binding agent in which at least one, and typically both, of the binding moieties is or comprises an antibody component. A variety of different bi-specific antibody structures are known in the art. In some embodiments, each binding moiety in a bispecific antibody that is or comprises an antibody component includes V_H and/or V_L regions; in some such embodiments, the V_H and/or V_L regions are those found in a particular monoclonal antibody. In some embodiments, where the bispecific antibody contains two antibody component-binding moieties, each includes V_H and/or V_L regions from different monoclonal antibodies. In some embodiments, where the bispecific antibody contains two antibody component binding moieties, wherein one of the two antibody component binding moieties includes an immunoglobulin molecule having V_H and/or V_L regions that contain CDRs from a first monoclonal antibody, and one of the two antibody component binding moieties includes an antibody fragment (e.g., Fab, F(ab'), F(ab')₂, Fd, Fv, dAB, scFv, etc.) having V_H and/or V_L regions that contain CDRs from a second monoclonal antibody.

[0067] "*Bispecific binding agent*", as used herein, refers to a polypeptide agent with two discrete binding moieties, each of which binds with a distinct target. In some embodiments, a bispecific binding agent is or comprises a single polypeptide; in some embodiments, a bispecific binding agent is or comprises a plurality of peptides which, in some such embodiments may be

covalently associated with one another, for example by cross-linking. In some embodiments, the two binding moieties of a bispecific binding agent recognize different sites (e.g., epitopes) the same target (e.g., antigen); in some embodiments, they recognize different targets. In some embodiments, a bispecific binding agent is capable of binding simultaneously to two targets that are of different structure.

[0068] "*Carrier*", as used herein, refers to a diluent, adjuvant, excipient, or vehicle with which a composition is administered. In some exemplary embodiments, carriers can include sterile liquids, such as, for example, water and oils, including oils of petroleum, animal, vegetable or synthetic origin, such as, for example, peanut oil, soybean oil, mineral oil, sesame oil and the like. In some embodiments, carriers are or include one or more solid components.

[0069] "*CDR*", as used herein, refers to a complementarity determining region within an antibody variable region. There are three CDRs in each of the variable regions of the heavy chain and the light chain, which are designated CDR1, CDR2 and CDR3, for each of the variable regions. A "*set of CDRs*" or "*CDR set*" refers to a group of three or six CDRs that occur in either a single variable region capable of binding the antigen or the CDRs of cognate heavy and light chain variable regions capable of binding the antigen. Certain systems have been established in the art for defining CDR boundaries (e.g., Kabat, Chothia, etc.); those skilled in the art appreciate the differences between and among these systems and are capable of understanding CDR boundaries to the extent required to understand and to practice the claimed invention.

[0070] "*CDR-grafted antibody*", as used herein, refers to an antibody whose amino acid sequence comprises heavy and light chain variable region sequences from one species but in which the sequences of one or more of the CDR regions of V_H and/or V_L are replaced with CDR sequences of another species, such as antibodies having murine V_H and V_L regions in which one or more of the murine CDRs (e.g., CDR3) has been replaced with human CDR sequences. Likewise, a "*CDR-grafted antibody*" may also refer to antibodies having human V_H and V_L regions in which one or more of the human CDRs (e.g., CDR3) has been replaced with mouse CDR sequences.

[0071] "*Chimeric antibody*", as used herein, refers to an antibody whose amino acid sequence includes V_H and V_L region sequences that are found in a first species and constant region sequences that are found in a second species, different from the first species. In many embodiments, a chimeric antibody has murine V_H and V_L regions linked to human constant regions. In some embodiments, an antibody with human V_H and V_L regions linked to non-human constant regions (e.g., a mouse constant region) is referred to as a "*reverse chimeric antibody*".

[0072] "*Comparable*", as used herein, refers to two or more agents, entities, situations, sets of conditions, etc. that may not be identical to one another but that are sufficiently similar to permit comparison there between so that conclusions may reasonably be drawn based on differences or similarities observed. Those of ordinary skill in the art will understand, in context, what degree of identity is required in any given circumstance for two or more such agents, entities, situations, sets of conditions, etc. to be considered comparable.

[0073] "*Corresponding to*", as used herein designates the position/identity of an amino acid residue in a polypeptide of interest. Those of ordinary skill will appreciate that, for purposes of simplicity, residues in a polypeptide are often designated using a canonical numbering system based on a reference related polypeptide, so that an amino acid "*corresponding to*" a residue at position 190, for example, need not actually be the 190th amino acid in a particular amino acid chain but rather corresponds to the residue found at 190 in the reference polypeptide; those of ordinary skill in the art readily appreciate how to identify "*corresponding*" amino acids.

[0074] "*Dosage form*" and "*unit dosage form*", as used herein, the term "*dosage form*" refers to physically discrete unit of a therapeutic agent for a subject (e.g., a human patient) to be treated. Each unit contains a predetermined quantity of active material calculated or demonstrated to produce a desired therapeutic effect when administered to a relevant population according to an appropriate dosing regimen. For example, in some embodiments, such quantity is a unit dosage amount (or a whole fraction thereof) appropriate for administration in accordance with a dosing regimen that has been determined to correlate with a desired or beneficial outcome when administered to a relevant population (i.e., with a therapeutic dosing regimen). It will be understood, however, that the total dosage administered to any particular patient will be selected by a medical professional (e.g., a medical doctor) within the scope of sound medical judgment.

[0075] "*Dosing regimen*" (or "*therapeutic regimen*"), as used herein is a set of unit doses (typically more than one) that are administered individually to a subject, typically separated by periods of time. In some embodiments, a given therapeutic agent has a recommended dosing regimen, which may involve one or more doses. In some embodiments, a dosing regimen comprises a plurality of doses each of which are separated from one another by a time period of the same length; in some embodiments, a dosing regime comprises a plurality of doses and at least two different time periods separating individual doses. In some embodiments, the therapeutic agent is administered continuously (e.g., by infusion) over a predetermined period. In some embodiments, a therapeutic agent is administered once a day (QD) or twice a day (BID). In some embodiments, a

dosing regimen comprises a plurality of doses each of which are separated from one another by a time period of the same length; in some embodiments, a dosing regimen comprises a plurality of doses and at least two different time periods separating individual doses. In some embodiments, all doses within a dosing regimen are of the same unit dose amount. In some embodiments, different doses within a dosing regimen are of different amounts. In some embodiments, a dosing regimen comprises a first dose in a first dose amount, followed by one or more additional doses in a second dose amount different from the first dose amount. In some embodiments, a dosing regimen comprises a first dose in a first dose amount, followed by one or more additional doses in a second dose amount same as the first dose amount. In some embodiments, a dosing regimen is correlated with a desired or beneficial outcome when administered across a relevant population (i.e., is a therapeutic dosing regimen).

[0076] "*Effector function*" as used herein refers a biochemical event that results from the interaction of an antibody Fc region with an Fc receptor or ligand. Effector functions include but are not limited to antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-mediated cytotoxicity (CMC). In some embodiments, an effector function is one that operates after the binding of an antigen, one that operates independent of antigen binding, or both.

[0077] "*Effector cell*" as used herein refers to a cell of the immune system that expresses one or more Fc receptors and mediates one or more effector functions. In some embodiments, effector cells may include, but may not be limited to, one or more of monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, large granular lymphocytes, Langerhans' cells, natural killer (NK) cells, T-lymphocytes, B-lymphocytes and may be from any organism including but not limited to humans, mice, rats, rabbits, and monkeys.

[0078] "*Epitope*", as used herein, includes any moiety that is specifically recognized by an immunoglobulin (e.g., antibody or receptor) binding component. In some embodiments, an epitope is comprised of a plurality of chemical atoms or groups on an antigen. In some embodiments, such chemical atoms or groups are surface-exposed when the antigen adopts a relevant three-dimensional conformation. In some embodiments, such chemical atoms or groups are physically near to each other in space when the antigen adopts such a conformation. In some embodiments, at least some such chemical atoms or groups are physically separated from one another when the antigen adopts an alternative conformation (e.g., is linearized).

[0079] "*Excipient*", as used herein, refers to a non-therapeutic agent that may be included in a pharmaceutical composition, for example to provide or contribute to a desired consistency or stabilizing effect. Suitable pharmaceutical excipients include, for example, starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like.

[0080] "*Fc ligand*" as used herein refers to a molecule, preferably a polypeptide, from any organism that binds to the Fc region of an antibody to form an Fc-ligand complex. Fc ligands include but are not limited to Fc γ RIIA (CD32A), Fc γ RIIB (CD32B), Fc γ RIIIA (CD16A), Fc γ RIIIB (CD16B), Fc γ RI (CD64), Fc ϵ RII (CD23), FcRn, Clq, C3, staphylococcal protein A, streptococcal protein G, and viral Fc γ R. Fc ligands may include undiscovered molecules that bind Fc.

[0081] "*Framework*" or "*framework region*", as used herein, refers to the sequences of a variable region minus the CDRs. Because a CDR sequence can be determined by different systems, likewise a framework sequence is subject to correspondingly different interpretations. The six CDRs divide the framework regions on the heavy and light chains into four sub-regions (FR1, FR2, FR3 and FR4) on each chain, in which CDR1 is positioned between FR1 and FR2, CDR2 between FR2 and FR3, and CDR3 between FR3 and FR4. Without specifying the particular sub-regions as FR1, FR2, FR3 or FR4, a framework region, as referred by others, represents the combined FRs within the variable region of a single, naturally occurring immunoglobulin chain. As used herein, a FR represents one of the four sub-regions, FR1, for example, represents the first framework region closest to the amino terminal end of the variable region and 5' with respect to CDR1, and FRs represents two or more of the sub-regions constituting a framework region.

[0082] "*Host cell*", as used herein, refers to a cell into which exogenous DNA (recombinant or otherwise) has been introduced. Persons of skill upon reading this disclosure will understand that such terms refer not only to the particular subject cell, but also to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "*host cell*" as used herein. In some embodiments, host cells include prokaryotic and eukaryotic cells selected from any of the Kingdoms of life that are suitable for expressing an exogenous DNA (e.g., a recombinant nucleic acid sequence). Exemplary cells include those of prokaryotes and eukaryotes (single-cell or multiple-cell), bacterial cells (e.g., strains of *E. coli*, *Bacillus* spp., *Streptomyces* spp., etc.), mycobacteria cells, fungal cells, yeast cells (e.g., *S. cerevisiae*, *S. pombe*, *P. pastoris*, *P. methanolica*, etc.), plant cells, insect cells (e.g., SF-9,

SF-21, baculovirus-infected insect cells, *Trichoplusia ni*, etc.), non-human animal cells, human cells, or cell fusions such as, for example, hybridomas or quadromas. In some embodiments, the cell is a human, monkey, ape, hamster, rat, or mouse cell. In some embodiments, the cell is eukaryotic and is selected from the following cells: CHO (e.g., CHO KI, DXB-1 CHO, Veggie-CHO), COS (e.g., COS-7), retinal cell, Vero, CV1, kidney (e.g., HEK293, 293 EBNA, MSR 293, MDCK, HaK, BHK), HeLa, HepG2, WI38, MRC 5, Colo205, HB 8065, HL-60, (e.g., BHK21), Jurkat, Daudi, A431 (epidermal), CV-1, U937, 3T3, L cell, C127 cell, SP2/0, NS-0, MMT 060562, Sertoli cell, BRL 3 A cell, HT1080 cell, myeloma cell, tumor cell, and a cell line derived from an aforementioned cell. In some embodiments, the cell comprises one or more viral genes, e.g., a retinal cell that expresses a viral gene (e.g., a PER.C6™ cell).

[0083] "*Human antibody*", as used herein, is intended to include antibodies having variable and constant regions generated (or assembled) from human immunoglobulin sequences. In some embodiments, antibodies (or antibody components) may be considered to be "*human*" even though their amino acid sequences include residues or elements not encoded by human germline immunoglobulin sequences (e.g., include sequence variations, for example that may (originally) have been introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo), for example in one or more CDRs and in particular CDR3.

[0084] "*Humanized*", as is known in the art, the term "*humanized*" is commonly used to refer to antibodies (or antibody components) whose amino acid sequence includes V_H and V_L region sequences from a reference antibody raised in a non-human species (e.g., a mouse), but also includes modifications in those sequences relative to the reference antibody intended to render them more "*human-like*", i.e., more similar to human germline variable sequences. In some embodiments, a "*humanized*" antibody (or antibody component) is one that immunospecifically binds to an antigen of interest and that has a framework (FR) region having substantially the amino acid sequence as that of a human antibody, and a complementary determining region (CDR) having substantially the amino acid sequence as that of a non-human antibody. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', F(ab')₂, FabC, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (i.e., donor immunoglobulin) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. In some embodiments, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin constant region. In some embodiments, a humanized antibody

contains both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include a C_H1, hinge, C_H2, C_H3, and, optionally, a C_H4 region of a heavy chain constant region. In some embodiments, a humanized antibody only contains a humanized V_L region. In some embodiments, a humanized antibody only contains a humanized V_H region. In some certain embodiments, a humanized antibody contains humanized V_H and V_L regions.

[0085] “*Improve*,” “*increase*” or “*reduce*,” as used herein or grammatical equivalents thereof, indicate values that are relative to a baseline measurement, such as a measurement in the same individual prior to initiation of a treatment described herein, or a measurement in a control individual (or multiple control individuals) in the absence of the treatment described herein. A “*control individual*” is an individual afflicted with the same form of disease or injury as the individual being treated.

[0086] “*In vitro*”, as used herein refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, etc., rather than within a multi-cellular organism.

[0087] “*In vivo*”, as used herein refers to events that occur within a multi-cellular organism, such as a human and a non-human animal. In the context of cell-based systems, the term may be used to refer to events that occur within a living cell (as opposed to, for example, in vitro systems).

[0088] “*Isolated*”, as used herein, refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) designed, produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is “*pure*” if it is substantially free of other components. In some embodiments, as will be understood by those skilled in the art, a substance may still be considered “*isolated*” or even “*pure*”, after having been combined with certain other components such as, for example, one or more carriers or excipients (e.g., buffer, solvent, water, etc.); in such embodiments, percent isolation or purity of the substance is calculated without including such carriers or excipients. To give but one example, in some embodiments, a biological polymer such as a polypeptide or polynucleotide that occurs in nature is considered to be

"isolated" when, a) by virtue of its origin or source of derivation is not associated with some or all of the components that accompany it in its native state in nature; b) it is substantially free of other polypeptides or nucleic acids of the same species from the species that produces it in nature; c) is expressed by or is otherwise in association with components from a cell or other expression system that is not of the species that produces it in nature. Thus, for instance, in some embodiments, a polypeptide that is chemically synthesized or is synthesized in a cellular system different from that which produces it in nature is considered to be an "isolated" polypeptide. Alternatively or additionally, in some embodiments, a polypeptide that has been subjected to one or more purification techniques may be considered to be an "isolated" polypeptide to the extent that it has been separated from other components a) with which it is associated in nature; and/or b) with which it was associated when initially produced.

[0089] " K_D ", as used herein, refers to the dissociation constant of a binding agent (e.g., an antibody or binding component thereof) from a complex with its partner (e.g., the epitope to which the antibody or binding component thereof binds).

[0090] " K_{off} ", as used herein, refers to the off rate constant for dissociation of a binding agent (e.g., an antibody or binding component thereof) from a complex with its partner (e.g., the epitope to which the antibody or binding component thereof binds).

[0091] " K_{on} ", as used herein, refers to the on rate constant for association of a binding agent (e.g., an antibody or binding component thereof) with its partner (e.g., the epitope to which the antibody or binding component thereof binds).

[0092] "*Linker*", as used herein, is used to refer to that portion of a multi-element polypeptide that connects different elements to one another. For example, those of ordinary skill in the art appreciate that a polypeptide whose structure includes two or more functional or organizational domains often includes a stretch of amino acids between such domains that links them to one another. In some embodiments, a polypeptide comprising a linker element has an overall structure of the general form S1-L-S2, wherein S1 and S2 may be the same or different and represent two domains associated with one another by the linker. In some embodiments, a linker is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids in length. In some embodiments, a linker is characterized in that it tends not to adopt a rigid three-dimensional structure, but rather provides flexibility to the polypeptide. A variety of different linker elements that can appropriately be used when engineering polypeptides (e.g., fusion polypeptides) known in the art (see e.g.,

Holliger, P., et al. (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak, R. J., et al. (1994) Structure 2: 1 121-1123).

[0093] "*Multivalent binding agent*", as used herein, refers a binding agent capable of binding to two or more antigens, which can be on the same molecule or on different molecules. Multivalent binding agents as described herein are, in some embodiments, engineered to have the three or more antigen binding sites, and are typically not naturally occurring proteins. Multivalent binding agents as described herein refer to binding agents capable of binding two or more related or unrelated targets. Multivalent binding agents may be composed of multiple copies of a single antibody component or multiple copies of different antibody components. Such binding agents are capable of binding to two or more antigens and are tetravalent or multivalent binding agents. Multivalent binding agents may additionally comprise a therapeutic agent, such as, for example, an immunomodulator, toxin or an RNase. Multivalent binding agents as described herein are, in some embodiments, capable of binding simultaneously to at least two targets that are of different structure, e.g., two different antigens, two different epitopes on the same antigen, or a hapten and/or an antigen or epitope. In many embodiments, multivalent binding agents of the present invention are proteins engineered to have characteristics of multivalent binding agents as described herein. Multivalent binding agents of the present invention may be monospecific (capable of binding one antigen) or multispecific (capable of binding two or more antigens), and may be composed of two heavy chain polypeptides and two light chain polypeptides. Each binding site, in some embodiments, is composed of a heavy chain variable domain and a light chain variable domain with a total of six CDRs involved in antigen binding per antigen binding site.

[0094] "*Nucleic acid*", as used herein, in its broadest sense, refers to any compound and/or substance that is or can be incorporated into an oligonucleotide chain. In some embodiments, a nucleic acid is a compound and/or substance that is or can be incorporated into an oligonucleotide chain via a phosphodiester linkage. As will be clear from context, in some embodiments, "*nucleic acid*" refers to individual nucleic acid residues (e.g., nucleotides and/or nucleosides); in some embodiments, "*nucleic acid*" refers to an oligonucleotide chain comprising individual nucleic acid residues. In some embodiments, a "*nucleic acid*" is or comprises RNA; in some embodiments, a "*nucleic acid*" is or comprises DNA. In some embodiments, a nucleic acid is, comprises, or consists of one or more natural nucleic acid residues. In some embodiments, a nucleic acid is, comprises, or consists of one or more nucleic acid analogs. In some embodiments, a nucleic acid analog differs from a nucleic acid in that it does not utilize a phosphodiester backbone. For example, in some

embodiments, a nucleic acid is, comprises, or consists of one or more "*peptide nucleic acids*", which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention. Alternatively or additionally, in some embodiments, a nucleic acid has one or more phosphorothioate and/or 5'-N-phosphoramidite linkages rather than phosphodiester bonds. In some embodiments, a nucleic acid is, comprises, or consists of one or more natural nucleosides (e.g., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxy guanosine, and deoxycytidine). In some embodiments, a nucleic acid is, comprises, or consists of one or more nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 0(6)-methylguanine, 2-thiocytidine, methylated bases, intercalated bases, and combinations thereof). In some embodiments, a nucleic acid comprises one or more modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose) as compared with those in natural nucleic acids. In some embodiments, a nucleic acid has a nucleotide sequence that encodes a functional gene product such as an RNA or protein. In some embodiments, a nucleic acid includes one or more introns. In some embodiments, nucleic acids are prepared by one or more of isolation from a natural source, enzymatic synthesis by polymerization based on a complementary template (*in vivo* or *in vitro*), reproduction in a recombinant cell or system, and chemical synthesis. In some embodiments, a nucleic acid is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 or more residues long. In some embodiments, a nucleic acid is single stranded; in some embodiments, a nucleic acid is double stranded. In some embodiments a nucleic acid has a nucleotide sequence comprising at least one element that encodes, or is the complement of a sequence that encodes, a polypeptide. In some embodiments, a nucleic acid has enzymatic activity.

[0095] "*Operably linked*", as used herein, refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "*operably linked*" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. "*Operably linked*" sequences include both expression control sequences that are contiguous with the gene of

interest and expression control sequences that act in trans or at a distance to control the gene of interest. The term "*expression control sequence*" as used herein refers to polynucleotide sequences that are necessary to effect the expression and processing of coding sequences to which they are ligated. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. The nature of such control sequences differs depending upon the host organism. For example, in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence, while in eukaryotes, typically, such control sequences include promoters and transcription termination sequence. The term "*control sequences*" is intended to include components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

[0096] "*Physiological conditions*", as used herein, has its art-understood meaning referencing conditions under which cells or organisms live and/or reproduce. In some embodiments, the term refers to conditions of the external or internal milieu that may occur in nature for an organism or cell system. In some embodiments, physiological conditions are those conditions present within the body of a human or non-human animal, especially those conditions present at and/or within a surgical site. Physiological conditions typically include, e.g., a temperature range of 20 - 40°C, atmospheric pressure of 1, pH of 6-8, glucose concentration of 1-20 mM, oxygen concentration at atmospheric levels, and gravity as it is encountered on earth. In some embodiments, conditions in a laboratory are manipulated and/or maintained at physiologic conditions. In some embodiments, physiological conditions are encountered in an organism.

[0097] "*Polypeptide*", as used herein, refers to any polymeric chain of amino acids. In some embodiments, a polypeptide has an amino acid sequence that occurs in nature. In some embodiments, a polypeptide has an amino acid sequence that does not occur in nature. In some embodiments, a polypeptide has an amino acid sequence that is engineered in that it is designed and/or produced through action of the hand of man. In some embodiments, a polypeptide may comprise or consist of natural amino acids, non-natural amino acids, or both. In some embodiments, a polypeptide may comprise or consist of only natural amino acids or only non-natural amino acids. In some embodiments, a polypeptide may comprise D-amino acids, L-amino

acids, or both. In some embodiments, a polypeptide may comprise only D-amino acids. In some embodiments, a polypeptide may comprise only L-amino acids. In some embodiments, a polypeptide may include one or more pendant groups or other modifications, e.g., modifying or attached to one or more amino acid side chains, at the polypeptide's N-terminus, at the polypeptide's C-terminus, or any combination thereof. In some embodiments, such pendant groups or modifications may be selected from the group consisting of acetylation, amidation, lipidation, methylation, pegylation, etc., including combinations thereof. In some embodiments, a polypeptide may be cyclic, and/or may comprise a cyclic portion. In some embodiments, a polypeptide is not cyclic and/or does not comprise any cyclic portion. In some embodiments, a polypeptide is linear. In some embodiments, a polypeptide may be or comprise a stapled polypeptide. In some embodiments, the term "polypeptide" may be appended to a name of a reference polypeptide, activity, or structure; in such instances it is used herein to refer to polypeptides that share the relevant activity or structure and thus can be considered to be members of the same class or family of polypeptides. For each such class, the present specification provides and/or those skilled in the art will be aware of exemplary polypeptides within the class whose amino acid sequences and/or functions are known; in some embodiments, such exemplary polypeptides are reference polypeptides for the polypeptide class. In some embodiments, a member of a polypeptide class or family shows significant sequence homology or identity with, shares a common sequence motif (e.g., a characteristic sequence element) with, and/or shares a common activity (in some embodiments at a comparable level or within a designated range) with a reference polypeptide of the class; in some embodiments with all polypeptides within the class). For example, in some embodiments, a member polypeptide shows an overall degree of sequence homology or identity with a reference polypeptide that is at least about 30-40%, and is often greater than about 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more and/or includes at least one region (i.e., a conserved region that may in some embodiments may be or comprise a characteristic sequence element) that shows very high sequence identity, often greater than 90% or even 95%, 96%, 97%, 98%, or 99%. Such a conserved region usually encompasses at least 3-4 and often up to 20 or more amino acids; in some embodiments, a conserved region encompasses at least one stretch of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more contiguous amino acids. In some embodiments, a useful polypeptide may comprise or consist of a fragment of a parent polypeptide. In some embodiments, a useful polypeptide as may comprise or consist of a plurality of fragments, each of which is found in the same parent polypeptide in a different spatial

arrangement relative to one another than is found in the polypeptide of interest (e.g., fragments that are directly linked in the parent may be spatially separated in the polypeptide of interest or vice versa, and/or fragments may be present in a different order in the polypeptide of interest than in the parent), so that the polypeptide of interest is a derivative of its parent polypeptide

[0098] “*Prevent*” or “*prevention*”, as used herein when used in connection with the occurrence of a disease, disorder, and/or condition, refers to reducing the risk of developing the disease, disorder and/or condition and/or to delaying onset of one or more characteristics or symptoms of the disease, disorder or condition. Prevention may be considered complete when onset of a disease, disorder or condition has been delayed for a predefined period of time.

[0099] “*Recombinant*”, as used herein, is intended to refer to polypeptides (e.g., antibodies or antibody components, or multispecific binding agents as described herein) that are designed, engineered, prepared, expressed, created or isolated by recombinant means, such as polypeptides expressed using a recombinant expression vector transfected into a host cell, polypeptides isolated from a recombinant, combinatorial human polypeptide library (Hoogenboom H. R., (1997) TIB Tech. 15:62-70; Azzazy H., and Highsmith W. E., (2002) Clin. Biochem. 35:425-445; Gavilondo J. V., and Larrick J. W. (2002) BioTechniques 29: 128-145; Hoogenboom H., and Chames P. (2000) Immunology Today 21:371-378), antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes (see e.g., Taylor, L. D., et al. (1992) Nucl. Acids Res. 20:6287-6295; Little M. et al. (2000) Immunology Today 21:364-370; Kellermann S-A., and Green L. L. (2002) Current Opinion in Biotechnology 13:593-597; Murphy, A.J. et al., (2014) Proc. Natl. Acad. Sci. U.S.A. 111(14):5153-5158) or polypeptides prepared, expressed, created or isolated by any other means that involves splicing selected sequence elements to one another. In some embodiments, one or more of such selected sequence elements is found in nature. In some embodiments, one or more of such selected sequence elements is designed *in silico*. In some embodiments, one or more such selected sequence elements results from mutagenesis (e.g., *in vivo* or *in vitro*) of a known sequence element, e.g., from a natural or synthetic source. For example, in some embodiments, a recombinant antibody polypeptide is comprised of sequences found in the germline of a source organism of interest (e.g., human, mouse, etc.). In some embodiments, a recombinant antibody has an amino acid sequence that resulted from mutagenesis (e.g., *in vitro* or *in vivo*, for example in a transgenic animal), so that the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while originating from and related to

germline V_H and V_L sequences, may not naturally exist within the germline antibody repertoire *in vivo*.

[00100] "*Recovering*", as used herein, refers to the process of rendering an agent or entity substantially free of other previously-associated components, for example by isolation, e.g., using purification techniques known in the art. In some embodiments, an agent or entity is recovered from a natural source and/or a source comprising cells.

[00101] "*Reference*", as used herein describes a standard or control agent, animal, individual, population, sample, sequence or value against which an agent, animal, individual, population, sample, sequence or value of interest is compared. In some embodiments, a reference agent, animal, individual, population, sample, sequence or value is tested and/or determined substantially simultaneously with the testing or determination of the agent, animal, individual, population, sample, sequence or value of interest. In some embodiments, a reference agent, animal, individual, population, sample, sequence or value is a historical reference, optionally embodied in a tangible medium. Typically, as would be understood by those skilled in the art, a reference agent, animal, individual, population, sample, sequence or value is determined or characterized under conditions comparable to those utilized to determine or characterize the agent, animal, individual, population, sample, sequence or value of interest.

[00102] "*Risk*", as will be understood from context, "*risk*" of a disease, disorder, and/or condition comprises likelihood that a particular individual will develop a disease, disorder, and/or condition (e.g., a radiation injury). In some embodiments, risk is expressed as a percentage. In some embodiments, risk is from 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 up to 100%. In some embodiments risk is expressed as a risk relative to a risk associated with a reference sample or group of reference samples. In some embodiments, a reference sample or group of reference samples have a known risk of a disease, disorder, condition and/or event (e.g., a radiation injury). In some embodiments a reference sample or group of reference samples are from individuals comparable to a particular individual. In some embodiments, relative risk is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more.

[00103] "*Specific binding*", as used herein, refers to a binding agent's ability to discriminate between possible partners in the environment in which binding is to occur. A binding agent that interacts with one particular target when other potential targets are present is said to "*bind specifically*" to the target with which it interacts. In some embodiments, specific binding is assessed by detecting or determining degree of association between the binding agent and its

partner; in some embodiments, specific binding is assessed by detecting or determining degree of dissociation of a binding agent-partner complex; in some embodiments, specific binding is assessed by detecting or determining ability of the binding agent to compete an alternative interaction between its partner and another entity. In some embodiments, specific binding is assessed by performing such detections or determinations across a range of concentrations.

[00104] "*Subject*", as used herein, means any mammal, including humans. In certain embodiments of the present invention the subject is an adult, an adolescent or an infant. In some embodiments, terms "*individual*" or "*patient*" are used and are intended to be interchangeable with "*subject*". Also contemplated by the present invention are the administration of the pharmaceutical compositions and/or performance of the methods of treatment in-utero.

[00105] "*Substantially*": As used herein, the term "*substantially*" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "*substantially*" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[00106] "*Substantial sequence homology*", as used herein refers to a comparison between amino acid or nucleic acid sequences. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be "*substantially homologous*" if they contain homologous residues in corresponding positions. Homologous residues may be identical residues. Alternatively, homologous residues may be non-identical residues will appropriately similar structural and/or functional characteristics. For example, as is well known by those of ordinary skill in the art, certain amino acids are typically classified as "*hydrophobic*" or "*hydrophilic*" amino acids, and/or as having "*polar*" or "*non-polar*" side chains. Substitution of one amino acid for another of the same type may often be considered a "*homologous*" substitution. Typical amino acid categorizations are summarized in Table 1 and 2.

TABLE 1

Alanine	Ala	A	Nonpolar	Neutral	1.8
Arginine	Arg	R	Polar	Positive	-4.5
Asparagine	Asn	N	Polar	Neutral	-3.5
Aspartic acid	Asp	D	Polar	Negative	-3.5
Cysteine	Cys	C	Nonpolar	Neutral	2.5

Glutamic acid	Glu	E	Polar	Negative	-3.5
Glutamine	Gln	Q	Polar	Neutral	-3.5
Glycine	Gly	G	Nonpolar	Neutral	-0.4
Histidine	His	H	Polar	Positive	-3.2
Isoleucine	Ile	I	Nonpolar	Neutral	4.5
Leucine	Leu	L	Nonpolar	Neutral	3.8
Lysine	Lys	K	Polar	Positive	-3.9
Methionine	Met	M	Nonpolar	Neutral	1.9
Phenylalanine	Phe	F	Nonpolar	Neutral	2.8
Proline	Pro	P	Nonpolar	Neutral	-1.6
Serine	Ser	S	Polar	Neutral	-0.8
Threonine	Thr	T	Polar	Neutral	-0.7
Tryptophan	Trp	W	Nonpolar	Neutral	-0.9
Tyrosine	Tyr	Y	Polar	Neutral	-1.3
Valine	Val	V	Nonpolar	Neutral	4.2

TABLE 2

Ambiguous Amino Acids	3-Letter	1-Letter
Asparagine or aspartic acid	Asx	B
Glutamine or glutamic acid	Glx	Z
Leucine or Isoleucine	Xle	J
Unspecified or unknown amino acid	Xaa	X

[00107] As is well known in this art, amino acid or nucleic acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST, and PSI-BLAST for amino acid sequences. Exemplary such programs are described in Altschul et al., Basic local alignment search tool, J. Mol. Biol., 215(3): 403-410, 1990; Altschul et al., Methods in Enzymology; Altschul et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402, 1997; Baxevanis et al., Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins, Wiley, 1998; and Misener et al., (eds.), Bioinformatics Methods and Protocols (Methods in Molecular Biology, Vol. 132), Humana Press, 1999; all of the foregoing of which are incorporated herein by reference. In addition to identifying homologous sequences, the programs mentioned above typically provide an indication of the degree of homology. In some embodiments, two sequences are considered to be substantially homologous if at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or more of their

corresponding residues are homologous over a relevant stretch of residues. In some embodiments, the relevant stretch is a complete sequence. In some embodiments, the relevant stretch is at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, at least 100, at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, at least 300, at least 325, at least 350, at least 375, at least 400, at least 425, at least 450, at least 475, at least 500 or more residues.

[00108] "*Substantial identity*", as used herein refers to a comparison between amino acid or nucleic acid sequences. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be "substantially identical" if they contain identical residues in corresponding positions. As is well known in this art, amino acid or nucleic acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST, and PSI-BLAST for amino acid sequences. Exemplary such programs are described in Altschul et al., Basic local alignment search tool, J. Mol. Biol., 215(3): 403-410, 1990; Altschul et al., Methods in Enzymology; Altschul et al., Nucleic Acids Res. 25:3389-3402, 1997; Baxevanis et al., Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins, Wiley, 1998; and Misener, et al, (eds.), Bioinformatics Methods and Protocols (Methods in Molecular Biology, Vol. 132), Humana Press, 1999. In addition to identifying identical sequences, the programs mentioned above typically provide an indication of the degree of identity. In some embodiments, two sequences are considered to be substantially identical if at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of their corresponding residues are identical over a relevant stretch of residues. In some embodiments, the relevant stretch is a complete sequence. In some embodiments, the relevant stretch is at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or more residues. In the context of a CDR, reference to "*substantial identity*" typically refers to a CDR having an amino acid sequence at least 80%, preferably at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to that of a reference CDR.

[00109] "*Surface plasmon resonance*", as used herein, refers to an optical phenomenon that allows for the analysis of specific binding interactions in real-time, for example through detection of alterations in protein concentrations within a biosensor matrix, such as by using a BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, N.J.). For further descriptions, see

Jonsson, U., et al. (1993) Ann. Biol. Clin. 51 : 19-26; Jonsson, U., et al. (1991) Biotechniques 11:620-627; Johnsson, B., et al. (1995) J. Mol. Recognit. 8: 125-131; and Johnson, B., et al. (1991) Anal. Biochem. 198:268-277.

[00110] "*Therapeutically effective amount*", as used herein, is meant an amount that produces the desired effect for which it is administered. In some embodiments, the term refers to an amount that is sufficient, when administered to a population suffering from or susceptible to a disease, disorder, and/or condition in accordance with a therapeutic dosing regimen, to treat the disease, disorder, and/or condition. In some embodiments, a therapeutically effective amount is one that reduces the incidence and/or severity of, and/or delays onset of, one or more symptoms of the disease, disorder, and/or condition. Those of ordinary skill in the art will appreciate that the term "*therapeutically effective amount*" does not in fact require successful treatment be achieved in a particular individual. Rather, a therapeutically effective amount may be that amount that provides a particular desired pharmacological response in a significant number of subjects when administered to patients in need of such treatment. In some embodiments, reference to a therapeutically effective amount may be a reference to an amount as measured in one or more specific tissues (e.g., a tissue affected by the disease, disorder or condition) or fluids (e.g., blood, saliva, serum, sweat, tears, urine, etc.). Those of ordinary skill in the art will appreciate that, in some embodiments, a therapeutically effective amount of a particular agent or therapy may be formulated and/or administered in a single dose. In some embodiments, a therapeutically effective agent may be formulated and/or administered in a plurality of doses, for example, as part of a dosing regimen.

[00111] "*Transformation*", as used herein, refers to any process by which exogenous DNA is introduced into a host cell. Transformation may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. In some embodiments, a particular transformation methodology is selected based on the host cell being transformed and may include, but is not limited to, viral infection, electroporation, mating, lipofection. In some embodiments, a "*transformed*" cell is stably transformed in that the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. In some embodiments, a transformed cell transiently expresses introduced nucleic acid for limited periods of time.

[00112] "*Vector*", as used herein, refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "*plasmid*", which refers to

a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "*expression vectors*."

[00113] Standard techniques may be used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures may be generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)), which is incorporated herein by reference for any purpose.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[00114] The present invention demonstrates the successful humanization of a murine antibody that binds an established melanoma associated tumor antigen. Thus, the present invention provides, among other things, humanized antibodies that bind to chondroitin sulfate proteoglycan 4 (CSPG4) or high molecular weight-melanoma associated antigen (HMW-MAA), also known as melanoma cell surface proteoglycan (MCSP) and neuron-glia protein 2 (NG2). The present invention specifically provides the first successful humanization of a murine anti-CSPG4 antibody, mouse 763.74 (referred to herein as mouse 763, m763 or 763), and furthermore provides multiple human IgG formats (e.g., IgG1 and IgG4) thereof.

[00115] Among other things, the present disclosure specifically demonstrates that humanized 763 antibodies described herein retain unusually slow k_{off} rates, high affinity (e.g., nanomolar) and immunoreactivity after iodination as compared to the parental mouse 763 antibody. Further, the present disclosure specifically demonstrates that humanized 763 antibodies are highly efficient at targeting tumors *in vivo*. To give one specific example, the present disclosure demonstrates that

unlike any other anti-CSPG4 antibodies, a specifically engineered variant glycoform of a humanized 763 antibody mediates efficient antibody-dependent cell-mediated cytotoxicity (ADCC).

[00116] The present inventors also demonstrate herein the first successful construction of a highly potent fully humanized bispecific antibody (referred to herein as hu763-BsAb) that retargets T cells to CSPG4⁺ tumors and, therefore, is useful in cancer therapy. Furthermore, as described herein, bispecific antibodies of the present invention provide an improvement over existing bispecific antibodies that engage T cells and address a common problem in the field.

Overstimulation of T cells resulting from engagement of CD3 by bispecific antibodies has been reported to contribute to the release of cytokines, which, in combination with Fc receptor binding and subsequent activation of complement, has a negative impact in patients resulting from a cytokine cascade. As described herein, bispecific antibodies of the present invention promote the release of cytokines by engaging CD3 on T cells only in the presence of tumor cells and, therefore, provide therapeutic bispecific antibodies with an improved safety profile.

[00117] Without wishing to be bound by theory, we note that data provided herein demonstrate that, in some embodiments, (e.g., where humanization of murine antibodies typically results in loss of affinity to antigen), detectable negative impact on affinity was not observed. Moreover, the present disclosure demonstrates, among other things, that humanization of murine 763 antibody as described herein did not negatively affect binding to the conformational epitope bound as compared to the parental murine antibody. The present disclosure also demonstrates, the design and construction of bispecific antibodies utilizing a specific format that combines bivalent binding to a tumor antigen and monovalent binding to T cells. We note that data provided herein demonstrate that, in some embodiments, (e.g., which includes variant Fc regions that do not bind FcRs and, therefore, do not activate complement), such a format provides efficient and potent targeting of T cells to CSPG4⁺ tumors without adverse effects of cytokine cascade (“cytokine storm”). Thus, in at least some embodiments, the present disclosure embraces the selection of a bispecific antibody format that eliminates the possibility of over stimulating T cells and achieves enhanced tumor targeting, and humanized antibodies that retain the high affinity binding to a conformational epitope of the parental murine antibody.

Tumors

[00118] In some embodiments, any tumor that expresses CSPG4 can be considered a CSPG4⁺ tumors. In some embodiments, a CSPG4⁺ tumor may arise from any tissue type. In some

embodiments, a CSPG4⁺ tumor may be a solid tumor. In some embodiments, a CSPG4⁺ tumor may include or comprise a soft tissue sarcoma, cerebral tumor, bone tumor, breast carcinoma, squamous cell carcinoma, pancreatic tumor, stomach tumor, melanoma and/or mesothelioma. In some embodiments, a CSPG4⁺ tumor may include or comprise a fibrosarcoma, leiomyosarcoma, pleomorphic sarcoma, liposarcoma, synovial sarcoma, chondrosarcoma, glioblastoma, chordoma, lobular breast carcinoma, TNBC breast carcinoma, ER⁺ breast carcinoma, HER2⁺ breast carcinoma, ductal breast carcinoma, oral cavity squamous cell carcinoma, pancreatic cystadenoma, pancreatic intraductal papillary mucinous neoplasm, pancreatic ductal malignancy, uveal melanoma, NS melanoma, acral lentiginous melanoma, nodular melanoma, superficial spreading melanoma, conjunctival melanoma, desmoplastic melanoma, sacromatoid mesothelioma, epithelioid mesothelioma, biphasic mesothelioma, osteosarcoma, head and neck cancer, glioblastoma multiforme, sarcoma, adenocarcinoma, or colorectal adenocarcinoma. In some embodiments, a CSPG4⁺ tumor is a melanoma, osteosarcoma, head and neck tumor, glioblastoma multiforme, sarcoma and/or mesothelioma.

Melanoma

[00119] The incidence of melanoma worldwide is rising rapidly with an annual increase by 3–7%. In the United States, the incidence almost tripled among males and more than doubled among females between 1973 and 1997, affecting approximately 22 per 100,000 males and 14 for 100,000 females. This translates to approximately 59,580 new diagnoses and 7770 deaths from melanoma in 2005 alone, according to the American Cancer Society. In the early stages of melanoma, surgery represents a potential curative modality. However, in nonresectable stage III or IV malignant melanoma, the prognosis remains very poor. The median survival of stage IV disease is approximately 6–10 months with only about 4–6% surviving to 5 years. As of 2010, less than 5 years ago, systemic chemotherapy is the mainstay of treatment, but it is generally considered palliative rather than curative. Until recently, few therapeutic agents have produced response rates >20%. In meta-analyses, the response to dacarbazine monotherapy ranged between 5.3% and 28.0% (with an average of 15.3%). Biochemotherapy, where high dose chemotherapy was combined with interferon or interleukin-2 (IL-2), did not improve survival. In the previous decade, of the seven completed randomized phase III adjuvant melanoma vaccine trials using self-antigens, none have shown a benefit. Induced cytotoxic T lymphocytes (CTLs) typically fail to home to the

site(s) of a tumor. Moreover, since these target antigens are non-essential for melanoma, tumor escape following single target vaccine is expected.

[00120] Two recent developments have begun to change the prognosis of high-risk melanoma, namely, small molecule inhibitors (e.g. BRAF inhibitors) and immune checkpoints manipulations (e.g. anti-CTLA4, anti-PD-L1, anti-PD1). Both human anti-CTLA-4 IgG1 monoclonal antibodies ipilimumab and tremelimumab have generated durable clinical responses in melanoma and other cancers, accompanied in some by autoimmune side effects. Adoptive T-cell therapy has also received much attention recently, and when combined with myeloablative therapy has produced unusually high response rates. These human experiments have repeatedly shown the potential for T cells in controlling and/or ameliorating melanoma.

[00121] Monoclonal antibodies can induce cell death, promote blockade of signaling pathways, induce antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). Several antibodies (R24, 9.2.27, 3F8 and CE7) have been successfully tested in the clinic against melanoma targeting GD3 (Houghton, A.N. et al. (1985) Proc. Natl. Acad. Sci. U.S.A. 82:1242-1246), HMW-MAA (or CSPG4; Raja, C. et al. (2007) Cancer Biol. Ther. 6:846-852), GD2 (Cheung, N.K. et al. (1987) Oncol. 5:1430-1440) and L1CAM (Meier, F. et al. (2006) Int. J. Cancer 119:549-555; Novak-Hofer, I. (2007) Cancer Biother. Radiopharm. 22:175-184), respectively.

Chondroitin Sulfate Proteoglycan 4

[00122] Chondroitin sulfate proteoglycan 4 (CSPG4, also known as HMW-MAA, MCSP, MCSPG, MEL-CSPG, MSK16, NG2) is a 250 kD glycoprotein that is expressed at high levels and in >85% of melanomas (Kantor, R.R. et al. (1982) Hybridoma 1:473-482), 70% of gliomas, 50% of chondromas and chondrosarcomas, 55% of acute lymphocytic leukemias (ALL), 100% of mesotheliomas (Rivera, Z. et al. (2012) Clin. Cancer Res. 18:5352-5363), 77% of invasive ductal breast carcinomas, 50% of head and neck squamous cell carcinomas (HNSCC), glioblastomas, clear cell renal carcinomas, neuroblastomas and sarcomas (Geldres, C. et al. (2014) Clin. Cancer Res. 20:962-971). Exemplary amino acid sequences of mouse and human CSPG4 are presented below (signal peptides are italicized).

Human CSPG4 (NP_001888)

MQSGPRPPLPAPGLALALTLTMLARLASAASFFGENHLEVPVATALTDIDLQLQFSTSQPEALL
 LLAAGPADHLLLQLYSGRLQVRLVLGQEELRLQTPAETLLSDSIPHTVVLTVEGWATLSV
 DGFLNASSAVPGAPLEVPYGLFVGGTGTLGLPYLRGTSRPLRGCLHAATLNGRSLRPLTP
 DVHEGCAEEFSASDDVALGFSGPHSLAAFPWGTQDEGTLEFTLTTQSRQAPLAFQAGGR

RGDFIYVDIFEGHLRAVVEKGQGTVLLHNSVPVADGQPHEVSVHINahrleISVDQYPTHT
 SNRGVLSYLEPRGSLLLGGLDAEASRHLQEHLRLGLTPEATNASLLGCMEDLSVNGQRRGL
 REALLTRNMAAGCRLEEEYEDDAYGHYEAFSTLAPEAWPAMELPEPCVPEPGLPPVFAN
 FTQLLTISPLVVAEGGTAWLEWRHVQPTLDLMEAE LRKSQVLFVTRGARHGELELDIPGA
 QARKMFTLLDVVNRKARFIHDGSEDTS DQLVLEVSVTARVPMPSCLRRGQTYLLPIQVNPV
 NDPPHIIFPHGSLMVILEHTQKPLGPEVFQAYDPDSACEGLTFQVLGTSSGLPVERRDQPGEP
 ATEFSCRELEAGSLVYVHRGGPAQDLTFRVSDGLQASPPATLKVVAIRPAIQIHRSTGLRLA
 QGSAMPILPANLSVETNAVGGQDVSVLFRVTGALQFGELQKQGAGGVEGAEWATQAFHQ
 RDVEQGRVRYLSTDPQHHA YDTVENLAL EVQVGQEILSNLSFPVTIQRATVWMLRLEPLH
 TQNTQQETLTTAHLEATLEEAGSPPTFH YEVVQAPRKGNLQLQGTRLSDGQGFTQDDIQA
 GRVTYGATARASEAVEDTFRFRVTAPPYFSPLYTFPIHIGGDPDAPVL TNVLLVPEGGEGV
 LSADHLFVKSLNSASYLYEVMERPRHGR LAWRTQDKTTMVTSTNEDLLRGRLVYQHD
 DSETTEDDIPFVATRQGESSGDMAWEEVRGVFRVAIQPVNDHAPVQTISRIFHVARGGRRL
 LTDDVAFSDADSGFADAQLVLTRKDLLFGSIVAVDEPTRPIYRFTQEDLRKRRVLFVHSG
 ADRGWIQLQVSDGQHQA TALLEVQASEPYLRVANGSSLVVPQGGQGTIDTAVLHLD TNLD
 IRSGDEVHYHV TAGPRWGQLVRAGQPATAFSQQDLLDGAVLYSHNGSLSPRDTMAFSVE
 AGPVHTDATLQVTIALEGPLAPLKLVRHKKIYVFQGEAAEIRRDQLEAAQEAVPPADIVFS
 VKSPPSAGYLVMSVRGALADEPPSLDPVQSFSQEA VDTGRVLYLHSRPEAWSDAFSLDVA
 SGLGAPLEGVLVELEVLPAAIPLEAQNFSVPEGGSLTAPPLLRVSGPYFPTLLGLSLQVLEP
 PQHGALQKEDGPQARTLSAFSWRMVEEQ LIRYVHDGSETLTDSFVL MANASEMDRQSHP
 VAFTVTVLVPVNDQPPILTTNTGLQM WEGATAPIPAEALRSTDGDSGSEDLVYTIEQPSNGR
 VVLRGAPGTEVRSFTQAQLDGGLVLF SHRGTLDDGGFRFRLSDGEHTSPGHFFRVTAQKQV
 LLSLKGSQTLTVCPGSGVQPLSSQTLRASSSAGTDPQLLLYRVVRGPQLGRLFHAQQDSTGE
 ALVNFTQAEVYAGNILYEHMPPEPFWEAHD TLELQLSSPPARDVAATLAVAVSFEAACP
 QRPSHLWKNKGLWVPEGQRARITVAALDASNLLASVPSPQRSEHDVLFQVTQFPSRGQLL
 VSEEPLHAGQPHFLQSQLAAGQLVYAHGGGGTQQDGFHFRAHLQGPAGASVAGPQTSEA
 FAITVRDVNERPPQPQASVPLRLTRGSRAPISRAQLSVVDPDSAPGEIEYEVQRAPHNGFLSL
 VGGGLGPVTRFTQADVD SGRLAFVANGSSVAGIFQLSMSDGASPLPMSLAVDILPSAIEV
 QLRAPLEVPPALGRSSLSQQQLRVVSDREEPEAA YRLIQGPQYGHLLVGGRPTSAFSQFQID
 QGEVVFAFTNFSSSHDHFRVLALARGVNASAVVNVTVRALLHVWAGGPWPQGATLRLDP
 TVLDAGELANRTGSPRFRLLLEGPRHGRVVRVPRARTEPGGSQ LVEQFTQQDLEDGRLGL
 EVGRPEGRAPGPAGDSL TLELWAQGVPPAVASLDFATEPYNAARPYSVALLSVPEAARTE
 AGKPESSTPTGEPGPMASSEP AVAKGGFLSFLEANMFSVIIPMCLVLLLLALILPLL FYLRK
 RNKTGKH DVQVLTAKPRNGLAGDTETFRKVEPGQAIPLTAVPGQGPPPGGQPDPELLQFCR
 TPNPALKNGQYWV (SEQ ID NO: 1)

Mouse CSPG4 (NP_620570)

MLLGPGHPLSAPA LALALTLALLVRSTAPASFFGENHLEVVPVSALTRVDLLLQFSTSQPEALL
 LLAAGQDDHLLLQLHSGCLQVRLALGQKELKLQTPADTVLSDSAPHTVVLT VSDSWAVLS
 VDGVLNTSAPIPRASHLKATYGLFVGSSGSLDLPYLGISRPLRGCLHSAILNGRNLLRPLTS
 DVHEGCAEEFSAGDEVGLGFSGPHSLAAFP AWSTREEGTLEFTLTTRSQQAPLAFQAGDKR
 GNFIYVDIFEGHLRAVVEKGQGTMLLRNSVPVADGQPHEVSVHIDVHRLEISVDQYPTRTF
 NRGVLSYLEPRGSLLLGGLDTEASRHLQEHLRLGLAPGAANISLVGCIEDFSVNGRRQGLRD
 AWLTRDMSAGCRPEEDEYEEYGYETFSTLAPEAWPAMELPEPCIPEPGLPAVFANFTQ
 LLTISPLVVAEGGTAWLEWRHVQPTLDL TEAE LRKSQVLFVSQSARHGDLELDILGAQTR
 KMFTLLDVVNRKARFVHDGSEDTS DQLMLEVSVTARAPVPSCLRRGQIYILPIQVNPVNDP
 PRIIFPHGSLMVILEHTQKPLGPEIFQAYDPDSACEGLTFQLLGVS SGVPVEHRDQPGEPATE
 FSCRELEVGDIVYVHRGGPAQDLTFRVSDGMQASAPATLKVVAVRPAIQILHNTGLHLAQ

GSAAAILPANLSVETNAVGGQDVSVLFRVTGTLQFGELQKQGAGGVEGTEWWDTLAFHQR
DVEQGRVRYLSTDPQHHTQDQTVEDLILEVQVGGQETLSNLSFPVTIQRATVWMLRLEPLHTQ
NPHQETLTPAHLEASLEEEEEEGSPQPHTFHYELVQAPRRGNLLLQGTRLSDGESFSQSDLQ
AGRVTYRATMRTSEAAADDSFRFRVTSPPHFSPLYTFPIHIGGDPNAPVLTNVLLMVPEGGEG
VLSADHLFLVKSLSNASYLYEVMEQPHHGKLAWRDPKKGKSTPVTSTFTNEDLLHGRLVYQH
DDSETIEDDIPFVATRQGGEGSGDMAWEEVRGVFRVAIQPVNDHAPVQTISR VFHVARGGQR
LLTTDDVAFSDADSGFSDAQLVLTRKDLLFGSIVAMEEPTPIYRFTQEDLRKKQVLFVHSG
ADHGWLQLQVSDGQHQAATAMLEVQASEPYLHVANSSSLVVPQGGGQTIDTAVLQLDNL
DIRSGNEVHYHVTAGPQWGQLLRDGGQSVTSFSQRDLLDGAILYSHNGSLSPQDTLAFSVAA
GPVHTNTFLQVTIALEGPLAPLQLVQHKKIYVFQGEAAEIRRDQLEVVQEAVLPADIMFSLR
SPPNAGYLVMSHGASAEPPSLDPVQSFSQEAVNSGRVLYLHSRPGAWSDFSGLDVASGL
GDPLEGISVELEVLPVPLDVQNFVPEGGTRTLAPPLVQITGPYFPTLPGLVLQVLEPPQH
GALQKEDHSQDGSLSSTFSWREVEEQLIRYVHDGSETQTD AFVLLANASEMDRQSQPVAFTI
TILPVNDQPPVLTNTTGLQIWEGAIVPIPEALRGTDNDSGPEDLVYTIEQPSNGRIALRVAP
DTEVHRFTQAQLDSGLVLFSHRGALEGGFHFDSLSDGAHTSPGHFFRVVAQKQALLSLEGTR
KLTVCPEVSVQPLSSQSLSASSSTGADPRHLLYRVVRGPQLGRLLHAQQGSAEEVLVNFTQA
EVNAGNILYEHEMSSEPFWEAHDITIGLLLSSPPARDLAATLAVMVSFDAACQQRPSRLWKN
KGLWVPEGQRAKITVAALDAANLLASVPASQRSRHDVLFQVTQFPTRGQLLVSEEPLHAR
RPYFLQSELAAGQLVYAHGGGGTQQDGFRRFRAHLQGPTGTSVAGPQTSEAFVITVRDVNE
RPPQPQASIPLRVTRGSRAPVSRAQLSVVDPDSAPGEIEYEVQGRAPHNGFLSLAGDNTGPVT
HFTQADVDAGRLAFVANGSSVAGVFQLSMSDGASPPIMSLAVDVLPSTIEVQLRAPLEV
QALGRTSLSRQQLQVISDREEDVAYRLTQGPLYQQLLVGGQPASAFSQLQVDQGDVVVFV
FTNFSSSQDHFVVALARGVNASATVNVTVQALLHVWAGGPWPQGTTLRDPTVLDASE
LANRTGSMPHFRLLAGPRYGRVVRVSQGRTESRSNQLVEHFTQRDLEEGQLGLEVGKPEG
RSTGPAGDRLTLELWAKGVPPAVALLDFAEPYHAAKSYSVALLSVPEAVRTEKPKGRSV
PTGQPGQAASSPVPTAAKGGFLGFLEANMFSIIIPVCLILLALLILPLLFLYLRKRKNTGKHD
VQVLTAKPRNGLAGDTETFRKVEPGQAIPLITVPGQGPPPGGQPDPELLQFCRTPNPALRNG
QYWV (SEQ ID NO: 2)

[00123] The biology of CSPG4 protein has been extensively annotated. CSPG4 has been reported to inhibit neurite outgrowth and growth cone collapse during axon regeneration (Bradbury, E.J. et al. (2002) *Nature* 416:636-640). As cell surface receptor for collagen alpha 2(VI), CSPG4 confers cells the ability to migrate on that substrate. CSPG4 binds through its extracellular N-terminus growth factors, extracellular matrix proteases modulating their activity (Stallcup, W.B. et al. (2008) *Cell Adh. Migr.* 2:192-201). CSPG4 also regulates MPP16-dependent degradation and invasion of type I collagen participating in melanoma cells invasion properties (Iida, J. et al. (2001) *J. Biol. Chem.* 276:18786-18794). CSPG4 has been reported to modulate the plasminogen system by enhancing plasminogen activation and inhibiting angiostatin (Kirsch, M. et al. (2004) *Cancer Treat. Res.* 117:285-304). Further, CSPG4 has been reported to function as a signal transducing protein by binding through its cytoplasmic C-terminus scaffolding and signaling proteins (Barritt, D.S. et al. (2000) *J. Cell Biochem.* 79:213-224; Stegmuller, J. et al. (2002) *J. Neurocytol.* 31:497-505; Chatterjee, N. et al. (2008) *J. Biol. Chem.* 283:8310-8317). CSPG4 also promotes retraction

fiber formation and cell polarization through Rho GTPase activation (Campoli, M.R. et al. (2004) Crit. Rev. Immunol. 24:267-296) and stimulates alpha-4, beta-1 integrin-mediated adhesion and spreading by recruiting and activating a signaling cascade through CDC42, ACK1 and BCAR1.17 (Eisenmann, K.M. et al. (1999) Nat. Cell Biol. 1:507-513). Still others have reported that CSPG4 activates FAK and ERK1/ERK2 signaling cascades (Yang, J. et al. (2004) J. Cell Biol. 165:881-891).

[00124] CSPG4 is an adhesion and migration protein on melanoma and tumor activated pericytes, highly conserved throughout evolution, and highly restricted in normal tissues. CSPG4 is expressed by basal breast cancer cell lines, but not by luminal breast cancer cell lines. CSPG4 was reported to be expressed in 73% of primary triple negative breast cancer tumors and cell lines as indicated by an anti-CSPG4 antibody (mAb 225.228), which inhibited tumor growth and metastasis *in vitro* and *in vivo* (Wang, X. et al. (2010) J. Natl. Cancer Inst. 102:1496-1512). CSPG4 was found in 57% of canine malignant melanoma (Mayayo, S.L. et al. (2011) Vet. J. 190:e26-30), and a recent vaccine trial significantly prolonged overall and disease-free survival times (Riccardo, F. et al. (2014) Clin. Cancer Res. 20:3753-3762). The first antibody against human HMW-MAA (CSPG4) described was 9.2.27, which is a mouse IgG2a antibody. Up to 200 mg were infused into humans without major side effects other than fever (Oldham, R.K. et al. (1984) J. Clin. Oncol. 2:1235-1244). Subsequent studies were mainly focused in radioimaging and radioimmunotherapy where the 9.2.27 antibody was conjugated to an alpha-emitter (Del Vecchio, S. et al. (1989) Cancer Res. 49:2783-2789). Among 22 patients with stage IV/in-transit metastatic melanoma treated with intravenous ²¹³Bi-9.2.27 (1.5-25.8 mCi; Raja, C. et al. (2007) Cancer Biol. Ther. 6:846-852), 14% had PR and 50% SD, and toxicity was negligible. Antibody 9.2.27 has also been used successfully as immunotoxin (Godal, A. et al. (1992) Int. J. Cancer 52:631-635) and for detecting osteosarcoma micrometastases (Bruland, O.S. et al. (2005) Clin. Cancer Res. 11:4666-4673). The mouse anti-CSPG4 antibody 763.74 was first described in the 1980's (Natali, P.G. et al. (1989) Cancer Res. 49:1269-1274) and shown to react with both cutaneous and uveal melanoma. The epitope of the mouse 763.74 antibody was mapped by phage display to an amino acid sequence (Luo, W. et al. (2005) J. Immunol. 174:7104-7110). An anti-idiotypic antibody to 763 has been used as a vaccine in clinic trials (Mittelman, A. et al. (1995) Clin. Cancer Res. 1:705-713). Mouse 763 antibody has been reported to significantly inhibit both basal breast tumor experimental and post-surgical lung metastases, and local tumor recurrence in mouse xenografts in mice (Wang, X. et al., supra). A chimeric antigen receptor (CAR) using an scFv constructed from the mouse 763 antibody has also

been described (Reinhold, U. et al. (1999) J. Investig. Dermatol. 112:744-750). CSPG4-CAR modified T cells (derived from antibodies 225.28 or 763) have demonstrated the ability to control tumor growth *in vitro* and *in vivo* in NSG mice xenografted with human melanoma, head and neck squamous cell carcinoma (HNSCC) and breast carcinoma (Geldres, C. et al., supra; Burns, W.R. et al. (2010) Cancer Res. 70:3027-3033). A human scFv derived from a phage library (scFv-FcC21) was recently described and engineered as an scFv-Fc form showing activity *in vitro* and *in vivo* against melanoma (Wang, X. et al. (2011) Cancer Res. 71:7410-7422). An anti-CSPG4 x anti-CD3 bispecific T-cell engager (BiTE) antibody has reportedly been developed (Bluemel, C. et al. (2010) Cancer Immunol. Immunother. 59:1197-1209; Torisu-Itakura, H. et al. (2011) J. Immunother. 34:597-605), however, the clinical status of this therapeutic is unknown. Currently, no bispecific antibodies derived from mouse 763.74 has been successfully developed.

[00125] As described herein, the inventors have developed humanized anti-CSPG4 antibodies based on mouse 763.74 (herein referred to as mouse 763 or m763). Twelve (12) particular such humanized anti-CSPG4 antibodies are explicitly exemplified herein. Without wishing to be bound by any particular theory, the inventors have developed humanized anti-CSPG4 antibodies provided herein on the insight that mouse 763 binds a peptide epitope (not a carbohydrate epitope) with high affinity (as described in the Examples section below), and has similar staining patterns in normal and melanoma tissue as compared to other anti-CSPG4 antibodies (see Tables 3 and 4; neg: negative, 1:positive +, 2:positive ++, 3:positive +++). Among the various heavy and light chain humanized sequences generated and described herein, one V_H and one V_L sequence was chosen based on antigen affinity and stability *in vitro*. Three humanized antibody formats were successfully engineered (hu763-IgG1, hu763-IgG4 and hu763-IgG1n [a special glycoform]). We note that data provided herein demonstrate that the humanized antibodies demonstrated antigen binding comparable to mouse 763, in particular, humanized 763 antibodies demonstrated slow k_{off} rates and highly favorable K_{DS} . Further, we note that unlike any of the published anti-CSPG4 antibodies or fusion proteins, humanized 763 antibodies provided herein mediate highly efficient antibody-dependent cell-mediated cytotoxicity (ADCC) against melanoma cells (e.g., hu763-IgG1n).

[00126] The present invention further provides bispecific antibodies based on humanized 763 antibody sequences, in particular, bispecific antibodies that redirect T cells to target CSPG4 on the surface of melanoma cells. To give two specific examples, the present disclosure demonstrates the successful linkage of an anti-CD3 antibody component (e.g., a humanized OKT3 scFv) to the

carboxyl end of a humanized 763 heavy chain to create an anti-CSPG4 x anti-CD3 bispecific antibody referred to herein as hu763-HC-OKT3 or to the carboxyl end of a humanized 763 light chain to create an anti-CSPG4 x anti-CD3 bispecific antibody referred to herein as hu763-LC-OKT3. Further modifications to bispecific antibodies were made to engineer additional effector functions. For example, an N297A mutation introduced into the Fc region thereby eliminating glycosylation. This elimination of glycosylation lead to a reduced complement activation due to abolishing Fc-receptor binding, which avoids nonspecific cytokine storm that has been reported to accompany engagement of T cells. We note that data provided herein specifically demonstrates that humanized anti-CSPG4 x anti-CD3 bispecific antibodies effectively activate T cells and directed T cells to lyse human tumor cell lines *in vitro*. Moreover, bispecific antibodies provided herein significantly inhibited tumor growth in murine melanoma xenograft models. The data provided herein confirms that humanized mono- and bispecific antibodies described herein represent cancer therapeutics characterized by improved efficacy and safety profiles.

TABLE 3

Comparison of anti-CSPG4 antibodies IHC on normal human tissues					
Name	MOPC21 (2 µg/mL)	225.28 (1 µg/mL)	D2.8.5-C4B8 (1 µg/mL)	9.2.2.7 (1 µg/mL)	m763 (1 µg/mL)
Cerebellum	neg	neg	neg	neg	neg
Frontal Lobe	neg	neg	neg	neg	neg
Pons	neg	neg	neg	neg	neg
Spinal Cord	neg	neg	neg	neg	neg
Muscle	neg	neg	neg	neg	neg
Skeletal	neg	neg	neg	neg	neg
Pancreas	neg	neg	neg	neg	neg
Liver	neg	neg	neg	neg	neg
Lung	neg	neg	neg	neg	neg
Spleen	neg	neg	neg	neg	neg
Thyroid	neg	neg	neg	neg	neg
Kidney	neg	neg	neg	neg	neg
Testes	neg	neg	neg	neg	neg
Adrenal	neg	neg	neg	neg	neg
Ileum	neg	2	1	2	2
Sigmoid Colon	neg	2	1	2	2
Stomach	neg	1	1	1	1

TABLE 4

Comparison of anti-CSPG4 antibodies IHC on human melanoma tissues					
Melanoma	MOPC21	225.28	D2.8.5-C4B8	9.2.2.7	m763

Sample #	(2 µg/mL)	(1 µg/mL)	(1 µg/mL)	(1 µg/mL)	(1 µg/mL)
619	neg	neg	neg	neg	neg
1926	neg	neg	neg	neg	neg
2665	neg	neg	neg	neg	neg
2673	neg	neg	neg	neg	neg
524	neg	1	neg to 1	neg to 1	1
2003	neg	1	neg to 1	1	1
2664	neg	1	neg to 1	1	1
319	neg	3	2	>2	3
508	neg	3	3	3	3
2655	neg	3	3	3	3
2657	neg	3	3	3	3
2658	neg	3	3	3	3
2659	neg	2	1	2	2
2667	neg	3	2	3	3
2668	neg	3	3	3	3
2669	neg	3	3	3	3
2671	neg	3	3	3	3
2715	neg	3	3	3	3
2716	neg	3	3	3	3

[00127] Exemplary humanized and chimeric CSPG4 antibodies of the present invention are presented in Table 5. Ch: chimeric; Hu: humanized; HC: heavy chain; LC: light chain.

TABLE 5

ch763 HC cDNA

CAGATCCAGTTGGTGCAGTCTGGACCTGAGCTGAAGAAGC
 CTGGAGAGACAGTCAAGATCTCCTGCAAGGCTTCTGGTTAT
 ACCTTCACAGACTATTCAATGCACTGGGTGAAGAAGACTCC
 AGGAAAGGGTTTAAAGTGGCTGGGCTGGATAAACACTGCG
 ACTGGTGAGCCAACATATGCAGATGACTTCAAGGGACGGT
 TTGCCATCTCTTTGGAAACCTCTGCCAGGACTGTCTATTTGC
 AGATCAATAATCTCAGAAATGAGGACACGGCTACATATTTCT
 TGTTTTAGTTACTACGACTACTGGGGCCAAGGCACCACTCT
 CACAGTTTCCGCCTCCACCAAGGGCCCATCGGTCTTCCCCC
 TGGCACCCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCC
 CTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGAC
 GGTGTCGTGGAACCTCAGGCGCCCTGACCAGCGGCGTGCAC
 ACCTTCCCGGCCGTCTACAGTCCTCAGGACTCTACTCCCTC
 AGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCA
 GACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACC
 AAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGACAAAA
 CTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGG
 GGACCGTCAGTCTTCTCTTCCCCCAAACCCAAGGACAC
 CCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGG

TGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTG
 GTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
 CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCA
 GCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAG
 GAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCC
 CCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCG
 AGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAG
 CTGACCAAGAACCAGGTGACGCTGACCTGCCTGGTCAAAG
 GCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAAT
 GGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGTGC
 TGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACC
 GTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCAT
 GCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAG
 AAGAGCCTCTCCCTGTCTCCGGGTAAATGA (SEQ ID NO: 3)

ch763 HC amino acid

QIQLVQSGPELKKPGETVKISCKASGYTFTDYSMHVVKKTPG
 KGLKWLGWINTATGEPTYADDFKGRFAISLETARTVYLQIN
 NLRNEDTATYFCFSYYDYWGQGTTLTVSASTKGPSVFPLAPSS
 KSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
 QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRV
 KSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
 VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV
 VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR
 EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
 ENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMH
 EALHNHYTQKSLSLSPGK (SEQ ID NO: 4)

ch763 LC cDNA

GACATCAAGCTGTCCCAGTCCCCCTCCATCCTGTCCGTGAC
 CCGCGGCGAGACCGTGTCCCTGTCCTGCCGGGCTCCCAGA
 CCATCTACAAGAACCTGCACTGGTACCAGCAGAAGTCCCAC
 CGGTCCCCCGGCTGCTGATCAAGTACGGCTCCGACTCCAT
 CTCCGGCATCCCCCTCCCGGTTCCACCGGCTCCGGCTCCGGCA
 CCGACTACACCCTGAACATCAACTCCGTGAAGCCCGAGGA
 CGAGGGCATCTACTACTGCCTGCAGGGCTACTCCACCCCT
 GGACCTTCGGCGGCGGCACCAAGCTGGAGATCAAGCGGAC
 CGTGGCCGCCCCCTCCGTGTTTCATCTTCCCCCCCCCTCCGACGA
 GCAGCTGAAGTCCGGCACCGCCTCCGTGGTGTGCCTGCTGA
 ACAACTTCTACCCCCGGGAGGCCAAGGTGCAGTGGAAGGT
 GGACAACGCCCTGCAGTCCGGCAACTCCCAGGAGTCCGTG
 ACCGAGCAGGACTCCAAGGACTCCACCTACTCCCTGTCTC
 CACCCTGACCCTGTCCAAGGCCGACTACGAGAAGCACAAAG
 GTGTACGCCTGCGAGGTGACCCACCAGGGCCTGTCTCCCC
 CGTGACCAAGTCCTTCAACCGGGGCGAGTGCTAG
 (SEQ ID NO: 5)

ch763 LC amino acid

DIKLSQSPSILSVTPGETVSLSCRASQTIYKNLHWYQQKSHRSP
 RLLIKYGSDSISGIPSRFTGSGSGTDYTLNINSVKPEDEGIYYCL
 QGYSTPWTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASV

VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
SLSSTLTLISKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
(SEQ ID NO: 6)

hu763 H1 IgG1 cDNA

CAGATCCAGCTGGTGCAGTCCGGCCCCGAGGTGAAGAAGC
CCGGCGCCTCCGTGAAGATCTCCTGCAAGGCCTCCGGCTAC
ACCTTCACCGACTACTCCATGCACTGGGTGAAGAAGGCCCC
CGGCCAGGGCCTGGAGTGGCTGGGCTGGATCAACACCGCC
ACCGGCGAGCCACCTACGCCGACGACTTCAAGGGCCGGT
TCACCATCACCTGGACACCTCCGCCCCGACCGTGTACCTG
CAGATCAACAACCTGCGGTCCGAGGACACCGCCACCTACTT
CTGCTTCTCCTACTACGACTACTGGGGCCAGGGCACCTG
TGACCGTGTCTCCTCCGCCTCCACCAAGGGCCCATCGGTCTT
CCCCTGGCACCTCCTCCAAGAGCACCTCTGGGGGCACAGC
GGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
TGACGGTGTCTGGAAGTCAAGGCGCCCTGACCAGCGGCGT
GCACACCTTCCCGGCCGTCCTACAGTCCTCAGGACTCTACT
CCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGC
ACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCA
ACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGA
CAAACTCACACATGCCCACCGTGCCACGACCTGAACTCC
TGGGGGGACCGTCAGTCTTCTTCCCCCAAAACCCAAG
GACACCTCATGATCTCCCGGACCCCTGAGGTCACATGCGT
GGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTC
AACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGA
CAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGT
GGTCAGCGTCCTACCGTCCTGCACCAGGACTGGCTGAATG
GCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCC
AGCCCCCATCGAGAAAACCATCTCCAAAGGCCAAAGGGCAG
CCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGA
TGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCA
AAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAG
CAATGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCC
GTGCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCT
CACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC
TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACAC
GCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA
(SEQ ID NO: 7)

hu763 H1 IgG1 amino acid

QIQLVQSGPEVKKPGASVKISCKASGYTFTDYSMHWVKKAPG
QGLEWLGWINTATGEPTYADDFKGRFTITLDTSAARTVYLQINN
LRSEDATYFCFSYYDYWGQGTLTSSASTKGPSVFPLAPSS
KSTSGGTAALGLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVKDKRVEP
KSCDKHTHTCPPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV
VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR
EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP

ENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPGK (SEQ ID NO: 8)

hu763 H2 IgG1 cDNA

CAGGTGCAGCTGGTGCAGTCCGGCCCCGAGGTGAAGAAGC
CCGGCGCCTCCGTGAAGATCTCCTGCAAGGCCTCCGGCTAC
ACCTTCACCGACTACTCCATGCACTGGGTGAAGAAGGCCCC
CGGCCAGGGCCTGAAGTGGCTGGGCTGGATCAACACCGCC
ACCGGCGAGCCACCTACGCCGACGACTTCAAGGGCCGGT
TCACCATCACCTGGACACCTCCGCCCCGACCGTGTACCTG
GAGATCTCCTCCCTGCGGTCCGAGGACACCGCCACCTACTT
CTGCTTCTCCTACTACGACTACTGGGGCCAGGGCACCTGC
TGACCGTGTCTCCTCCGCCTCCACCAAGGGCCCCATCGGTCTTC
CCCCTGGCACCTCCTCCAAGAGCACCTCTGGGGGACACAGC
GGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
TGACGGTGTCTGGAAGTCAAGGCGCCCTGACCAGCGGCGT
GCACACCTTCCCGGCCGTCCTACAGTCCTCAGGACTCTACT
CCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGC
ACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCA
ACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGA
CAAACTCACACATGCCACCGTGCCAGCACCTGAACTCC
TGGGGGGACCGTCAGTCTTCTTCCCCCAAACCCAAAG
GACACCTCATGATCTCCCGGACCCCTGAGGTACATGCGT
GGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTC
AACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGA
CAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGT
GGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATG
GCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCC
AGCCCCCATCGAGAAAACCATCTCCAAGGCCAAAGGGCAG
CCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGA
TGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCA
AAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAG
CAATGGGCAGCCGGAGAACAATAAGACCACGCCTCCC
GTGCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCT
CACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC
TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACAC
GCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA
(SEQ ID NO: 9)

hu763 H2 IgG1 amino acid

QVQLVQSGPEVKKPGASVKISCKASGYTFTDYSMHVKKAP
GQGLKWLGWINTATGEPTYADDFKGRFTITLDT SARTVYLEIS
SLRSED TATYFCFSYYDYWGQGTLLTVSSASTKGPSVFPLAPS
SKSTSGGTAA LGCLVKDYFPEPVT VSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT
CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVS VLT VLVH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSV

MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 10)

hu763 L1 Igk cDNA

GAGATCAAGCTGACCCAGTCCCCCTCCATCCTGTCCGTGTC
 CCCC GGCGAGACCGTGACCTGTCTGCGGGCCTCCCAGA
 CCATCTACAAGAACCTGCACTGGTACCAGCAGAAGTCCCAC
 CGGTCCCCCGGCTGCTGATCAAGTACGGCTCCGACTCCAT
 CTCCGGCATCCCCGCCCGGTTCTCCGGCTCCGGCTCCGGCA
 CCGACTACACCCTGACCATCAACTCCGTGAAGCCCCGAGGAC
 GAGGGCATCTACTACTGCCTGCAGGGCTACTCCACCCCCCTG
 GACCTTCGGCCAGGGCACCAAGCTGGAGATCAAGCGGACC
 GTGGCCGCCCCCTCCGTGTTTCATCTTCCCCCCTCCGACGA
 GCAGCTGAAGTCCGGCACCGCCTCCGTGGTGTGCCTGCTGA
 ACAACTTCTACCCCCGGGAGGCCAAGGTGCAGTGGAAGGT
 GGACAACGCCCTGCAGTCCGGCAACTCCCAGGAGTCCGTG
 ACCGAGCAGGACTCCAAGGACTCCACCTACTCCCTGTCTC
 CACCCTGACCCTGTCCAAGGCCGACTACGAGAAGCACAAG
 GTGTACGCCTGCGAGGTGACCCACCAGGGCCTGTCTCCCC
 CGTGACCAAGTCCTTCAACCGGGGCGAGTGCTAG
 (SEQ ID NO: 11)

hu763 L1 Igk amino acid

EIKLTQSPSILSVSPGETVTLSCRASQTIYKNLHWYQQKSHRSP
 RLLIKYGSDSISGIPARFSGSGSGTDYTLTINSVKPEDEGIYYCL
 QGYSTPWTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASV
 VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
 SLSSTLTLSKADPGVRDRAGLQGLHLLPSSTLTLSKADYEKHK
 VYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 12)

hu763 L2 Igk cDNA

GAGATCGTGCTGACCCAGTCCCCCGCCACCCTGTCCGTGTC
 CCCC GGCGAGACCGTGACCTGTCTGCGGGCCTCCCAGA
 CCATCTACAAGAACCTGCACTGGTACCAGCAGAAGTCCGG
 CCTGTCCCCCGGCTGCTGATCAAGTACGGCTCCGACTCCA
 TCTCCGGCATCCCCGCCCGGTTCTCCGGCTCCGGCTCCGGC
 ACCGACTACACCCTGACCATCAACTCCGTGGAGCCCGAGG
 ACGAGGGCATCTACTACTGCCTGCAGGGCTACTCCACCCCC
 TGGACCTTCGGCCAGGGCACCAAGCTGGAGATCAAGCGGA
 CCGTGGCCGCCCCCTCCGTGTTTCATCTTCCCCCCTCCGACG
 AGCAGCTGAAGTCCGGCACCGCCTCCGTGGTGTGCCTGCTG
 AACAACTTCTACCCCCGGGAGGCCAAGGTGCAGTGGAAGG
 TGGACAACGCCCTGCAGTCCGGCAACTCCCAGGAGTCCGTG
 ACCGAGCAGGACTCCAAGGACTCCACCTACTCCCTGTCTC
 CACCCTGACCCTGTCCAAGGCCGACTACGAGAAGCACAAG
 GTGTACGCCTGCGAGGTGACCCACCAGGGCCTGTCTCCCC
 CGTGACCAAGTCCTTCAACCGGGGCGAGTGCTAG
 (SEQ ID NO: 13)

hu763 L2 Igk amino acid

EIVLTQSPATLSVSPGETVTLSCRASQTIYKNLHWYQQKSGLSP
 RLLIKYGSDSISGIPARFSGSGSGTDYTLTINSVEPEDEGIYYCL
 QGYSTPWTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASV

VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
SLSSTLTLISKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
(SEQ ID NO: 14)

hu763 H1 IgG4 cDNA

CAGATCCAGCTGGTGCAGTCCGGCCCCGAGGTGAAGAAGC
CCGGCGCCTCCGTGAAGATCTCCTGCAAGGCCTCCGGCTAC
ACCTTCACCGACTACTCCATGCACTGGGTGAAGAAGGCCCC
CGGCCAGGGCCTGGAGTGGCTGGGCTGGATCAACACCGCC
ACCGGCGAGCCACCTACGCCGACGACTTCAAGGGCCGGT
TCACCATCACCTGGACACCTCCGCCCCGACCGTGTACCTG
CAGATCAACAACCTGCGGTCCGAGGACACCGCCACCTACTT
CTGCTTCTCCTACTACGACTACTGGGGCCAGGGCACCCCTGC
TGACCGTGTCTCCTCCGCCTCCACCAAGGGCCCCCTCCGTGTT
CCCCTGGCCCCCTGCTCCCGGTCCACCTCCGAGTCCACCGC
CGCCCTGGGCTGCCTGGTGAAGGACTACTTCCCCGAGCCCG
TGACCGTGTCTCCTGGAACCTCCGGCGCCCTGACCTCCGGCGTG
CACACCTTCCCCGCCGTGCTGCAGTCCTCCGGCCTGTACTC
CCTGTCCTCCGTGGTGACCGTGCCCTCCTCCTCCCTGGGCAC
CAAGACCTACACCTGCAACGTGGACCACAAGCCCTCCAAC
ACCAAGGTGGACAAGCGGGTGGAGTCCAAGTACGGCCCCC
CCTGCCCTCCTGCCCGCCCCCGAGTTCTTGGGCGGCCCC
TCCGTGTTCTGTTCCCCCCCCAAGCCCAAGGACACCCTGAT
GATCTCCCCGACCCCCGAGGTGACCTGCGTGGTGGTGGACG
TGTCCCAGGAGGACCCCGAGGTGCAGTTCAACTGGTACGTG
GACGGCGTGGAGGTGCACAACGCCAAGACCAAGCCCCGGG
AGGAGCAGTTCAACTCCACCTACCGGGTGGTGTCCGTGCTG
ACCGTGCTGCACCAGGACTGGCTGAACGGCAAGGAGTACA
AGTGCAAGGTGTCCAACAAGGGCCTGCCCTCCTCCATCGAG
AAGACCATCTCCAAGGCCAAGGGCCAGCCCCGGGAGCCCC
AGGTGTACACCCTGCCCCCCTCCCAGGAGGAGATGACCAA
GAACCAGGTGTCCCTGACCTGCCTGGTGAAGGGCTTCTACC
CCTCCGACATCGCCGTGGAGTGGGAGTCCAACGGCCAGCC
CGAGAACAACACTACAAGACCACCCCCCCCCGTGCTGGACTCC
GACGGCTCCTTCTTCTGTACTCCCGGCTGACCGTGGACAA
GTCCCGGTGGCAGGAGGGCAACGTGTTCTCCTGCTCCGTGA
TGCACGAGGCCCTGCACAACCACTACACCCAGAAGTCCCTG
TCCCTGTCCCTGGGCAAG (SEQ ID NO: 15)

hu763 H1 IgG4 amino acid

QIQLVQSGPEVKKPGASVKISCKASGYTFTDYSMHVVKAPG
QGLEWLGWINTATGEPTYADDFKGRFTITLDTSAARTVYLQINN
LRSEDTATYFCFSYYDYWGQGTLTSSASTKGPSVFPLAPCS
RSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ
SSGLYSLSVTVTPSSSLGKTKYTCNVDPKPSNTKVDKRVESK
YGPCCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVL
TVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQV
YTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN
YKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMEAL

HNHYTQKSLSLSLGK (SEQ ID NO: 16)

hu763 H2 IgG4 cDNA

CAGGTGCAGCTGGTGCAGTCCGGCCCCGAGGTGAAGAAGC
 CCGGCGCCTCCGTGAAGATCTCCTGCAAGGCCTCCGGCTAC
 ACCTTCACCGACTACTCCATGCACTGGGTGAAGAAGGCCCC
 CGGCCAGGGCCTGAAGTGGCTGGGCTGGATCAACACCGCC
 ACCGGCGAGCCACCTACGCCGACGACTTCAAGGGCCGGT
 TCACCATCACCTGGACACCTCCGCCCCGACCGTGTACCTG
 GAGATCTCCTCCCTGCGGTCCGAGGACACCGCCACCTACTT
 CTGCTTCTCCTACTACGACTACTGGGGCCAGGGCACCTGC
 TGACCGTGTCTCCTCCGCCTCCACCAAGGGCCCCCTCCGTGTTT
 CCCCTGGCCCCCTGCTCCCGGTCCACCTCCGAGTCCACCGC
 CGCCCTGGGCTGCCTGGTGAAGGACTACTTCCCCGAGCCCCG
 TGACCGTGTCTGGAACCTCCGGCGCCCTGACCTCCGGCGTG
 CACACCTTCCCCGCCGTGCTGCAGTCTCCTCCGGCCTGTACTC
 CCTGTCTCCTCCGTGGTGACCGTGCCCTCCTCCTCCCTGGGCAC
 CAAGACCTACACCTGCAACGTGGACCACAAGCCCTCCAAC
 ACCAAGGTGGACAAGCGGGTGGAGTCCAAGTACGGCCCCC
 CCTGCCCTCCTGCCCGCCCCCGAGTTCTTGGGCGGCCCC
 TCCGTGTTCTGTTCCCCCCCCAAGCCCAAGGACACCCTGAT
 GATCTCCCGGACCCCCGAGGTGACCTGCGTGGTGGTGGACG
 TGTCCCAGGAGGACCCCGAGGTGCAGTTCAACTGGTACGTG
 GACGGCGTGGAGGTGCACAACGCCAAGACCAAGCCCCGGG
 AGGAGCAGTTCAACTCCACCTACCGGGTGGTGTCCGTGCTG
 ACCGTGCTGCACCAGGACTGGCTGAACGGCAAGGAGTACA
 AGTGCAAGGTGTCCAACAAGGGCCTGCCCTCCTCCATCGAG
 AAGACCATCTCCAAGGCCAAGGGCCAGCCCCGGGAGCCCC
 AGGTGTACACCCTGCCCCCTCCAGGAGGAGATGACCAA
 GAACCAGGTGTCCCTGACCTGCCTGGTGAAGGGCTTCTACC
 CCTCCGACATCGCCGTGGAGTGGGAGTCCAACGGCCAGCC
 CGAGAACAACTACAAGACCACCCCCCGTGCTGGACTCC
 GACGGCTCCTTCTTCTGTACTCCCGGTGACCGTGGACAA
 GTCCCGGTGGCAGGAGGGCAACGTGTTCTCCTGCTCCGTGA
 TGCACGAGGCCCTGCACAACCACTACACCCAGAAGTCCCTG
 TCCCTGTCCCTGGGCAAG (SEQ ID NO: 17)

hu763 H2 IgG4 amino acid

QVQLVQSGPEVKKPGASVKISCKASGYTFTDYSMHVVKAP
 GQGLKWLGWINTATGEPTYADDFKGRFTITLDTSAARTVYLEIS
 SLRSEDATYFCFSYYDYWGQGTLLTVSSASTKGPSVFPLAPC
 SRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
 QSSGLYSLSSVTVPSSSLGKTYTCNVDHKPSNTKVDKRVES
 KYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVV
 DVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSV
 LTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQ
 VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN
 NYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEA
 LHNHYTQKSLSLSLGK (SEQ ID NO: 18)

hu763-HC-huOKT3 cDNA CAGGTGCAGCTGGTGCAGTCCGGCCCCGAGGTGAAGAAGC
CCGGCGCCTCCGTGAAGATCTCCTGCAAGGCCTCCGGGCTAC
ACCTTCACCGACTACTCCATGCACTGGGTGAAGAAGGCCCC
CGGCCAGGGCCTGAAGTGGCTGGGCTGGATCAACACCGCC
ACCGGCGAGCCACCTACGCCGACGACTTCAAGGGCCGGT
TCACCATCACCTGGACACCTCCGCCCCGACCGTGTACCTG
GAGATCTCCTCCCTGCGGTCCGAGGACACCGCCACCTACTT
CTGCTTCTCCTACTACGACTACTGGGGCCAGGGCACCTGC
TGACCGTGTCTCCTCCGCCTCCACCAAGGGCCCCATCGGTCTT
CCCCTGGCACCTCCTCCAAGAGCACCTCTGGGGGACACAGC
GGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
TGACGGTGTCTGTGGAAGTCAAGGCGCCCTGACCAGCGGCGT
GCACACCTTCCCGGCCGTCCTACAGTCCTCAGGACTCTACT
CCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGC
ACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCA
ACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGA
CAAACTCACACATGCCACCGTGCCAGCACCTGAACTCC
TGGGGGGACCGTCAGTCTTCTTCCCCCAAACCCAAG
GACACCTCATGATCTCCCGGACCCCTGAGGTCACATGCGT
GGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTC
AACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGA
CAAAGCCGCGGGAGGAGCAGTACGCCAGCACGTACCGTGT
GGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATG
GCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCC
AGCCCCCATCGAGAAAACCATCTCCAAGGCCAAAGGGCAG
CCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGA
TGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCA
AAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAG
CAATGGGCAGCCGGAGAACAATAAGACCACGCCTCCC
GTGCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCT
CACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC
TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACAC
GCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGATCCGGA
GGAGGAGGTAGCGGAGGAGGAGGTTCTGGCGGAGGGGGTT
CCCAGGTGCAGCTGGTGCAGAGCGGAGGAGGAGTGGTGCA
GCCAGGAAGGAGCCTGCGACTGTCTTGCAAGGCTAGTGGC
TACACCTTCACACGATATACTATGCACTGGGTGAGGCAGGC
ACCTGGTAAAGGCCTGGAGTGGATCGGCTACATTAACCCCT
CTAGGGGATACACCAACTATAATCAGAAGTTCAAAGACAG
GTTACCATCTCACGCGATAACTCCAAGAATACCGCCTTCC
TGCAGATGGACTCCCTGCGGCCCCGAAGATACAGGCGTGTAT
TTTTGCGCTAGATACTATGACGATCATTACTGTCTGGACTAT
TGGGGACAGGGGACCCCTGTGACAGTGTCCAGCGGTGGAG
GAGGGTCAGGTGGAGGAGGGAGCGGTGGCGGAGGGTCTGA
CATCCAGATGACCCAGTCCCCATCTAGTCTGAGCGCCTCTG
TGGGCGATAGAGTGACTATTACCTGCAGTGCTTCATCCAGC
GTGAGCTACATGAACTGGTATCAGCAGACACCCGGAAAGG
CACCTAAACGCTGGATCTACGATACTAGCAAGCTGGCCTCT

GGCGTGCCCAGTCGATTCAGTGGTTCAGGCTCCGGAACCGA
CTATACCTTCACCATCTCTAGTCTGCAGCCTGAGGATATTG
CCACATACTATTGTCAGCAGTGGTCATCCAATCCATTCACT
TTTGGGCAGGGTACCAAACCTGCAGATTACAAGGTAGGGAT
CCGAGCTCGGTACAAACCG (SEQ ID NO: 19)

hu763-HC-huOKT3 amino
acid

QVQLVQSGPEVKKPGASVKISCKASGYTFTDYSMHWVKKAP
GQGLKWLGWINTATGEPTYADDFKGRFTITLDTSAARTVYLEIS
SLRSEDATYFCFSYYDYWGQGTLTSSASTKGPSVFPLAPS
SKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVE
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT
CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYR
VVSVELTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM
HEALHNHYTQKSLSLSPGKSGGGGSGGGGSGGGGSGVQLV
QSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGKGLE
WIGYINPSRGYTNYNQKFKDRFTISRDNKNTAFLQMDSLRPE
DTGVYFCARYYDDHYCLDYWGQGPVTVSSGGGGSGGGGS
GGGGSDIQMTQSPSSLSASVGDRVTITCSASSSVSYMNWYQQ
TPGKAPKRWIYDTSKLASGVPSRFSGSGSGTDYFTISLQPED
IATYYCQQWSSNPFTFGQGTKLQITR (SEQ ID NO: 20)

hu763-LC-huOKT3 cDNA

GAGATCGTGCTGACCCAGTCCCCCGCCACCCTGTCCGTGTC
CCCCGGCGAGACCGTGACCCTGTCCTGCCGGGCCTCCCAGA
CCATCTACAAGAACCTGCACTGGTACCAGCAGAAGTCCGG
CCTGTCCCCCGGCTGCTGATCAAGTACGGCTCCGACTCCA
TCTCCGGCATCCCCGCCCCGGTTCTCCGGCTCCGGCTCCGGC
ACCGACTACACCCTGACCATCAACTCCGTGGAGCCCCGAGG
ACGAGGGCATCTACTACTGCCTGCAGGGCTACTCCACCCCC
TGGACCTTCGGCCAGGGCACCAAGCTGGAGATCAAGCGGA
CCGTGGCCCGCCCCCTCCGTGTTTCATCTTCCCCCCCCTCCGACG
AGCAGCTGAAGTCCGGCACCGCCTCCGTGGTGTGCCTGCTG
AACAACTTCTACCCCCGGGAGGCCAAGGTGCAGTGGAAGG
TGGACAACGCCCTGCAGTCCGGCAACTCCCAGGAGTCCGTG
ACCGAGCAGGACTCCAAGGACTCCACCTACTCCCTGTCTCTC
CACCCTGACCCTGTCCAAGGCCGACTACGAGAAGCACAAAG
GTGTACGCCTGCGAGGTGACCCACCAGGGCCTGTCTCCCC
CGTGACCAAGTCCTTCAACCGGGGCGAGTGCACTAGTGGA
GGAGGAGGTAGCGGAGGAGGAGGTTCTGGCGGAGGGGGTT
CCCAGGTGCAGCTGGTGCAGAGCGGAGGAGGAGTGGTGCA
GCCAGGAAGGAGCCTGCGACTGTCTTGCAAGGCTAGTGGC
TACACCTTCACACGATATACTATGCACTGGGTGAGGCAGGC
ACCTGGTAAAGGCCTGGAGTGGATCGGCTACATTAACCCCT
CTAGGGGATACACCAACTATAATCAGAAGTTCAAAGACAG
GTTACCATCTCACGCGATAACTCCAAGAATACCGCCTTCC
TGCAGATGGACTCCCTGCGGCCCGAAGATACAGGCGTGTAT

TTTTGCGCTAGATACTATGACGATCATTACTGTCTGGACTAT
 TGGGGACAGGGGACCCCTGTGACAGTGTCCAGCGGTGGAG
 GAGGGTCAGGTGGAGGAGGGAGCGGTGGCGGAGGGTCTGA
 CATCCAGATGACCCAGTCCCCATCTAGTCTGAGCGCCTCTG
 TGGGCGATAGAGTGACTATTACCTGCAGTGCTTCATCCAGC
 GTGAGCTACATGAACTGGTATCAGCAGACACCCGGAAAGG
 CACCTAAACGCTGGATCTACGATACTAGCAAGCTGGCCTCT
 GGCGTGCCAGTCGATTCAGTGGTTCAGGCTCCGGAACCGA
 CTATACCTTCACCATCTCTAGTCTGCAGCCTGAGGATATTG
 CCACATACTATTGTCAGCAGTGGTCATCCAATCCATTCACT
 TTTGGGCAGGGTACCAAACCTGCAGATTACAAGGTAGTCTAG
 AGCTTGCCCTCGAGCAGCGCTGCTCGAGAGATCTACGGGTGG
 (SEQ ID NO: 21)

hu763-LC-huOKT3 amino
acid

EIVLTQSPATLSVSPGETVTLSCRASQTIYKNLHWYQQKSGLS
 RLLIKYGSDSISGIPARFSGSGSGTDYTLTINSVEPEDEGIYYCL
 QGYSTPWTFTGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASV
 VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
 SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECTS
 GGGSGGGSGGGSGGGSQVQLVQSGGGVVPGRSLRLSCKASG
 YTFTRYTMHWVRQAPGKGLEWIGYINPSRGYTNYNQKFKDR
 FTISRDNSKNTAFLQMDSLRPEDTGVYFCARYYDDHYCLDYW
 GQGTPVTVSSGGGGSGGGSGGGGSDIQMTQSPSSLSASVGD
 RVTITCSASSSVSYMNWYQQTPGKAPKRWIYDTSKLASGVPS
 RFSGSGSGTDYFTFTISLQPEDATYYCQQWSSNPFTFGQGTKL
 QITR (SEQ ID NO: 22)

[00128] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the heavy chain variable region contains at least one of the CDRs found in the heavy chain variable region of murine 763 antibody and the light chain variable region contains at least one of the CDRs found in the light chain variable region of murine 763 antibody.

[00129] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the heavy chain variable region contains at least two of the CDRs found in the heavy chain variable region of murine 763 antibody and the light chain variable region contains at least two of the CDRs found in the light chain variable region of murine 763 antibody.

[00130] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the heavy chain variable region contains the three CDRs found in the heavy chain variable region of murine 763 antibody

and the light chain variable region contains the three CDRs found in the light chain variable region of murine 763 antibody.

[00131] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the heavy chain variable region contains the three CDRs found in the heavy chain variable region of murine 763 antibody.

[00132] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the heavy chain variable region contains three CDRs, which CDRs each have a sequence that is at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to heavy chain CDRs that appear in Table 5.

[00133] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the heavy chain variable region contains three CDRs, which CDRs each have a sequence that is identical to heavy chain CDRs that appear in Table 5.

[00134] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the light chain variable region contains the three CDRs found in the light chain variable region of murine 763 antibody.

[00135] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the light chain variable region contains three CDRs, which CDRs each have a sequence that is at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to light chain CDRs that appear in Table 5.

[00136] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the light chain variable region contains three CDRs, which CDRs each have a sequence that is identical to light chain CDRs that appear in Table 5.

[00137] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the heavy chain variable region has a sequence that is at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to a heavy chain variable region that appears in Table 5.

[00138] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the heavy chain variable region has a sequence that is identical to a heavy chain variable region that appears in Table 5.

[00139] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the light chain variable region has a sequence that is at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to a light chain variable region that appears in Table 5.

[00140] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, wherein the light chain variable region has a sequence that is identical to a light chain variable region that appears in Table 5.

[00141] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, which heavy chain variable region has a sequence that is at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to a heavy chain variable region that appears in Table 5, and which light chain variable region has a sequence that is at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to a light chain variable region that appears in Table 5.

[00142] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions, which heavy chain variable region has a sequence that is identical to a heavy chain variable region that appears in Table 5, and which light chain variable region has a sequence that is identical to a light chain variable region that appears in Table 5.

[00143] In various embodiments, a humanized anti-CSPG4 antibody according to the present invention is composed of heavy and light chain variable regions that are selected from heavy and light chain variable region sequences that appear in Table 5.

[00144] In various embodiments, a bispecific binding agent (e.g., a bispecific antibody) according to the present invention is composed of a first binding component and a second binding component. In many embodiments, first and second binding components of a bispecific binding agent as described herein are each composed of antibody components characterized by different specificities. In many embodiments, antibody components are selected from Table 5.

[00145] In various embodiments, a bispecific binding agent according to the present invention comprises a first binding component, a second binding component. In various embodiments, a bispecific binding agent according to the present invention comprises a first binding component, a second binding component and a linker that is connected to both the first and second binding component (e.g., positioned between the first and second binding components).

[00146] In various embodiments, first and/or second binding components as described herein comprise or are antibody components. In various embodiments, first and/or second binding components as described herein comprise a linker sequence.

[00147] In various embodiments, first and/or second binding components as described herein comprise or are immunoglobulins (e.g., IgGs). In various embodiments, first and/or second binding components binding components as described herein comprise or are antibody fragments (e.g., scFvs). In various embodiments, first binding components as described herein comprise or are immunoglobulins and second binding components comprise or are antibody fragments. In some certain embodiments, first binding components are immunoglobulins and second binding components are antibody fragments. In some certain embodiments, first binding components are IgGs and second binding components are scFvs.

[00148] In some certain embodiments, a bispecific binding agent according to the present invention comprises an immunoglobulin, which immunoglobulin comprises a heavy chain and a light chain, and an scFv. In some certain embodiments, scFvs are linked to the C-terminal end of the heavy chain of the immunoglobulin. In some certain embodiments, scFvs are linked to the C-terminal end of the light chain of the immunoglobulin. In various embodiments, scFvs are linked to heavy or light chains via a linker sequence.

[00149] In some embodiments, a bispecific binding agent of the present invention comprises a sequence at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to a sequence that appears in Table 5.

[00150] In some embodiments, a bispecific binding agent of the present invention comprises a sequence that is substantially identical to a sequence that appears in Table 5.

[00151] In some embodiments, a bispecific binding agent of the present invention comprises a sequence that is identical to a sequence that appears in Table 5.

[00152] In some embodiments, a bispecific binding agent of the present invention is selected from a sequence that appears in Table 5.

[00153] In various embodiments, a first binding component of a bispecific binding agent as described herein comprises an antibody component having a sequence at least 50% (e.g., 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to an antibody component that appears in Table 5.

[00154] In various embodiments, a first binding component of a bispecific binding agent as described herein comprises an antibody component having a sequence that is identical to an antibody component that appears in Table 5.

[00155] In various embodiments, a second binding component of a bispecific binding agent as described herein comprises an antibody component having a sequence at least 50% (e.g., 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to an antibody component that appears in Table 5.

[00156] In various embodiments, a second binding component of a bispecific binding agent as described herein comprises an antibody component having a sequence that is identical to an antibody component that appears in Table 5.

Humanized Antibodies

[00157] In some embodiments, the antibodies provided by the present invention are monoclonal antibodies, in particular, humanized versions of cognate anti-CSPG4 antibodies derived from other species. A humanized antibody is, in some embodiments, an antibody produced by recombinant DNA technology, in which some or all of the amino acids of a human immunoglobulin light or heavy chain that are not required for antigen binding (e.g., the constant regions and the framework regions of the variable domains) are used to substitute for the corresponding amino acids from the light or heavy chain of the cognate, nonhuman antibody. By way of example, a humanized version of a murine antibody to a given antigen has on both of its heavy and light chains (1) constant regions of a human antibody; (2) framework regions from the variable domains of a human antibody; and (3) CDRs from the murine antibody. When necessary, one or more residues in the human framework regions can be changed to residues at the corresponding positions in the murine antibody so as to preserve the binding affinity of the humanized antibody to the antigen. This change is sometimes called "back mutation." Similarly, forward mutations may be made to revert back to murine sequence for a desired reason, e.g. stability or affinity to antigen. Humanized antibodies generally are less likely to elicit an immune response in humans as compared to chimeric human antibodies because the former contain considerably fewer non-human components.

[00158] Suitable methods for making humanized antibodies of the present invention are described in, e.g., Winter EP 0 239 400; Jones et al. (1986) Nature 321:522-525; Riechmann et al. (1988) Nature 332:323-327; Verhoeyen et al. (1988) Science 239:1534-1536; Queen et al. (1989) Proc. Nat. Acad. Sci. U.S.A. 86:10029; U.S. Patent No. 6, 180,370; and Orlandi et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:3833; the disclosures of all of which are incorporated by reference herein in their entireties. Generally, the transplantation of murine (or other non-human) CDRs onto a human antibody is achieved as follows. The cDNAs encoding heavy and light chain variable domains are isolated from a hybridoma. The DNA sequences of the variable domains, including the CDRs, are determined by sequencing. The DNAs, encoding the CDRs are inserted into the corresponding regions of a human antibody heavy or light chain variable domain coding sequences, attached to human constant region gene segments of a desired isotype (e.g., $\gamma 1$ for C_H and κ for C_L), are gene synthesized. The humanized heavy and light chain genes are co-expressed in mammalian host cells (e.g., CHO or NSO cells) to produce soluble humanized antibody. To facilitate large-scale production of antibodies, it is often desirable to select for a high expressor using a DHFR gene or GS gene in the producer line. These producer cell lines are cultured in bioreactors, or hollow fiber culture system, or WAVE technology, to produce bulk cultures of soluble antibody, or to produce transgenic mammals (e.g., goats, cows, or sheep) that express the antibody in milk (see, e.g., U.S. Patent No. 5,827,690).

[00159] Using the above-described approaches, humanized and chimeric versions of the murine 763 antibody, were generated. The cDNAs encoding the murine 763 variable regions of the light and heavy chains were used to construct vectors for expression of murine-human chimeras in which the murine 763 variable regions were linked to human IgG1 (for heavy chain) and human kappa (for light chain) constant regions, as described previously. In addition, novel forms of humanized 763 with variant glycosylation were created, in order to enhance binding to the Fc receptor and enhance antigen affinity.

[00160] In order to produce humanized 763 antibodies, the human acceptor framework domains were chosen by homology matching to human germline sequences. Using these chosen human acceptor frameworks, the light and heavy chain variable domains were designed and a number of variants/versions of each were generated and expressed, as described below in Examples.

[00161] Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human

immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716, 11 1; and International Patent Application Publications WO 98/46645, WO 98/60433, WO 98/24893, WO 98/16664, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety. The techniques of Cole et al., and Boerder et al., are also available for the preparation of human monoclonal antibodies (Cole et al. (1985) *Monoclonal Antibodies and Cancer Therapy*, ed. R.A. Reisfeld & S. Sell, pp.77-96, New York, Alan R. Liss; Boerner et al. (1991) *J. Immunol.* 147(1):86-95).

[00162] Human antibodies produced using other techniques but retaining the variable regions of the anti-CSPG4 antibody of the present invention are included herein. Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous mouse immunoglobulins, but which can express human immunoglobulin genes (e.g., see Lonberg and Huszar (1995) *Int. Rev. Immunol.* 13:65-93; Taylor, L. D., et al. (1992) *Nucl. Acids Res.* 20:6287-6295; Kellermann S-A., and Green L. L. (2002) *Current Opinion in Biotechnology* 13:593-597; Little M. et al. (2000) *Immunol. Today* 21:364-370; Murphy, A.J. et al. (2014) *Proc. Natl. Acad. Sci. U. S. A.* 111(14):5153-5158). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., International Patent Application Publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,886,793; 5,916,771; 5,939,598; and 8,502,018, which are incorporated by reference herein in their entirety.

[00163] Also human monoclonal antibodies could be made by immunizing mice transplanted with human peripheral blood leukocytes, splenocytes or bone marrows (e.g., Trioma techniques of XTL). Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope (Jespers et al. (1988) *Biotechnology* 12:899-903).

[00164] As used herein, an "anti-CSPG4 antibody", "anti-CSPG4 antibody portion," or "anti-CSPG4 antibody fragment" and/or "anti-CSPG4 antibody variant" and the like include any protein or peptide containing molecule that comprises at least a portion of an immunoglobulin molecule, containing at least one complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof derived from any of the monoclonal antibodies described herein, in combination with a heavy chain or light chain variable region, a heavy chain or light chain constant

region, a framework region, or any portion thereof, of non-murine origin, preferably of human origin, which can be incorporated into an antibody of the present invention. Alternatively, the term "anti-CSPG4 antibody" shall refer collectively or individually to hu763IgG1 H1-L1, hu763IgG1 H2-L2, hu763IgG4 H1-L1, hu763IgG4 H2-L2, hu763IgG1n H1-L1, hu763IgG1n H2-L2, hu763IgG1 H1-L2, hu763IgG4 H1-L2, hu763IgG1n H1-L2, hu763IgG1 H2-L1, hu763IgG4 H2-L1, hu763IgG1n H2-L1, and combinations thereof, as well fragments and regions thereof such as single chain variable fragments of the present invention including hu763H1-L1 scFv, hu763H2-L2 scFv, hu763H1-L2 scFv, hu763H2-L1 scFv, and combinations thereof. Such humanized antibody is capable of modulating, decreasing, antagonizing, mitigating, alleviating, blocking, inhibiting, abrogating and/or interfering with at least one cell function *in vitro*, *in situ* and/or *in vivo*, wherein said cell expresses CSPG4. As a non-limiting example, a suitable anti-CSPG4 antibody, specified portion or variant of the present invention can bind with high affinity to an epitope, in particular a peptide epitope, of human CSPG4.

[00165] Antibody fragments can be produced by enzymatic cleavage, synthetic or recombinant techniques, as known in the art and/or as described herein. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a combination gene encoding a F(ab')₂ heavy chain portion can be designed to include DNA sequences encoding the C_H1 domain and/or hinge region of the heavy chain. The various portions of antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques.

[00166] In some embodiments, chimeric or humanized antibodies of the present invention include those wherein the CDRs are derived from one or more of the anti-CSPG4 antibodies described herein and at least a portion, or the remainder of the antibody is derived from one or more human antibodies. Thus, the human part of the antibody may include the framework, C_L, C_H domains (e.g., C_H1, C_H2, C_H3), hinge, V_L, V_H regions which are substantially non-immunogenic in humans. The regions of the antibody that are derived from human antibodies need not have 100% identity with human antibodies. In some embodiments, as many of the human amino acid residues as possible are retained in order for the immunogenicity to be negligible, however, the human residues may be modified as necessary to support the antigen binding site formed by the CDRs while simultaneously maximizing the humanization of the antibody. Such changes or variations, in some embodiments, retain or reduce the immunogenicity in humans or other species relative to non-

modified antibodies. It is pointed out that a humanized antibody can be produced by a non-human animal or prokaryotic or eukaryotic cell that is capable of expressing functionally rearranged human immunoglobulin (e.g., heavy chain and/or light chain) genes. Further, when the antibody is a single chain antibody, it can comprise a linker peptide that is not found in native human antibodies. For example, an Fv can comprise a linker peptide, such as two to about twenty glycine or other amino acid residues, preferably 8-15 glycine or other amino acid residues, which connects the variable region of the heavy chain and the variable region of the light chain. Such linker peptides are considered to be of human origin.

[00167] Antibody humanization can be performed by, for example, synthesizing a combinatorial library comprising the six CDRs of a non-human target monoclonal antibody fused in frame to a pool of individual human frameworks. A human framework library that contains genes representative of all known heavy and light chain human germline genes can be utilized. The resulting combinatorial libraries can then be screened for binding to antigens of interest. This approach can allow for the selection of the most favorable combinations of fully human frameworks in terms of maintaining the binding activity to the parental antibody. Humanized antibodies can then be further optimized by a variety of techniques.

[00168] Antibody humanization can be used to evolve mouse or other non-human antibodies into "fully human" antibodies. The resulting antibody contains only human sequence and no mouse or non-human antibody sequence, while maintaining similar binding affinity and specificity as the starting antibody.

[00169] In some embodiments, anti-CSPG4 humanized or chimeric antibodies of the present invention comprise a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region (or the parental Fc region), such that said molecule has an altered affinity for an Fc receptor (e.g., an Fc γ R), provided that said variant Fc region does not have a substitution at positions that make a direct contact with Fc receptor based on crystallographic and structural analysis of Fc-Fc receptor interactions such as those disclosed by Sonderrmann et al. (2000, Nature, 406:267-273, which is incorporated herein by reference in its entirety). Examples of positions within the Fc region that make a direct contact with an Fc receptor such as an Fc γ R are amino acids 234-239 (hinge region), amino acids 265-269 (B/C loop), amino acids 297-299 (C'/E loop), and amino acids 327-332 (F/G) loop. In some embodiments, the anti-CSPG4 antibodies of the present invention comprising variant Fc regions comprise modification of

at least one residue that makes a direct contact with an Fc γ R based on structural and crystallographic analysis.

[00170] In some embodiments, anti-CSPG4 antibodies of the present invention includes a humanized 763 antibody with an altered affinity for activating and/or inhibitory receptors, having variant Fc regions with one or more amino acid modifications, wherein said one or more amino acid modification is a substitution at position 297 with alanine; in some embodiments, a substitution at 239D, 330L, 332E to enhance FcR affinity. In some embodiments, anti-CSPG4 antibodies of the present invention have an Fc region with variant glycosylation as compared to a parent Fc region; in some embodiments, variant glycosylation includes absence of fucose; in some embodiments, variant glycosylation results from expression in GnT1-deficient CHO cells.

[00171] In some embodiments, the present invention includes molecules comprising a variant Fc region with additions, deletions, and/or substitutions to one or more amino acid in the Fc region of an antibody of the present invention in order to alter effector function, or enhance or diminish affinity of antibody to FcR. These mutations are within the skill of a person in the art. Therefore, the present invention includes molecules comprising variant Fc regions that bind with a greater affinity to one or more Fc γ Rs. Such molecules preferably mediate effector function more effectively as discussed infra. In some embodiments, the present invention includes molecules comprising a variant Fc region that bind with a weaker affinity to one or more Fc γ Rs. Reduction or elimination of effector function is desirable in certain cases for example in the case of antibodies whose mechanism of action involves blocking or antagonism but not killing of the cells bearing a target antigen. Further, elimination of effector function is desirable, in some embodiments, when making bispecific antibodies as discussed infra. Reduction or elimination of effector function would be desirable in cases of autoimmune disease where one would block Fc γ R activating receptors in effector cells (This type of function would be present in the host cells). Generally, increased effector function may be directed to tumor and foreign cells; in some embodiments, effector function may be directed away from tumor cells.

[00172] Fc variants of the present invention may be combined with other Fc modifications, including but not limited to modifications that alter effector function. The invention encompasses combining an Fc variant of the invention with other Fc modifications to provide additive, synergistic, or novel properties in antibodies or Fc fusions. Preferably the Fc variants of the invention enhance the phenotype of the modification with which they are combined. For example, if an Fc variant is combined with a mutant known to bind Fc γ RIIIA with a higher affinity than a

comparable molecule comprising a wild type Fc region, the combination with the mutant results in a greater fold enhancement in FcγRIIIA affinity. In some embodiments, Fc variants of the present invention are incorporated into an antibody or Fc fusion that comprises one or more engineered glycoforms, i.e., a carbohydrate composition that is covalently attached to a molecule comprising an Fc region, wherein said carbohydrate composition differs chemically from that of a parent molecule comprising an Fc region. In some embodiments, Fc variants of the present invention are incorporated into an antibody or Fc fusion that comprises variant glycosylation. For example, antibodies may be expressed in glycosylation deficient cell line (e.g., a GnT1-deficient CHO cell) such that the antibody is produced with an Fc region lacking glycosylation as compared to a wild type Fc region, or an Fc region expressed in a cell line not deficient in glycosylation.

[00173] The present invention includes antibodies with modified glycosylation sites, preferably without altering the functionality of the antibody, e.g., binding activity CSPG4. As used herein, "*glycosylation sites*" include any specific amino acid sequence in an antibody to which an oligosaccharide (i.e., carbohydrates containing two or more simple sugars linked together) will specifically and covalently attach. Oligosaccharide side chains are typically linked to the backbone of an antibody via either N-or O-linkages. N-linked glycosylation refers to the attachment of an oligosaccharide moiety to the side chain of an asparagine residue. O-linked glycosylation refers to the attachment of an oligosaccharide moiety to a hydroxyamino acid, e.g., serine, threonine. For example, an Fc-glycoform, hu763-H2L2-IgG1n (or hu763-IgG1n), that lacked certain oligosaccharides including fucose and terminal N-acetylglucosamine was produced in special CHO cells and exhibited enhanced ADCC effector function.

[00174] In some embodiments, the present invention encompasses methods of modifying the carbohydrate content of an antibody of the invention by adding or deleting a glycosylation site. Methods for modifying the carbohydrate content of antibodies are well known in the art and are included within the present invention, see, e.g., U.S. Patent No. 6,218,149; EP 0 359 096 B1; U.S. Patent Publication No. US 2002/0028486; International Patent Application Publication WO 03/035835; U.S. Patent Publication No. 2003/0115614; U.S. Patent No. 6,218,149; U.S. Patent No. 6,472,511; all of which are incorporated herein by reference in their entirety. In some embodiments, the present invention includes methods of modifying the carbohydrate content of an antibody of the invention by deleting one or more endogenous carbohydrate moieties of the antibody. In some certain embodiments, the present invention includes deleting the glycosylation site of the Fc region of an antibody, by modifying position 297 from asparagine to alanine.

[00175] Engineered glycoforms may be useful for a variety of purposes, including but not limited to enhancing or reducing effector function. Engineered glycoforms may be generated by any method known to one skilled in the art, for example by using engineered or variant expression strains, by co-expression with one or more enzymes, for example DI N-acetylglucosaminyltransferase III (GnTIII), by expressing a molecule comprising an Fc region in various organisms or cell lines from various organisms, or by modifying carbohydrate(s) after the molecule comprising Fc region has been expressed. Methods for generating engineered glycoforms are known in the art, and include but are not limited to those described in Umana et al. (1999) Nat. Biotechnol. 17:176-180; Davies et al. (2001) Biotechnol. Bioeng. 74:288-294; Shields et al. (2002) J. Biol. Chem. 277:26733-26740; Shinkawa et al. (2003) J. Biol. Chem. 278:3466-3473) U.S. Patent No. 6,602,684; U.S. Patent Application Serial No. 10/277,370; U.S. Patent Application Serial No. 10/113,929; International Patent Application Publications WO 00/61739A1; WO 01/292246A1; WO 02/311140A1; WO 02/30954A1; POTILLEGENT™ technology (Biowa, Inc. Princeton, N.J.); GLYCOMAB™ glycosylation engineering technology (GLYCART biotechnology AG, Zurich, Switzerland); each of which is incorporated herein by reference in its entirety. See, e.g., International Patent Application Publication WO 00/061739; EA01229125; U.S. Patent Application Publication No. 2003/0115614; Okazaki et al. (2004) JMB, 336:1239-49, each of which is incorporated herein by reference in its entirety.

Multivalent Binding Agents

[00176] As those skilled in the art are aware, a multivalent binding agent is a molecular entity or complex that includes binding components that bind specifically to two or more targets (e.g., epitopes). Such multivalent binding agents find a variety of uses in the art, including therapeutic uses. To give but one example, as those skilled in the art are aware, multivalent binding agents have been engineered to facilitate killing of tumor cells by directing (or recruiting) cytotoxic T cells to a tumor site. Examples of tumor antigens include, but are not limited to, alpha fetoprotein (AFP), CA15-3, CA27-29, CA19-9, CA-125, calretinin, carcinoembryonic antigen, CD34, CD99, CD117, chromogranin, cytokeratin, desmin, epithelial membrane protein (EMA), Factor VIII, CD31 FL1, glial fibrillary acidic protein (GFAP), gross cystic disease fluid protein (GCDFP-15), HMB-45, human chorionic gonadotropin (hCG), inhibin, keratin, CD45, a lymphocyte marker, MART-1 (Melan-A), Myo D1, muscle-specific actin (MSA), neurofilament, neuron-specific enolase (NSE),

placental alkaline phosphatase (PLAP), prostate-specific antigen, S100 protein, smooth muscle actin (SMA), synaptophysin, thyroglobulin, thyroid transcription factor- 1, tumor M2-PK, and vimentin.

[00177] The potential efficacy of multispecific binding agents that engage T cells lies in the ability of these agents to direct T cells to a tumor site for T-cell mediated killing. T cells are the most potent effector cells in the immune system at killing aberrant cells and are not capable of Fc-mediated antibody dependent cellular cytotoxicity (ADCC). The mechanism by which such multivalent binding agents direct T cells to a tumor site is through binding of a tumor antigen on the surface of a tumor and a co-receptor on the surface of T cells, CD3. CD3 is a complex of three chains (γ , δ , and ϵ) expressed on the surface of all mature T cells. Expression of CD3 is almost exclusively restricted to T cells. The anti-CD3 component of a bispecific binding agent can transform a previously unstimulated and uncommitted nonclonal T cell to become potent serial killer of tumor cells (Wolf et al. (2005) *Drug Discov. Today* 10:1237-1244). Binding agents of this type have demonstrated efficacy in animal xenograft studies of solid tumors expressing the epithelial cell adhesion molecule (EpCAM) antigens in addition to other targets (Bargou et al. (2008) *Science* 321:974-977; Brischwein et al. (2006) *Mol. Immunol.* 43:1129-1143; Baeuerle and Reinhardt (2009) *Cancer Res.* 69:4941-4944).

[00178] In some embodiments, multivalent binding agents for use in accordance with the present invention are bispecific binding agents. In many embodiments, such bispecific binding agents are capable of binding to T cells. In many embodiments, such bispecific binding agents are capable of binding to CD3 on T cells.

[00179] In some embodiments, multivalent or bispecific binding agents for use in accordance with the present invention are or comprise antibody components. A variety of technologies are known in the art for designing, constructing, and/or producing multispecific or bispecific binding agents comprising antibody components.

[00180] For example, bispecific binding agents have been constructed that either utilize the full immunoglobulin framework (e.g., IgG), single chain variable fragment (scFv), or combinations thereof. Bispecific binding agents composed of two scFv units in tandem has been shown to be one of the most clinically successful bispecific antibody formats. In the case of anti-tumor immunotherapy, bispecific binding agents that comprise two single chain variable fragments (scFvs) in tandem have been designed such that an scFv that binds a tumor antigen is linked with an scFv that engages T cells by binding CD3. In this way, T cells are recruited to a tumor site in the hope that they can mediate killing of the tumor cells making up the tumor by the cytotoxic

properties that certain T cells have. An example of such a bispecific binding agent has been made that targets CD19 and CD3 for lymphoma (termed Bispecific T cell Engaging, or BiTE; e.g., see Dreier et al. (2003) *J. Immunol.* 170:4397-4402; Bargou et al. (2008) *Science* 321:974-977), which has been successful in preventing tumor growth in animal xenograft studies. In human studies, this bispecific binding agent demonstrated objective tumor response, including five partial and two complete remissions.

[00181] Bispecific binding agents (e.g., bispecific antibodies) of the present invention are based on the particular insight that certain formats may be more beneficial for certain targets (e.g., a tumor antigen) when engaging T cells via CD3. For example, bispecific antibodies provided herein utilize a combination of a full IgG and an scFv. Such bispecific antibodies demonstrate bivalent binding via the IgG component (e.g., anti-CSPG4) and monovalent binding via the scFv component (e.g., anti-CD3). As described herein, bispecific antibodies having this format demonstrate a very high potency to kill tumor cells (i.e., have a very low EC₅₀). This high potency is due, in part, to the increased avidity resulting from the combination of bivalent and monovalent binding components into a single molecule and results in enhanced targeting of T cells to tumor cells. Moreover, by employing monovalent binding for the anti-CD3 component, overstimulation of T cells in the absence of tumors is avoided, thereby eliminating cytokine storm, which is a tremendous safety concern for patients and very common side effect for bispecific agents that target CD3 on T cells.

[00182] Exemplary bispecific binding agents include those with a first antibody component specific for a tumor antigen and a second antibody component specific for a cytotoxic marker, e.g., an Fc receptor (e.g., FcγRI, FcγRII, FcγRIII, etc.) or a T cell marker (e.g., CD3, CD28, etc.). Further, the second antibody component can be substituted with an antibody component having a different desired specificity. For example, a bispecific binding agent with a first antibody component specific for a tumor antigen and a second antibody component specific for a toxin can be paired so as to deliver a toxin (e.g., saporin, vinca alkaloid, etc.) to a tumor cell. Other exemplary bispecific binding agents include those with a first antibody component specific for an activating receptor (e.g., B cell receptor, FcγRI, FcγRIIA, FcγRIIIA, FcγRI, T cell receptor, etc.) and a second antibody component specific for an inhibitory receptor (e.g., FcγRIIB, CD5, CD22, CD72, CD300a, etc.). Yet another example includes a second antibody component specific to a different antigen on the same cell type for which a first antibody component is specific, for example, CD20, CD19, CD21, CD23, CD46, CD80, HLA-DR, CD74, MUC1, and CD22 on B-cells. Such bispecific binding agents can be constructed for therapeutic conditions associated with

cell activation (e.g. allergy and asthma). Bispecific binding agents can be made, for example, by combining heavy chains and/or light chains that recognize different epitopes of the same or different antigen. In some embodiments, by molecular function, a bispecific binding agent binds one antigen (or epitope) on one of its two binding arms (one V_H/V_L pair), and binds a different antigen (or epitope) on its second arm (a different V_H/V_L pair). By this definition, a bispecific binding agent has two distinct antigen binding arms (in both specificity and CDR sequences), and is monovalent for each antigen to which it binds.

[00183] In some embodiments, bispecific binding agents of the present invention are characterized by the ability to can bind simultaneously to two targets which are of different structure. In some embodiments, bispecific binding agents of the present invention have at least one component that specifically binds to, for example, a B-cell, T-cell, myeloid, plasma, or a mast cell antigen or epitope and at least one other component that specifically binds to a targetable conjugate that bears a therapeutic or diagnostic agent.

[00184] The tumor antigen CSPG4 is highly expressed in several melanomas, and there have been no successful humanized antibodies nor bispecific humanized antibodies based on the murine 763 antibody. Humanized 763 antibodies as described herein demonstrate high affinity to CSPG4 and bind to a non-carbohydrate (peptide) epitope and usually low k_{off} rates as measured by Biacore. Also, bispecific binding proteins employing humanized 763 antibodies as described herein are capable of bivalent binding to CSPG4 and monovalent binding to CD3 which results in enhanced potency for killing CSPG4⁺ tumors and increased safety from a lack of overstimulation of CD3. As such, the strategy for employing the format of the bispecific binding proteins as described represents a unique approach for enhanced tumor killing, reduced adverse effects, and demonstrates a potent therapeutic for the treatment of several CSPG4-positive cancers.

Targets

[00185] Among other things, the present invention encompasses the recognition that multispecific binding agents, and particularly bispecific binding agents such as bispecific antibodies, are particularly useful and/or effective to facilitate cell killing. In particular, the present invention demonstrates that activity of multivalent binding agents that bind specifically to both a target-cell-associated epitope (e.g., a melanoma-associated tumor antigen) and a lymphocyte-associated epitope (e.g., a T cell surface protein) can be an effective immunotherapy for melanoma-associated cancers.

[00186] For example, in some embodiments of the present invention, a multivalent binding agent binds specifically to a tumor-cell-associated epitope and a T-cell epitope. In accordance with such embodiments, the multivalent binding agent can facilitate binding of the agent to one or both of its target epitopes and/or can enhance killing of the target tumor cell as mediated by the target T cell.

[00187] In some embodiments, target cells to be killed include, for example, cells that express a tumor antigen (e.g., a melanoma-associated tumor antigen). Those of ordinary skill in the art will be aware of appropriate target epitopes on such cells to which multivalent binding agents as described herein desirably bind.

[00188] In some embodiments, lymphocyte cells that can mediate killing of target cells as described herein include T cells (e.g., CD8⁺ T cells), natural killer (NK) cells, macrophages, granulocytes and antibody-dependent cytotoxic cells. Those of ordinary skill in the art will be aware of appropriate target epitopes on such lymphocytes to which multivalent binding agents as described herein desirably bind. Representative such epitopes can be found on antigens such as, for example, Fc receptor of IgG (e.g., FcγRIIB), CD Id, CD3, CD4, CD7, CD8, CD13, CD14, CD16, CD31, CD38, CD56, CD68, MAC-1/MAC-3, IL-2Ra, OX40, Ly49, and CD94.

Nucleic Acid Construction and Expression

[00189] Humanized antibodies and multispecific binding agents (e.g., bispecific antibodies) as described herein may be produced from nucleic acid molecules using molecular biological methods known to the art. Nucleic acid molecules are inserted into a vector that is able to express the fusion proteins in when introduced into an appropriate host cell. Appropriate host cells include, but are not limited to, bacterial, yeast, insect, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the fusion proteins of the present invention under control of transcriptional/translational control signals. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (See Sambrook et al. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al, Greene Publ. Assoc., Wiley-Interscience, NY).

[00190] Expression of nucleic acid molecules in accordance with the present invention may be regulated by a second nucleic acid sequence so that the molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the nucleic acid molecules of the invention may be controlled by a promoter and/or enhancer element, which are known in the art.

[00191] Nucleic acid constructs include regions that encode multispecific binding proteins generated from antibodies and/or antibody components. Typically, such multispecific binding proteins will be generated from V_H and/or V_L regions. After identification and selection of antibodies exhibiting desired binding and/or functional properties, variable regions of each antibody are isolated, amplified, cloned and sequenced. Modifications may be made to the V_H and V_L nucleotide sequences, including additions of nucleotide sequences encoding amino acids and/or carrying restriction sites, deletions of nucleotide sequences encoding amino acids, or substitutions of nucleotide sequences encoding amino acids. The antibodies and/or antibody components may be generated from human, humanized or chimeric antibodies.

[00192] Nucleic acid constructs of the present invention are inserted into an expression vector or viral vector by methods known to the art, and nucleic acid molecules are operatively linked to an expression control sequence.

[00193] Where appropriate, nucleic acid sequences that encode humanized antibodies and multispecific binding agents as described herein may be modified to include codons that are optimized for expression in a particular cell type or organism (e.g., see U.S. Patent No's 5,670,356 and 5,874,304). Codon optimized sequences are synthetic sequences, and preferably encode the identical polypeptide (or a biologically active fragment of a full length polypeptide which has substantially the same activity as the full length polypeptide) encoded by the non-codon optimized parent polynucleotide. In some embodiments, the coding region of the genetic material encoding antibody components, in whole or in part, may include an altered sequence to optimize codon usage for a particular cell type (e.g., a eukaryotic or prokaryotic cell). For example, the coding sequence for a humanized heavy (or light) chain variable region as described herein may be optimized for expression in a bacterial cells. Alternatively, the coding sequence may be optimized for expression in a mammalian cell (e.g., a CHO). Such a sequence may be described as a codon-optimized sequence.

[00194] An expression vector containing a nucleic acid molecule is transformed into a suitable host cell to allow for production of the protein encoded by the nucleic acid constructs. Exemplary host cells include prokaryotes (e.g., E. coli) and eukaryotes (e.g., a COS or CHO cell). Host cells transformed with an expression vector are grown under conditions permitting production of a humanized antibody or multispecific binding agent of the present invention followed by recovery of the humanized antibody or multispecific binding agent.

[00195] Humanized antibodies and/or multispecific binding agents of the present invention may be purified by any technique, which allows for the subsequent formation of a stable antibody or binding agent molecule. For example, not wishing to be bound by theory, antibodies and/or multispecific binding agents may be recovered from cells either as soluble polypeptides or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify antibodies and/or multispecific binding agents of the present invention, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used. Humanized antibodies and/or multispecific binding agents of the present invention may also be recovered from conditioned media following secretion from eukaryotic or prokaryotic cells.

Screening and Detection Methods

[00196] Humanized antibodies and/or multispecific binding agents of the present invention may also be used in *in vitro* or *in vivo* screening methods where it is desirable to detect and/or measure one or more activities of a cell or cells (e.g., apoptosis or cell growth). Screening methods are well known to the art and include cell-free, cell-based, and animal assays. *In vitro* assays can be either solid state or soluble target molecule detection may be achieved in a number of ways known to the art, including the use of a label or detectable group capable of identifying a humanized antibody or a multispecific binding agent which is bound to a target molecule (e.g., cell surface antigen). Detectable labels may be used in conjunction with assays using humanized antibodies or multispecific binding agents of the present invention.

Therapeutic Methods

[00197] The ability of humanized antibodies and/or multispecific binding agents of the present invention to exhibit high affinity binding for one of the target antigens makes them therapeutically useful for efficiently targeting cells expressing the target antigen. Thus, in some embodiments, it may be desirable to increase the affinity of a humanized antibody or multispecific binding agent for one target antigen and not the other target antigen that is also bound by the multispecific binding agent (or an Fc receptor in the case of a humanized antibody). For example, in the context of tumor killing, certain conditions may benefit from an increase in affinity to a tumor antigen but not to an antigen on the surface of a cell capable of mediating killing of the tumor (e.g., a T cell). Thus, it may be beneficial to increase the binding affinity of a humanized antibody or multispecific binding

agent to a tumor antigen in a patient having a tumor that expresses the tumor antigen through the use of a humanized antibody or multispecific binding agent as described herein.

[00198] The present invention provides a humanized antibody and/or multispecific binding agent as described herein as a therapeutic for the treatment of patients having a tumor that expresses an antigen that is capable of being bound by such a multispecific binding agent. Such humanized antibodies and/or multispecific binding agents may be used in a method of treatment of the human or animal body, or in a method of diagnosis.

Administration

[00199] The present invention provides methods of administering an effective amount of a therapeutic active described herein (e.g., a humanized antibody or multispecific binding agent) to a subject in need of treatment.

[00200] Humanized antibodies or multispecific binding agents as described herein may be administered through various methods known in the art for the therapeutic delivery of agents, such as proteins or nucleic acids can be used for the therapeutic delivery of a humanized antibody or multispecific binding agent or a nucleic acid encoding a humanized antibody or multispecific binding agent of the present invention for killing or inhibiting growth of target cells in a subject, e.g., cellular transfection, gene therapy, direct administration with a delivery vehicle or pharmaceutically acceptable carrier, indirect delivery by providing recombinant cells comprising a nucleic acid encoding a multispecific binding agent of the present invention.

[00201] Various delivery systems are known and can be used to administer a humanized antibody or multispecific binding agent of the present invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Routes of administration can be enteral or parenteral and include, but are not limited to, intravenous, subcutaneous, intramuscular, parenteral, transdermal, or transmucosal (e.g., oral or nasal). In some embodiments, multispecific binding agents of the present invention are administered intravenously. In some embodiments, multispecific binding agents of the present invention are administered subcutaneously. In some embodiments, multispecific binding agents are administered together with other biologically active agents.

Pharmaceutical Compositions

[00202] The present invention further provides pharmaceutical compositions comprising humanized antibodies or multispecific binding agents of the present invention and a pharmaceutically acceptable carrier or excipient. The composition, if desired, can also contain one or more additional therapeutically active substances.

[00203] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions that are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation.

[00204] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with a diluent or another excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, shaping and/or packaging the product into a desired single- or multi-dose unit.

[00205] A pharmaceutical composition in accordance with the present invention may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "*unit dose*" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient that would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[00206] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the invention will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient.

[00207] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's The Science and Practice of Pharmacy, 21st Edition, A. R.

Gennaro (Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference) discloses various excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention.

[00208] In some embodiments, a pharmaceutically acceptable excipient is at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use in humans and for veterinary use. In some embodiments, an excipient is approved by the United States Food and Drug Administration. In some embodiments, an excipient is pharmaceutical grade. In some embodiments, an excipient meets the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[00209] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in pharmaceutical formulations. Excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents can be present in the composition, according to the judgment of the formulator.

[00210] General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in Remington: The Science and Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference).

Kits

[00211] The present invention further provides a pharmaceutical pack or kit comprising one or more containers filled with at least one humanized antibody or multispecific binding agent (e.g., a bispecific antibody) as described herein. Kits may be used in any applicable method, including, for example, diagnostically. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects (a) approval by the agency of manufacture, use or sale for human administration, (b) directions for use, or both.

[00212] Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments, which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

[00213] The following examples are provided so as to describe to those of ordinary skill in the art how to make and use methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Unless indicated otherwise, temperature is indicated in Celsius, and pressure is at or near atmospheric.

Example 1. Selection of murine 763 as anti-CSPG4 antibody of interest

[00214] Among other things, the present invention encompasses the insight that the murine anti-CSPG4 known as 763 (as an abbreviation for clone 763.74) was of particular interest for humanization. Without wishing to be bound by any particular theory, present inventors proposed that high affinity (particularly single digit nanomolecular affinity) and slow k_{off} (desirably 10^{-5} or slower) would be particularly desirable for an antibody to be humanized, and/or to be incorporated into a multispecific format.

[00215] We tested four candidate anti-CSPG4 antibodies with nonoverlapping epitope specificities for their leaking after binding to CSPG4⁺ tumor cell line M14. As shown in Table 6 and Figure 1, 763 was found to have the slowest k_{off} rate, based on the binding kinetics as determined using a BIOCORE T-100 machine. The slow k_{off} of antibodies translated into a slower wash-off when antibodies were reacted with CSPG4⁺ M14 tumor cells and then washed multiple times in wash buffer. With each wash, the remaining antibodies on the cell surface were detected using a secondary FITC-labeled goat anti-mouse antibody and mean fluorescent intensity determined by flow cytometry. Based on MFI, 763 and 225.28s had stronger reactivity compared to D2.8.5 or 9.2.27.

[00216] We also defined as a selection parameter that a desirable mouse antibody for humanization would not have excessive affinity (e.g., 9.2.27) since a 0.03 nM affinity could lead to affinity barrier issues (Weinstein et al. (1992) Cancer Res. 52:2747s-2751s).

[00217] Still further, we defined as a selection parameter that a desirable mouse antibody for humanization would bind to a peptide epitope (and particularly to a conformational epitope), rather than to carbohydrate (9.2.27; Hwang et al. (1985) Cancer Res. 45:4150-4155) or linear peptide

(225.28s) epitope. We note that previous reports of peptide mimics that reacted with 763 had homology (position 289-294) with CSPG4 but very low affinity (Geiser et al. Cancer Res. 59:905,1999), and therefore not the cognate epitope recognized by 763.

TABLE 6

Antibody	Parental Ab isotype/format	K_{on}	K_{off}	K_d	Epitope
763.74	mouse IgG1	2.96E+04	3.81E-05	1.3	Conformation epitope 1289-1760 (D2.8.11)
225.28s	mouse IgG2a	1.20E-04	1.44E-04	1.2	Linear epitope 1705-1712 ^{5,6}
9.2.27	mouse IgG2a	-	-	0.03	Carbohydrate epitope ⁷
D2.8.5	scFv	1.32E+05	3.50E-04	2.65	peptide (D2.8.5)

Example 2. Humanization of murine anti-CSPG4 antibody 763

[00218] CSPG4 (Chondroitin sulfate proteoglycan 4) or HMW-MAA (high molecular weight melanoma associated antigen) is an established melanoma associated tumor antigen. In fresh melanoma tissues, it is homogeneously and strongly expressed and yet highly restricted in normal human tissues. More recently, CSPG4 was found to be overexpressed in triple negative breast cancer stem cells (Wang et al., JNCI 102:1496-1512). CSPG4 has been successfully targeted using monoclonal antibodies carrying α -emitting isotopes in patients with melanoma (Raja, C. et al. (2007) Cancer Biol. Ther. 6:846-852). The present Example describes production of humanized antibodies based on murine antibody 763, which is specific for chondroitin sulfate proteoglycan 4 (CSPG4). Although murine 763 antibody has been previously described, it has not been used as a basis for the construction of chimeric or humanized antibodies. The data presented herein describes the successful production of several humanized 763 antibodies in multiple formats such as, for example, a humanized 763 IgG1, a humanized 763 IgG4 and a humanized IgG1n (a special glycoform) that was expressed in an engineered CHO cell.

[00219] Additional Examples presented herein demonstrate that all humanized 763 antibody formats showed antigen binding comparable to murine 763, kept favorable K_D and unusually K_{off} , mediate antibody-dependent cell-mediated cytotoxicity (ADCC) with high potency to melanoma cells (e.g., humanized 763-IgG1n), and are able to engage T cells to specifically target CSPG4⁺ tumor cells when in the context of bispecific antibodies.

[00220] Briefly, humanized formats of murine 763 antibody (humanized 763-IgG1, humanized 763-IgG4, and humanized 763-IgG1n) were constructed. Sequence design was based on human IgG homology calculations while conserving critical mouse amino acid residues. The CDRs of the

heavy and light chains of murine 763 were grafted onto human IgG1 frameworks based on their homology with human frameworks IgGHV3-33 and IGLKV3-15, respectively, and of the allotypes Km3 and G1m3, respectively. Two different heavy chain and two different light chain sequences were expressed as full IgGs and tested for binding and stability. The most stable combination (H2/L2), without forming aggregates by HPLC on repeat freeze/thaw cycles, was chosen for the final form of humanized 763 for the rest of the experiments. Additional constructs were made using a human IgG4 framework. In addition a chimeric 763 antibody was made using human C_L of kappa (κ) light chain and human C_H1-C_H2-C_H3 of gamma1 heavy chain constant regions. Exemplary antibodies made in accordance with this Example are set forth in Table 7.

[00221] Humanized 763 antibodies were packaged in a single vector (for balanced heavy chain and light chain secretion) and transduced into CHO-DG44 cells using bluescript vectors. Hu763-IgG1n is a humanized 763 IgG1 antibody glycoform expressed in CHO cells with variant glycosylation from a GnT1 deficiency (Jefferis, R. (2009) Nat Rev Drug Discov 8:226-234; Idusogie, E.E. et al. (2000) J. Immunol. 164:4178-4184). Humanized 763 IgG1, humanized 763 IgG4 and humanized 763 IgG1n were purified using standard protein A affinity chromatography. Sugar analysis confirmed that humanized 763 IgG1n had 78.3% (Mol%) Mannose, 20.5% (Mol%) N-Acetyl Glucosamine and 1.2% (Mol%) Glucose. On SDS gel, humanized 763 migrated as IgG with the appropriate size heavy and light chains; and by HPLC, they all eluted as whole IgG with < 5% aggregate formation (Figure 2).

TABLE 7

Name	Description
hu763IgG1 H1-L1	humanized 763 H1 and L1 in IgG1 format
hu763IgG1 H2-L2	humanized 763 H2 and L2 in IgG1 format
hu763IgG4 H1-L1	humanized 763 H1 and L1 in IgG4 format
hu763IgG4 H2-L2	humanized 763 H2 and L2 in IgG4 format
hu763IgG1n H2-L2	humanized 763 H2 and L2 in IgG1 format with variant glycosylation
ch763IgG1	chimeric 763 HC and LC in IgG1 format

Example 3. Antigen binding kinetics of humanized 763 antibodies

[00222] This Example illustrates the effect of humanization made in accordance with Example 1 on the functional affinity to CSPG4. In some cases, humanized 763 antibodies may bind to CSPG4 for short periods of time (e.g., poor retention due to size). In this example, humanized 763 antibodies demonstrate favorable K_D and unusually slow k_{off} rates.

[00223] Briefly, antigen (D2.8.11, a peptide epitope for murine 763) or anti-763 idiotype antibody MK2-23 was immobilized onto CM5 chips, kinetics of antibody binding (k_{on} , k_{off} and K_D) were compared by surface plasma resonance (SPR) using Biacore T-100 (Figure 3A). For binding to D2.8.11, humanized 763 antibodies including humanized IgG1 and IgG4 demonstrated slower k_{off} than murine 763, and better K_D than murine 763 (see Table 8; 1.34 pM for mouse 763, 1.29 pM for hu763-IgG1, 1.15 pM for hu763-IgG1n and 1.22 pM for hu763-IgG4). Similarly, for binding to MK2-23, both chimeric 763 and humanized 763 antibodies showed comparable binding to murine 763 (Figures 3B and 3C), as well as slightly better K_D (Tables 9 and 10).

[00224] Antigen binding was also analyzed by FACS analysis using CSPG4 positive melanoma M14 cells. For humanized 763 antibodies, cell binding was determined using a FITC-labeled goat anti-human secondary antibody. For murine 763, a FITC-labeled goat anti-mouse secondary antibody was used (Figure 3D). Data were expressed as mean fluorescent intensity determined by flow cytometry and normalized as percentage of binding of the highest concentration of antibody used ($5 \mu\text{g}/10^6$ cells). Hu763-IgG1 showed better EC_{50} ($0.02 \mu\text{g}/1$ million cells) than that of murine 763 ($0.07 \mu\text{g}/1$ million cells). Hu763-IgG1 is quite stable, its EC_{50} of antigen binding is $0.03 \mu\text{g}/1$ million cells after subjected to five cycles of freezing and thawing process.

[00225] An ELISA method using coated M14 as antigen was also used to assay hu763-IgG1 and hu763-IgG1n binding (Figure 3E). Both hu763-IgG1 and hu763-IgG1n showed comparable binding

to M14 cells. Hu763-IgG1n still bound to M14 cells well after subjected to five cycles of freezing and thawing process.

TABLE 8

Biacore analysis of antigen binding on peptide D2.8.11			
Antibody	k_{on} (1/Ms)	k_{off} (1/s)	$K_D=k_{off}/k_{on}$ (M)
murine 763	2.90E+04	3.89E-05	1.34E-09
ch763-IgG1	3.51E+04	2.95E-03	8.40E-08
hu763-IgG1	2.96E+04	3.81E-05	1.29E-09
hu763-IgG1n	2.85E+04	3.28E-05	1.15E-09
hu763-IgG4	2.87E+04	3.50E-05	1.22E-09

TABLE 9

Biacore analysis of antigen binding on anti-idiotypic MK2-23			
Antibody	k_{on} (1/Ms)	k_{off} (1/s)	$K_D=k_{off}/k_{on}$ (M)
murine 763	1.46E+05	8.50E-05	5.82E-10
hu763-IgG1n	1.68E+05	7.54E-05	4.49E-10
hu763-IgG4	1.67E+05	8.01E-05	4.80E-10

TABLE 10

Biacore analysis of antigen binding on anti-idiotypic MK2-23			
Antibody	k_{on} (1/Ms)	k_{off} (1/s)	$K_D=k_{off}/k_{on}$ (M)
murine 763	7.82E+04	1.63E-04	2.09E-09
hu763-IgG1	1.03E+05	1.81E-04	1.75E-09
ch763-IgG1	1.79E+05	3.39E-04	1.89E-09

Example 4. Antibody-dependent cell-mediated cytotoxicity (ADCC) with humanized 763 antibodies

[00226] This Example demonstrates the enhanced ability of humanized 763 antibodies to mediate ADCC via NK cells on target cells. Further, the data described in the present Example illustrates the benefit of humanizing the murine 763 is not solely to reduce immunogenicity.

[00227] Mouse 763 antibody is a mouse IgG1, which does not mediate ADCC due to a lack of binding to human Fc receptor. To determine the ADCC potential of humanized 763 antibodies, a CD16-transduced NK92Mi cell line was first generated. This NK92 cell was transduced with both IL-2 and human CD16 (FcγRIIIA), an activating Fc receptor. The human CD16 used contained a high-affinity polymorphism (F158V), which leads to an enhancement in ADCC and clinical response to IgG1-based immunotherapy. ADCC of humanized 763 was evaluated using the

NK92Mi cell line described above. The specific lysis of target cells by NK cells activated by humanized 763 antibodies is shown in Figure 4.

[00228] As shown in Figure 4, only hu763-IgG1n was able to mediated ADCC. EC_{50} for M14 cells was 0.1 $\mu\text{g/mL}$, while EC_{50} for U2OS cells was 0.65 $\mu\text{g/mL}$. When toxicity was tested on hepatocytes and cardiac myocytes, neither hu763-IgG4 nor hu763-IgG1n triggered ADCC or CMC in the presence of PBMC or NK92Mi (CD16) cells at antibody concentrations from 1 ng/mL to 10 $\mu\text{g/mL}$.

Example 5. Biodistribution of humanized 763 antibodies in mice bearing tumor xenografts

[00229] The humanized 763 antibodies described in the prior Examples were tested for their *in vivo* efficacy. Biodistribution of radioiodinated antibody in mice implanted with SKMEL-28 tumor cells was determined.

[00230] Hu763-IgG1, hu763-IgG4 and hu763-IgG1n were radiolabeled with ^{131}I or ^{124}I . All demonstrated comparable immunoreactivity of ~80-90%. Biodistributions of humanized 763 antibodies at 48 hours were analyzed using mice bearing subcutaneous SKMEL-28 xenografts. Tumor uptake was measured by %ID/gm. Treatment with mouse 763 antibody resulted in 27.4%, hu763-IgG1 in 13.55%, hu763-IgG4 in 10.24%, and hu763-IgG1n in 10.38% (Figure 5). Tumor to non-tumor ratios were comparable among the tested antibodies.

Example 6. Design, construction and expression of bispecific antibodies based on humanized 763

[00231] This Example describes production of bispecific antibodies composed of a first antigen-binding site based on a humanized 763 antibody and a second antigen-binding site that binds to T cells. The data presented herein describes the successful production of bispecific antibodies (termed hu763-BsAbs) to retargeting T cells to melanoma cells. As described herein, an anti-CD3 single chain Fv fragment (ScFv) based on a humanized OKT3 antibody was linked to the carboxyl end of a humanized 763 heavy chain (hu763-HC-OKT3) or linked to the carboxyl end of light chain (hu763-LC-OKT3). A major drawback in the development of T-cell engaging bispecific antibodies has been overstimulation of T cells resulting from CD3 engagement. Such engagement can lead to excessive release of cytokines (known as cytokine storm), which results in serious adverse effects in patients. Therefore, the inventors have introduced an N297A substitution in the Fc region to remove glycosylation and, therefore, eliminating Fc-receptor binding, which also reduces

complement activation thereby reducing cytokine storm. As demonstrated below, hu763-BsAbs described herein effectively redirected T cells to lyse CSPG4⁺ tumor cells *in vitro* and significantly inhibited tumor growth in murine melanoma xenografts. Such hu763-BsAbs provide both Fc-dependent and T cell-dependent immunotherapeutic possibilities for metastatic tumors such as melanoma.

[00232] The inventors have designed hu763-BsAbs using the IgG-scFv format set forth in Figure 6. For the hu763-HC-OKT3 format, the light chain was identical to that of a hu763-IgG1, the heavy chain was constructed by extending a hu763 IgG1 heavy chain with a C-terminal (G₄S)₃ linker followed by huOKT3 scFv; for hu763-LC-OKT3 format, the heavy chain was identical to that of a hu763 IgG1, the light chain was constructed by extending a hu763 light chain with a C-terminal (G₄S)₃ linker followed by huOKT3 scFv. For both formats, a N297A mutation was introduced to hIgG1 Fc region to remove glycosylation (as described above). The DNA encoding both heavy chain and light chain was inserted into a mammalian expression vector, transfected into CHO-S cells, and stable clones of highest expression were selected. Supernatants were collected from shaker flasks and purified on protein A affinity chromatography. Proteins were further purified to >90% monomer by size exclusion chromatography.

[00233] Biochemical purity analysis of the BsAb is shown in Figure 7. Under reducing SDS-PAGE conditions, Hu763-HC-OKT3 format gave rise to two bands (~75 KDa and ~25 KDa; the huOKT3 scFv fusion to hu763 heavy chain increases the molecular weight to ~75 KDa). Hu763-LC-OKT3 format gave rise to two bands at around 50 KDa (the huOKT3 scFv fusion to hu763 light chain increases the molecular weight to ~50 KDa). SEC-HPLC showed a major peak (97% by UV analysis) with an approximate molecular weight of 210 KDa for both formats, as well as a minor peak of multimers removable by gel filtration.

Example 7. Bispecific antibodies based on humanized 763 bind to tumor cells and T cells

[00234] This Example demonstrates bispecific antibodies as described herein are characterized by binding to tumor cells and T cells thereby directing effector T cells to kill target tumor cells. The data presented in this Example confirms that such bispecific antibodies are useful for killing and/or inhibiting the growth of tumor cells.

[00235] The binding of hu763-BsAbs to both target cells and effector cells was tested by FACS immunostaining. As shown in Figure 8A, hu763-BsAbs both bound to CSPG4⁺ breast carcinoma cell M14 as good as parental hu763. Further, as shown in Figure 8B, hu763-BsAbs bound to CD3⁺

T cells as well, however, hu763-HC-OKT3 bound slightly worse than humanized OKT3 IgG1 and hu763-LC-OKT3 bound 20-30 fold weaker than parental hu763 or hu763-HC-OKT3. These data are consistent with our observation that light chain anchored scFvs have lower avidity for T cells than regular humanized OKT3 IgG1, which are purposely designed to minimize cytokine release in the absence of target tumor cells.

[00236] The lower avidity of hu763-BsAb for T cells was further confirmed by binding affinity analysis by Biacore as previously described (Table 11; Cheung, N.K. et al. (2012) *OncoImmunology* 1:477-486; Law, C.L. et al. (2002) *Int. Immunol.* 14:389-400). For CD3 antigen, hu763-HC-OKT3 had a k_{on} at $3.02 \times 10^5 \text{ M}^{-1}\text{S}^{-1}$, a k_{off} at $6.96 \times 10^{-2} \text{ s}^{-1}$, and overall K_D at 231 nm, which is comparable to parental humanized OKT3 IgG1-aGlyco at k_{off} ($1.05 \times 10^{-1} \text{ s}^{-1}$), but less at k_{on} ($1.71 \times 10^6 \text{ M}^{-1}\text{S}^{-1}$) and overall K_D (61.7 nM). Hu763-LC-OKT3 had a k_{on} at $1.75 \times 10^5 \text{ M}^{-1}\text{S}^{-1}$, a k_{off} at $9.01 \times 10^{-2} \text{ s}^{-1}$, and overall K_D at 515 nm. Taken together, hu763-BsAbs, in particular, hu763-LC-OKT3, had much lower k_{on} than parental humanized OKT3-aGlyco and larger overall K_D , which suggests that hu763BsAbs have much lower avidity to binding CD3 and, therefore, are less likely to bind and activate T cells under same conditions. Under these circumstances, hu763BsAbs yield hence less cytokine release and would provide an improved safety benefit to patients.

TABLE 11

CD3 binding of hu763 BsAbs measured by surface plasma resonance

Two State Reaction	k_{on} (1/Ms)	k_{off} (1/s)	$K_D = k_{off}/k_{on}$
huOKT3-aGlyco	1.71E+06	1.05E-01	6.17E-08
hu763-HC-OKT3	3.02E+05	6.96E-02	2.31E-07
hu763-LC-OKT3	1.75E+05	9.01E-02	5.15E-07

Example 8. Humanized 763 bispecific antibody-directed T cell killing of human tumor cell lines

[00237] This Example demonstrates the enhanced ability of bispecific antibodies based on humanized 763 to initiate tumor cell killing mediated through T cells. Typically, bispecific binding proteins that engage T cells are able to direct T cell to a tumor site for T cell mediated killing of the tumor. In this example, exemplary bispecific antibodies are shown to effectively mediate T cell killing of tumor cells more effectively as compared to control bispecific antibodies.

[00238] Briefly, to evaluate whether hu763-BsAbs could redirect T cells to kill tumor cells, T cell cytotoxicity on CSPG4⁺ cancer cell lines (M14, HTB-63 and SKMEL-28) was tested in a 4-hour ⁵¹Cr release assay. Exemplary results are presented in Figure 9.

[00239] Compared to a control BsAb (one antigen-binding domain specific to CSPG4 and one antigen-binding domain specific to an organic compound), both hu763-HC-OKT3 and hu763-LC-OKT3 were able to mediated substantial killing of all three types of tumor cells in the presence of T cells.

[00240] This Example just confirms, among other things, that bispecific antibodies based on humanized 763 that also bind to T cells can effectively mediate T cell killing of multiple tumor cells that express CSPG4.

Example 9. *In vivo* efficacy of CSPG4 bispecific antibodies

[00241] This Example illustrates the *in vivo* efficacy of humanized 763 bispecific antibodies described in the prior Examples.

[00242] Briefly, BALB-Rag2-KO-IL-2R- γ c-KO mice were used to evaluate the *in vivo* effect of humanized 763 bispecific antibodies. M14-Luciferase cells were inoculated intravenously to mimic a metastatic model. Treatment with humanized 763 bispecific antibody (hu763-HC-OKT3) was initiated four days post implantation and at two doses per week for a total of two weeks. Effector cells ATC were intravenously administered on day six at one dose (5×10^6 cells) per week for two weeks. Tumor luciferin bioluminescence signal was recorded and quantified weekly. Exemplary results are presented in Figure 10.

[00243] As shown in Figure 10, mice treated with hu763-BsAb alone demonstrated tumor growth equivalent to control treated mice. ATC only (T cell only) had a moderate effect on tumor growth. When ATC and hu763-HC-huOKT3 were combined, substantial suppression of tumor growth was recorded. On day 24, treatment with hu763-HC-huOKT3 demonstrated near complete eradication of tumor cells.

Materials and Methods for Examples

Construction of the hu763-IgG1, hu763-IgG4, hu763-IgG1n Antibody Producer Lines

[00244] Based on human homologues of murine 763, CDR sequences of both heavy and light chains of humanized 763 were grafted into the human IgG1 framework and optimized. The humanized 763 genes were synthesized for CHO cells (Blue Heron Biotechnology, Bothell, WA or

Genscript, Piscataway, NY). Using the bluescript vector (Eureka, CA), the heavy and light chain genes of humanized 763 were transfected into DG44 cells and selected with G418 (Invitrogen, CA). Similarly, human V_H and V_L sequences were grafted onto IgG4 frameworks to make humanized 763-IgG4 recombinant antibodies.

Purification of humanized 763

[00245] Humanized 763 producer lines were cultured in Optichro serum free medium (Invitrogen, CA) and the mature supernatant harvested. Protein A affinity column was preequilibrated with 25mM sodium citrate buffer with 0.15 M NaCl, pH 8.2. Bound humanized 763 was eluted with 0.1 M citric acid/sodium citrate buffer, pH 3.9 and alkalinized (1:10 v/v ratio) in 25mM sodium citrate, pH 8.5. It was passed through a Sartobind-Q membrane and concentrated to 5-10 mg/mL in 25 mM sodium citrate, 0.15 M NaCl, pH 8.2. 2 µg each of the proteins was analyzed by SDS-PAGE under non-reducing or reducing conditions using 4-15% Tris-Glycine Ready Gel System (Bio-Rad, Hercules, CA). Invitrogen SeeBlue Plus2 Pre-Stained Standard was used as the protein molecular weight marker. After electrophoresis, the gel was stained using PIERCE's GelCode Blue Stain Reagent. The gel was scanned using Bio-Rad Fluor-S MultiImager (Bio-Rad), and the band intensity quantified with Quantity One software (Bio-Rad).

Humanized 763 Bispecific Antibody Design, Production, and Purification Analyses

[00246] The humanized 763 bispecific antibody format was designed as a humanized OKT3 scFv fusion to the C-terminus of the heavy chain (hu763-Hc-OKT3) or C-terminus of the light chain (hu763-Lc-OKT3) of a humanized 763-IgG1. For the hu763-Hc-OKT3 format, the V_L was identical to that of humanized 763 IgG1, while the heavy chain is constructed as V_H-Cκ-(G₄S)₃-(huOKT3) scFv including an N297A mutation in a wild-type IgG1 Fc region. For the hu763-Lc-OKT3 format, the V_H was identical to that of humanized 763 IgG1 except an N297A mutation in a wild-type human IgG1 Fc region, while the light chain is constructed as V_L-Cκ-(G₄S)₃-(huOKT3) scFv. Nucleotide sequences encoding V_H and V_L domains from humanized 763, and the humanized OKT3 scFv were synthesized by GenScript with appropriate flanking restriction enzyme sites, and were subcloned into a standard mammalian expression vector. Linearized plasmid DNA was used to transfect CHO-S cells (Invitrogen) for stable production of bispecific antibody. 2x10⁶ cells were transfected with 5 µg of plasmid DNA by Nucleofection (Lonza) and then recovered in CD OptiCHO medium supplemented with 8 mM L-glutamine (Invitrogen) for two days at 37°C in 6-

well culture plates. Stable pools were selected with 500 µg/mL hygromycin for approximately two weeks and single clones were then selected out with limited dilution. Humanized 763 bispecific antibody titer was determined by CSPG4⁺ M14 cell and CD3⁺ Jurkat cell ELISA, respectively, and stable clones with highest expression were selected. The bispecific antibody producer line was cultured in OptiCHO medium and the mature supernatant harvested. A protein A affinity column (GE Healthcare) was pre-equilibrated with 25 mM sodium citrate buffer with 0.15 M NaCl, pH 8.2. Bound bispecific antibody was eluted with 0.1 M citric acid/sodium citrate buffer, pH 3.9 and neutralized with 25 mM sodium citrate, pH 8.5 (1:10 v/v ratio). For storage, bispecific antibody was dialyzed into 25 mM sodium citrate, 0.15 M NaCl, pH 8.2 and frozen in aliquots at -80°C. Two micrograms of the protein was analyzed by SDS-PAGE under reducing conditions using 4-15% Tris-Glycine Ready Gel System (Bio-Rad). The purity of humanized 763 bispecific antibody was also evaluated by size-exclusion high-performance liquid chromatography (SE-HPLC). Approximately 20 µg of protein was injected into a TSK-GEL G3000SWXL 7.8 mm x 30 cm, 5 µm column (TOSOH Bioscience) with 0.4 M NaClO₄, 0.05 M NaH₂PO₄, pH 6.0 buffer at flow rate of 0.5 mL/min, and UV detection at 280 nm. Ten microliters of gel-filtration standard (Bio-Rad) was analyzed in parallel for MW markers.

In Vitro Binding Kinetics by Biacore T-100 Biosensor

[00247] CM5 sensor chip (Research grade) and related reagents were purchased from Biacore USA (Piscataway, NJ). Antigen D2.8.11 or anti-763 idiotype antibody MK2-23 was directly immobilized onto the CM5 sensor chip via hydrophobic interaction. The system first run 20X cycles of buffer only to get stable baseline levels. Purified antibodies (murine 763, humanized 763-IgG1, humanized 763-IgG1n, humanized 763-IgG4) were diluted in HBS-E buffer containing 250 mM NaCl at varying concentrations (41.7 ~ 666.7 nM) prior to analysis. Samples (60 µL) were injected over the sensor surface at a flow rate of 30 µL/min over 2 min. Association time was set for one minute; dissociation time was set for from five minutes to 30 minutes. Following completion of the association phase, dissociation was monitored in HBS-E buffer containing 250 mM NaCl at the same flow rate. At the end of each cycle, the surface was regenerated using 50 µL 20 mM NaOH at a flow rate of 50 µL/min over one minute and 100 µL 4M MgCl₂ at a flow rate of 50 µL/min over two minutes. The data were analyzed by the bivalent analyte model (for antigen D2.8.11 binding) or monovalent analyte model (for anti-763 idiotype antibody MK2-23 binding) and default parameter setting for the rate constants using the Biacore T-100 (Biacore AB of GE

Healthcare, Uppsala, Sweden) evaluation software, and the apparent association on rate constant (k_{on}), dissociation off rate constant (k_{off}) and equilibrium dissociation constant ($K_D = k_{off}/k_{on}$) were calculated.

FACS Analyses

[00248] Cells were incubated with different concentration of primary antibody (humanized 763-IgG1, hu763-Hc-OKT3, hu763-Lc-OKT3 and humanized OKT3) for thirty minutes at 4°C in PBS, and a secondary phycoerythrin-labeled antibody specific for human Fc was used after wash of excess primary antibody. Cells were fixed with 1% paraformaldehyde (PFA) prior to analysis on FACS Calibur cytometer (BD biosciences). Controls were cells with control human IgG1 antibody (non-specific for CSPG4 or T cells), for which the mean fluorescent intensity (MFI) was set to five.

⁵¹Chromium Release Assay

[00249] For Antibody-Dependent cell-mediated cytotoxicity (ADCC), effector cells were NK-92MI cells stably transfected with human CD16 Fc receptor. E:T ratio was 20:1. For T cell cytotoxicity assay, effector T cells cultured *in vitro* in the presence of anti-CD3 and anti-CD28 for about 14 days, and used at E:T ratio of 10:1. All target tumor cells were harvested with 2 mM EDTA in PBS, labeled with ⁵¹Cr (Amersham, Arlington Height, IL) at 100 μ Ci/ 10^6 cells at 37°C for one hour. 5000 target cells/well were mixed with 50,000 effector cells and bispecific antibodies in 96-well polystyrene round-bottom plates (BD Biosciences) to a final volume of 250 μ L/well. The plates were incubated at 37°C for four hours. The released ⁵¹Cr in supernatant was counted in a γ -counter (Packed Instrument, Downers Grove, IL). Percentage of specific release was calculated using the formula: (experimental cpm - background cpm)/(total cpm - background cpm) x 100%, where cpm represented counts per minute of ⁵¹Cr released. Total release was assessed by lysis with 10% SDS (Sigma, St Louis, Mo), and background release was measured in the absence of effector cells. EC50 was calculated using Sigmaplot software.

Immunohistochemistry (IHC)

[00250] Stage 4 melanoma tumors and normal tissues were obtained at Memorial Sloan-Kettering Cancer Center with institutional review board approval. Five- to seven-micrometer sections of snap-frozen tissues were fixed in acetone for 30 min at -20°C. Endogenous biotin-binding activity was blocked by sequential treatment with avidin and biotin (Vector avidin-biotin

blocking kit; Invitrogen) for 20 minutes each followed by blocked with 10% horse serum for one hour at room temperature. Sections were then sequentially reacted with primary antibody, biotinylated horse anti-mouse IgG (H+L) (Vector Laboratories) and Avidin-Biotin Complex (Vectastain ABC kit) for 60 minutes respectively at room temperature, and washed between each reaction. Subsequently, sections were stained with dye (DAB Peroxidase substrate kit) for two minutes, washed, counterstained with Myer's hematoxylin, washed, dehydrated in 95% ethyl alcohol.

Animals and In Vivo Assays

[00251] For *in vivo* studies, BALB-Rag2-KO-IL-2R- γ c-KO (DKO) mice (derived from colony of Dr. Mamoru Ito, CIEA, Kawasaki, Japan; Koo GC, et al. (2009) Expert Rev. Vaccines 8:113-120; Andrade, D. et al. (2011) Arthritis Rheum. 63:2764-2773; Cheng, M. et al. (2014) Int. J. Cancer). M14 cells expressing luciferase were administered to DKO mice intravenously. Four days post administration, mice were treated with 20 μ g of hu763-Hc-OKT3 with intravenous administration ATC for 1×10^6 . Tumor growth was assessed by luciferin bioluminescence once a week. Bioluminescence imaging was conducted using the Xenogen In Vivo Imaging System (IVIS) 200 (Caliper LifeSciences). Briefly, mice were injected intravenously with 0.1 mL solution of D-luciferin (Gold Biotechnology, 30 mg/mL stock in PBS). Images were collected 1-2 min post injection using the following parameters: a 10-60 seconds exposure time, medium binning, and an 8 f/stop. Bioluminescence image analysis was performed using Living Image 2.6 (Caliper LifeSciences).

Antibody Biodistribution in Xenografted Mice

[00252] Female athymic nude mice were purchased from Harlan Sprague Dawley, Inc. All procedures were carried out in accordance with the protocols approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee and institutional guidelines for the proper and humane use of animals in research. SKMEI-28 tumor cells were harvested and resuspended in Matrigel (BD Biosciences). Cells ($2 \sim 10 \times 10^6$) were implanted subcutaneously (sc) to the flank of the mice in 0.1 mL volume using 22-gauge needles. Tumors were allowed to grow to the size of $\sim 200 \text{ mm}^3$ before initiating treatment. Mice with established tumors were randomly separated into treatment groups. 100 μ Ci of radioiodinated antibody per mouse was injected intravenously and animals sacrificed usually at 48 hours, and their organs removed and counted in a

gamma counter (Packard Instruments, Perkin Elmer). These organs included skin, liver, spleen, kidney, adrenal, stomach, small intestine, large intestine, bladder, femur, muscle, tumor, heart, lung, spine, and brain. Based on the μCi accumulated in the organ and the organ weight, % injected dose (ID)/gm of mouse was calculated. Tumor to non-tumor ratios of % ID/gm was also calculated.

[00253] Having thus described several aspects of at least one embodiment of this invention, it is to be appreciated that various alterations, modifications, and improvements will readily be apparent to those skilled in the art. Such alterations, modifications, and improvements are intended to be part of this disclosure, and are intended to be within the spirit and scope of the invention. Accordingly, the foregoing description and drawings are by way of example only and the invention is described in detail by the claims that follow.

EQUIVALENTS

[00254] Use of ordinal terms such as "first," "second," "third," etc., in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed, but are used merely as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term) to distinguish the claim elements.

[00255] The articles "a" and "an" as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to include the plural referents. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or the entire group members are present in, employed in, or otherwise relevant to a given product or process. Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the listed claims is introduced into another claim dependent on the same base claim (or, as relevant, any other claim) unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise. Where elements are presented as lists, (e.g., in Markush group or similar format) it is to be understood that each subgroup of the elements is also disclosed,

and any element(s) can be removed from the group. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, etc. For purposes of simplicity those embodiments have not in every case been specifically set forth in so many words herein. It should also be understood that any embodiment or aspect of the invention can be explicitly excluded from the claims, regardless of whether the specific exclusion is recited in the specification. The publications, websites and other reference materials referenced herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference.

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We claim:

1. A humanized or chimeric antibody or fragment thereof that binds CSPG4, wherein the humanized or chimeric antibody or fragment thereof comprises at least one of the complementarity determining regions (CDRs) found in the light chain variable region of murine antibody 763 and/or at least one of the CDRs found in the heavy chain variable region of murine antibody 763.
2. The antibody of claim 1, wherein the antibody is humanized.
3. The antibody of claim 2, wherein the humanized antibody comprises a light chain variable region sequence of SEQ ID NO: 12.
4. The antibody of claim 2, wherein the humanized antibody comprises a light chain variable region sequence of SEQ ID NO: 14.
5. The antibody of claim 2, wherein the humanized antibody comprises a heavy chain variable region sequence of SEQ ID NO: 8.
6. The antibody of claim 2, wherein the humanized antibody comprises a heavy chain variable region sequence of SEQ ID NO: 10.
7. The antibody of claim 1, wherein the antibody is chimeric.
8. The antibody of claim 7, wherein the chimeric antibody comprises a light chain variable region sequence of SEQ ID NO: 6.
9. The antibody of claim 7, wherein the chimeric antibody comprises a heavy chain variable region sequence of SEQ ID NO: 4.
10. The antibody of any one of claims 1-9, wherein the antibody is characterized in that it inhibits tumor uptake of SKMEI-28 xenographs by about 50% as compared to a reference antibody.

11. The antibody of any one of claims 1-9, wherein the antibody is glycosylated with terminal mannose, N-acetylglucose or glucose, but no fucose.
12. The antibody of any one of claims 1-9, wherein the antibody is a human IgG1 or a human IgG4.
13. The antibody of claim 12, wherein the antibody is a human IgG1 that has a variant glycosylation.
14. The antibody of claim 13, wherein the variant glycosylation results from an amino acid substitution at residue 297 of the human IgG1 Fc.
15. An isolated nucleic acid molecule that encodes any one of SEQ ID NOs: 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22.
16. The isolated nucleic acid molecule of claim 15, wherein the coding sequence is codon-optimized.
17. A recombinant vector comprising the nucleic acid molecule of claim 15 or 16.
18. A host cell comprising the recombinant vector of claim 17.
19. A method for producing a humanized or chimeric antibody or fragment thereof according to any one of claims 1-14 comprising a step of
 - culturing the host cell according to claim 18 in a culture medium under conditions allowing the expression of the humanized or chimeric antibody or fragment thereof and
 - separating the humanized or chimeric antibody or fragment thereof from the culture medium.
20. A composition comprising the humanized or chimeric antibody or fragment thereof of any of claims 1-14.

21. The composition of claim 20, wherein the antibody is conjugated to a cytotoxic agent.
22. A pharmaceutical composition comprising the antibody or fragment the composition of claims 20 or 21, and further comprising a pharmaceutically acceptable carrier or diluent.
23. A method of treating a medical condition in a subject, wherein the medical condition characterized by CSPG4 expression, comprising
administering a therapeutically effective amount of an antibody or fragment thereof according to any one of claims 1-14 to said subject.
24. The method of claim 23, wherein the medical condition includes a CSPG4-positive tumor.
25. A method of treating medical condition in a subject according to claim 23, wherein said medical condition is melanoma, breast cancer, osteosarcoma, head and neck cancers, glioblastoma multiforme, sarcoma, and mesothelioma.
26. A bispecific antibody comprising a first antigen-binding site derived from a humanized 763 antibody and a second antigen-binding site.
27. The bispecific antibody of claim 26, wherein the first and second antigen-binding sites are single chain variable fragments (scFvs).
28. The bispecific antibody of claim 26, wherein the first antigen-binding site is composed of an immunoglobulin molecule and the second antigen-binding site is composed of an scFv, scFab, Fab or Fv.
29. The bispecific antibody of claim 26, wherein the second antigen-binding site binds an immune cell selected from the group consisting of a T cell, NK cell, B cell, dendritic cell, monocyte, macrophage, neutrophil, mesenchymal stem cell and neural stem cell.
30. The bispecific antibody of claim 26, wherein the second antigen-binding site binds CD3.

31. The bispecific antibody of claim 28, wherein the second antigen-binding site is an scFv.
32. The bispecific antibody of claim 31, wherein the scFv is linked to the C-terminal end of the heavy chain of the immunoglobulin.
33. The bispecific antibody of claim 32, wherein the bispecific antibody comprises the sequence of SEQ ID NO: 20.
34. The bispecific antibody of claim 31, wherein the scFv is linked to the C-terminal end of the light chain of the immunoglobulin.
35. The bispecific antibody of claim 34, wherein the bispecific antibody comprises the sequence of SEQ ID NO: 22.
36. An isolated nucleic acid molecule comprising a coding sequence for part or all of a polypeptide chain of the bispecific antibody of any one of claims 26-35.
37. The isolated nucleic acid molecule of claim 36, wherein the coding sequence is codon optimized.
38. An expression vector comprising the nucleic acid sequence of claim 36 or 37.
39. A host cell comprising the expression vector of claim 38.
40. A composition comprising the bispecific antibody of any one of claims 26-35.
41. A pharmaceutical composition comprising the composition of claim 40 or the bispecific antibody of any one of claims 26-35.
42. A chimeric antigen receptor comprising an antigen-binding domain of a humanized 763 antibody.

43. The chimeric antigen receptor of claim 42, wherein the antigen-binding domain is an scFv.
44. An immune effector cell that expresses the chimeric antigen receptor of claim 42 or 43.
45. Use of the chimeric antigen receptor of claim 42 or 43 for the treatment or detection of a condition related to CSPG4 expression.
46. A bispecific T-cell engaging monoclonal antibody comprising an antigen-binding site based on a humanized 763 antibody.
47. A method of killing tumor cells, the method comprising steps of
contacting the tumor cells with a bispecific antibody, which bispecific antibody is composed of a first antigen-binding site based on a humanized 763 antibody and a second antigen-binding site that binds CD3, the contacting being performed under conditions and for a time sufficient that T cells to which the bispecific antibody has bound mediate killing of the tumor cells.
48. The method of claim 47, wherein the first and second antigen-binding sites are scFvs.
49. The method of claim 47, wherein the first antigen-binding site is composed of an immunoglobulin molecule and the second antigen-binding site is composed of an scFv.
50. The method of claim 49, wherein the scFv is linked to the immunoglobulin molecule at the C-terminal end of the heavy chain.
51. The method of claim 49, wherein the scFv is linked to the immunoglobulin molecule at the C-terminal end of the light chain.
52. A method of inhibiting tumor growth, the method comprising the steps of
contacting a tumor with a bispecific antibody, which bispecific antibody is composed of a first antigen-binding site based on a humanized 763 antibody and a second antigen-binding site that binds CD3 on T cells, the contacting being performed under conditions and for a time sufficient that T cells to which the bispecific antibody has bound inhibit growth of a tumor.

53. The method of claim 52, wherein the first and second antigen-binding sites are scFvs.
54. The method of claim 52, wherein the first antigen-binding site is composed of an immunoglobulin molecule and the second antigen-binding site is composed of an scFv.
55. The method of claim 54, wherein the scFv is linked to the immunoglobulin molecule at the C-terminal end of the heavy chain.
56. The method of claim 54, wherein the scFv is linked to the immunoglobulin molecule at the C-terminal end of the light chain.
57. A bispecific antibody comprised of
an immunoglobulin molecule that binds CSPG4,
an scFv that binds to CD3 on T cells,
wherein the bispecific antibody is characterized by an increased efficiency to mediate T cell killing of tumor cells as compared to a reference bispecific antibody.
58. The bispecific antibody of claim 57, wherein immunoglobulin molecule is a humanized antibody based on murine 763 antibody.
59. The bispecific antibody of claim 57 or 58, wherein the scFv is based on a humanized OKT3 antibody.
60. The bispecific antibody of any one of claims 57-59, wherein the scFv is linked to the immunoglobulin molecule at the C-terminal end of the heavy chain.
61. The bispecific antibody of any one of claims 57-59, wherein the scFv is linked to the immunoglobulin molecule at the C-terminal end of the light chain.
62. The bispecific antibody of any one of claims 57-59, wherein the bispecific antibody comprises SEQ ID NO: 20.

63. The bispecific antibody of any one of claims 57-59, wherein the bispecific antibody comprises SEQ ID NO: 22.

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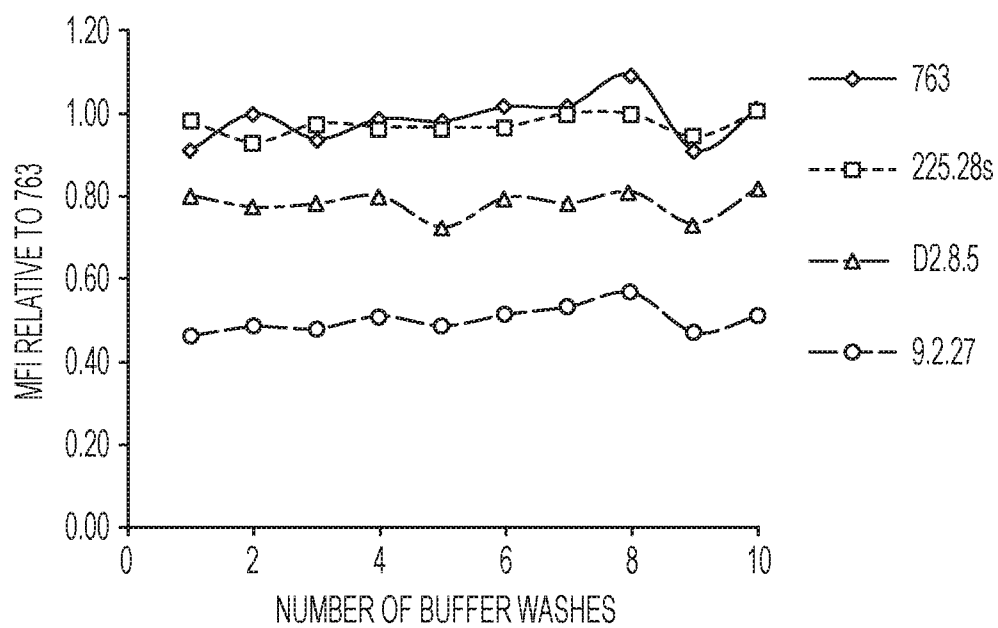


FIG. 1

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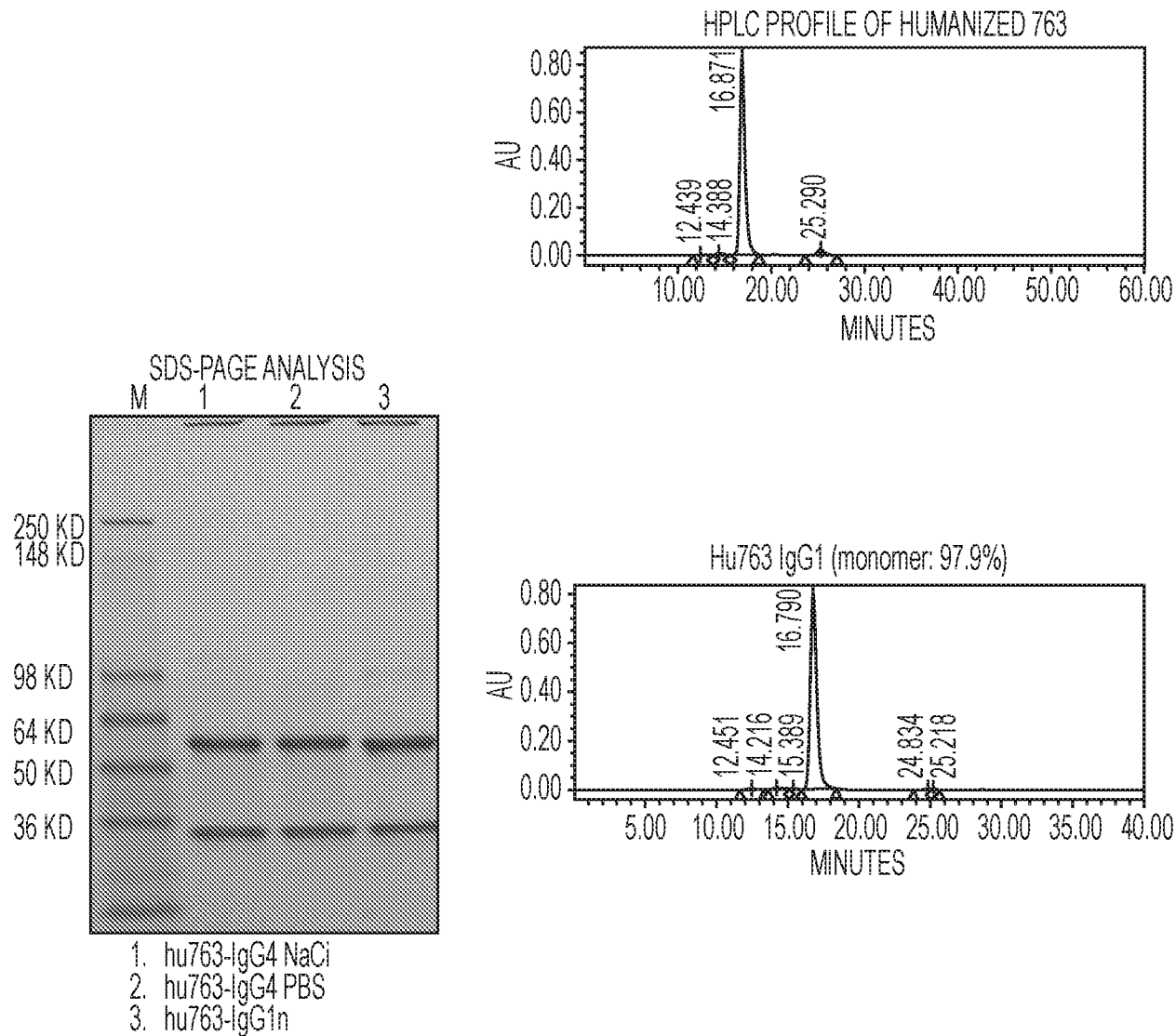


FIG. 2A

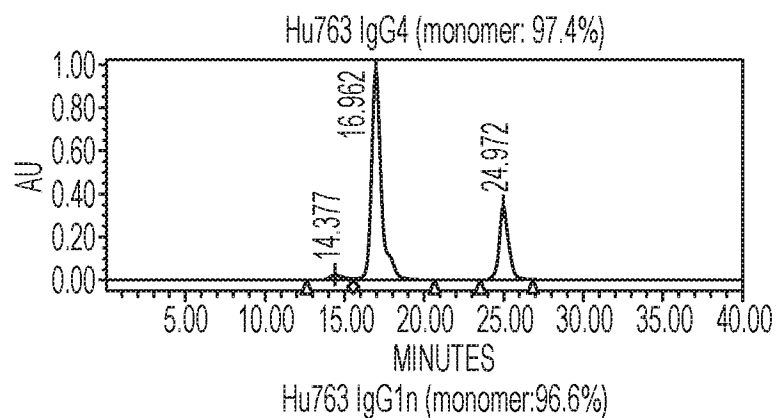


FIG. 2B

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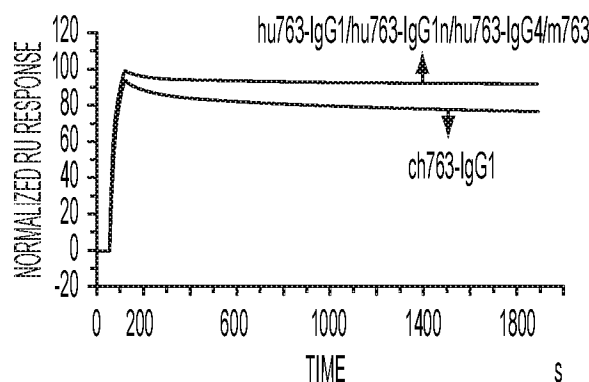


FIG. 3A

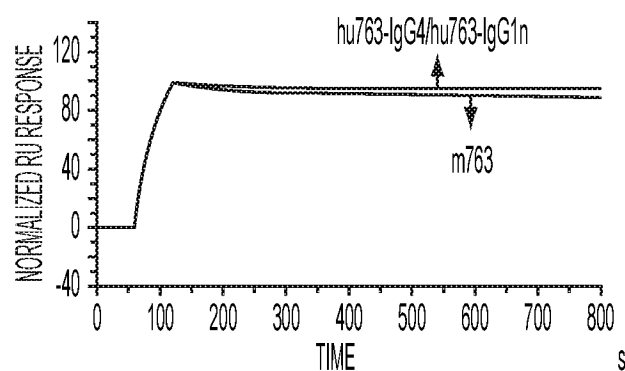


FIG. 3B

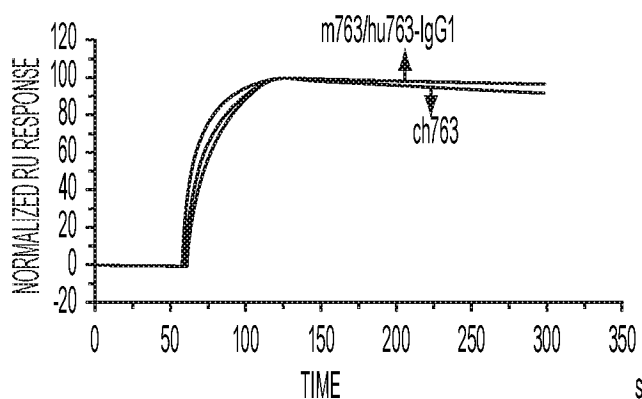


FIG. 3C

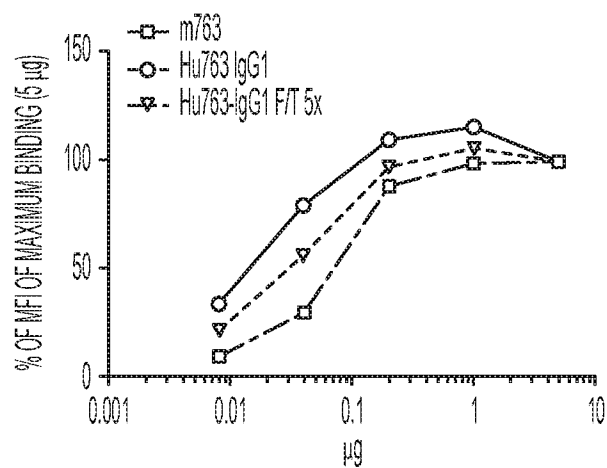


FIG. 3D

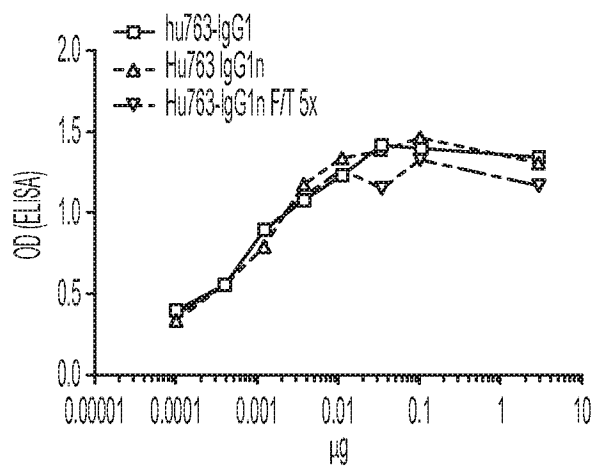


FIG. 3E

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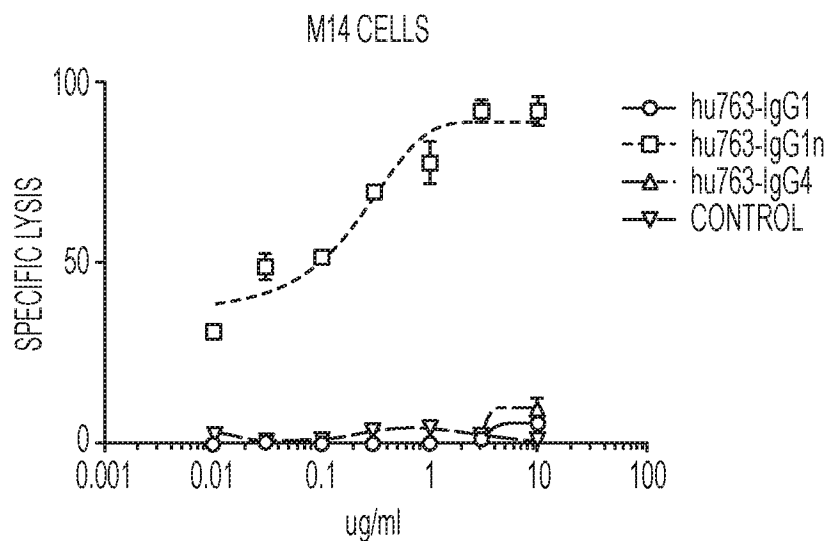


FIG. 4A

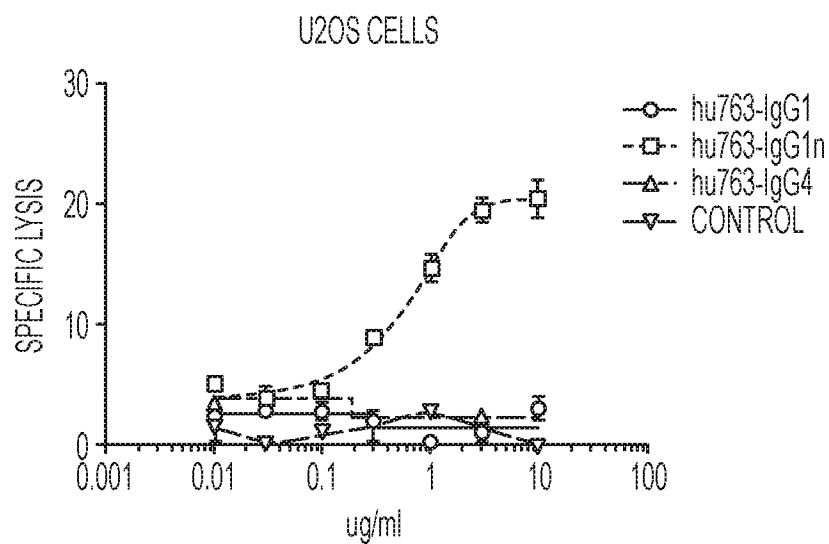


FIG. 4B

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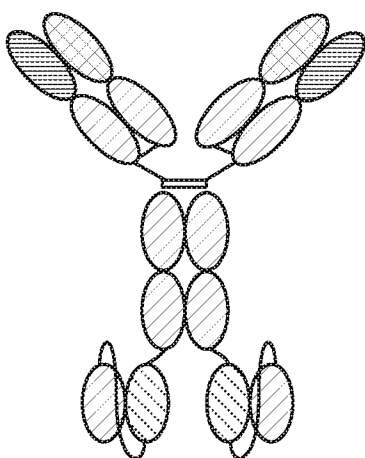
TARGETING OF ^{124}I -LABELLED HUMANIZED 763 ANTIBODIES TO SKMEL-28 XENOGRAFTS

N	% ID/gm								TUMOR TO NON-TUMOR RATIO							
	MOUSE h763		hu763-IgG1		hu763-IgG4		hu763-IgG1n		MOUSE h763		hu763-IgG1		hu763-IgG4		hu763-IgG1n	
	4	6	6	6	8	6	6	6	4	6	8	6	6	6	6	6
ORGAN	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM
SKIN	3.96	0.87	2.91	0.94	1.85	0.45	1.51	0.33	7.15	0.70	3.67	0.89	5.44	0.42	7.78	1.48
LIVER	5.33	1.10	3.12	1.07	1.77	0.41	1.52	0.31	5.26	0.58	3.49	0.66	5.84	0.94	7.44	1.10
SPLEEN	3.30	0.78	1.62	0.41	0.99	0.20	1.22	0.27	9.16	2.24	6.82	2.03	9.34	1.53	9.97	2.29
KIDNEY	3.85	0.90	2.69	0.97	1.75	0.41	1.19	0.24	7.30	0.54	4.32	0.53	5.86	0.95	9.36	1.29
ADRENAL	5.37	1.11	3.27	1.37	2.23	0.59	1.74	0.59	5.55	1.43	3.09	1.52	1.56	3.20	8.46	1.93
STOMACH	1.85	0.27	1.51	0.49	1.28	0.28	0.76	0.10	14.84	1.52	7.34	2.09	7.44	1.32	14.47	2.54
ST CONT	0.89	0.35	0.56	0.08	1.17	0.26	0.64	0.09	40.38	12.13	26.51	10.51	8.30	1.66	19.09	5.53
SM INT	1.34	0.26	0.97	0.33	0.64	0.14	0.42	0.07	20.59	1.03	11.49	3.24	14.74	2.02	26.52	3.81
LG INT	1.31	0.11	1.26	0.36	0.91	0.22	0.52	0.11	20.52	2.02	8.51	2.47	11.70	1.95	22.19	3.26
BLADDER	3.92	0.62	2.20	0.83	1.58	0.42	1.31	0.28	7.03	0.64	2.02	3.60	7.13	1.65	8.70	1.23
FEMUR	1.15	0.28	0.99	0.36	0.67	0.17	0.45	0.11	25.15	3.80	11.48	3.21	15.93	2.70	25.61	3.61
MUSCLE	1.01	0.17	0.81	0.27	0.51	0.14	0.38	0.07	27.22	1.99	13.56	2.66	22.86	5.10	29.25	3.05
TUMOR	27.39	5.12	13.55	5.76	10.24	2.67	10.38	1.38	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
HEART	4.66	1.30	2.71	0.94	1.58	0.41	1.32	0.29	6.20	0.72	4.37	0.50	6.97	0.69	8.83	1.68
LUNG	6.27	1.35	4.63	1.56	2.46	0.66	1.96	0.47	4.49	0.43	2.53	0.42	4.72	0.76	6.14	1.14
SPINE	2.23	0.31	1.61	0.51	1.05	0.29	0.64	0.13	12.45	1.85	6.98	1.52	11.36	2.23	17.99	2.92
BRAIN	0.36	0.08	0.26	0.09	0.14	0.04	0.12	0.03	77.43	3.10	45.73	5.55	90.00	27.52	94.74	16.54
BLOOD	11.08	3.38	6.69	2.56	3.52	0.92	3.07	0.61	2.63	0.24	2.20	0.41	3.41	0.56	3.67	0.56

FIG. 5

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HC format (Hu763-HC-hOKT3)



LC format (Hu763-LC-hOKT3)

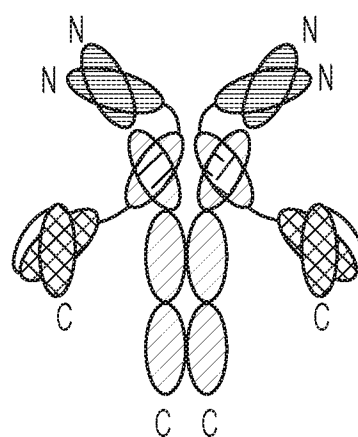


FIG. 6

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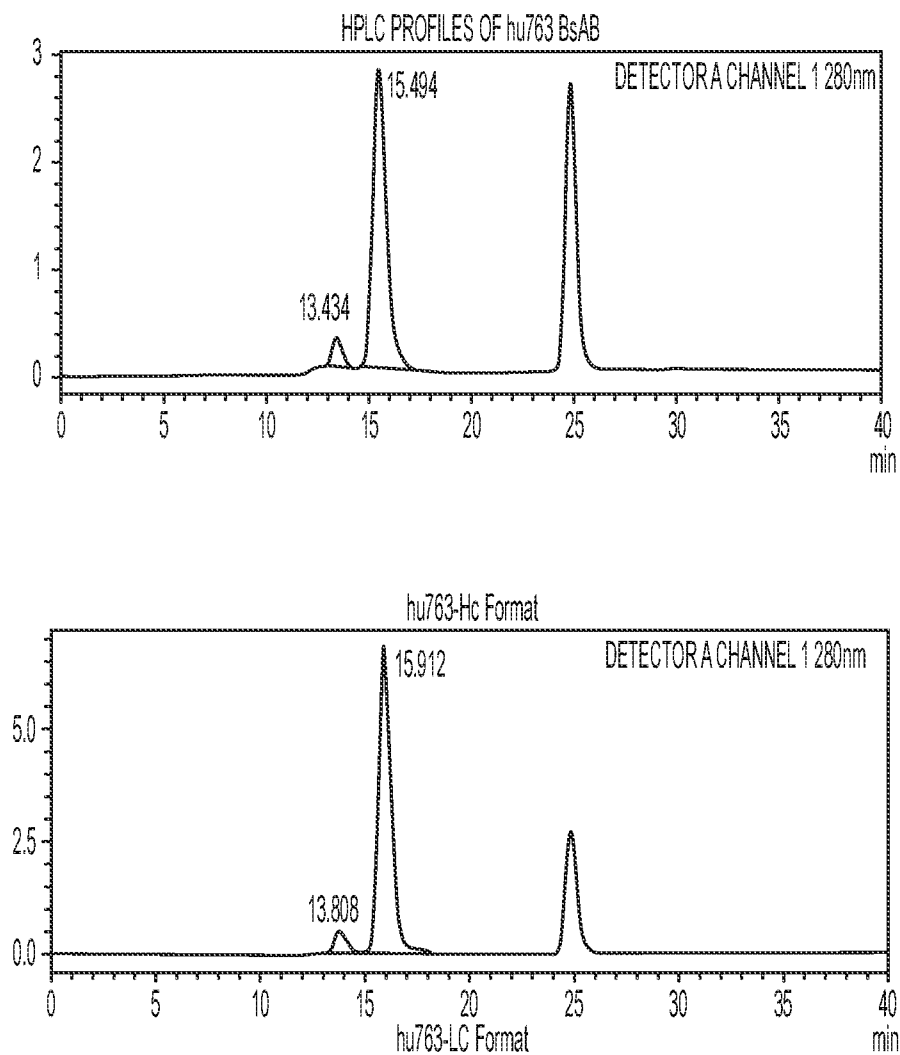


FIG. 7A

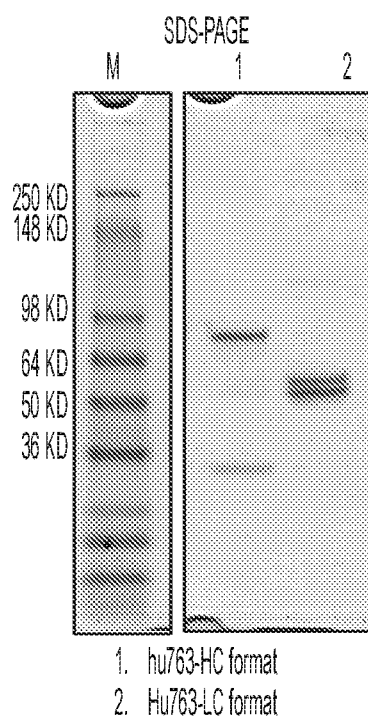


FIG. 7B

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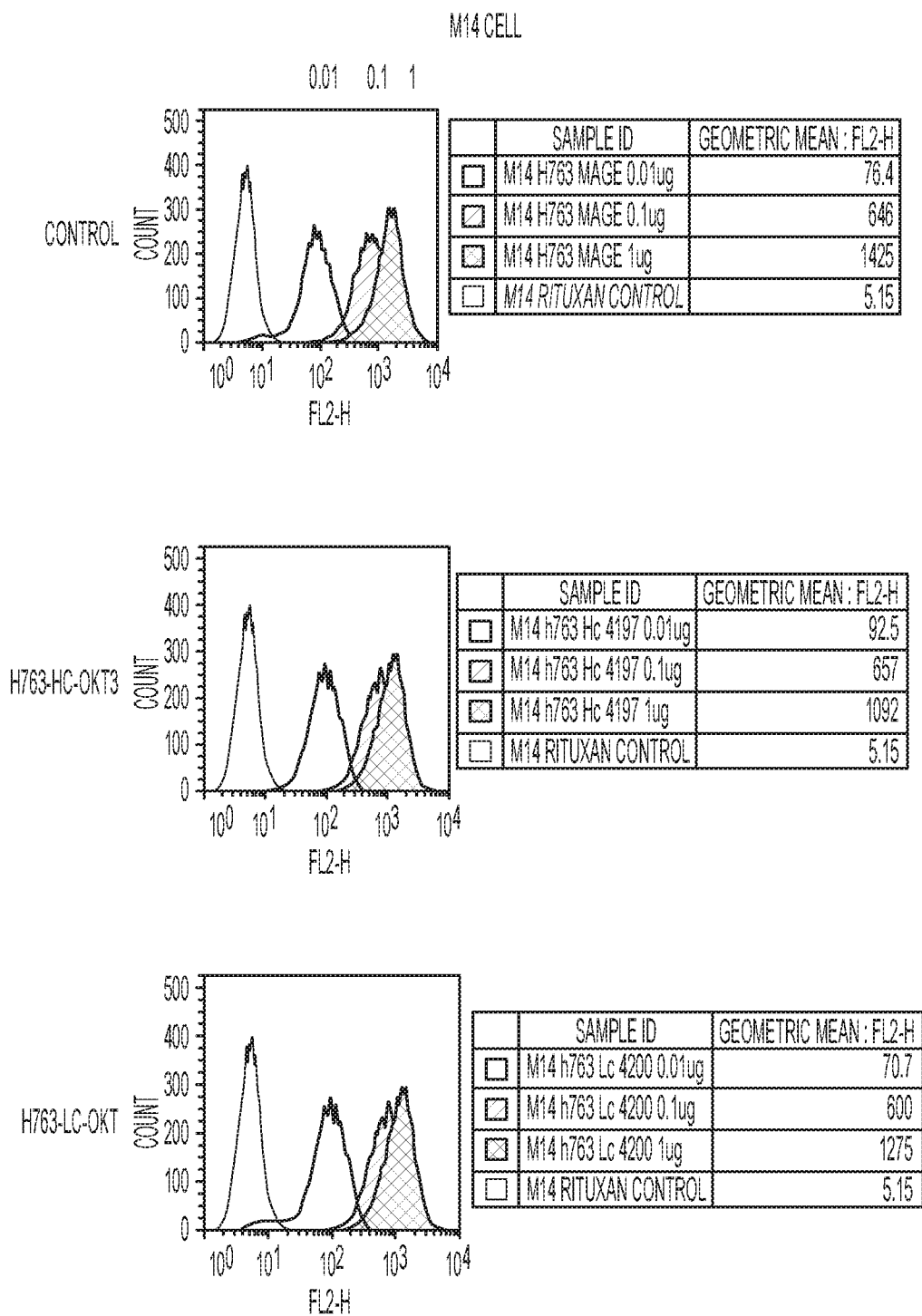


FIG. 8A

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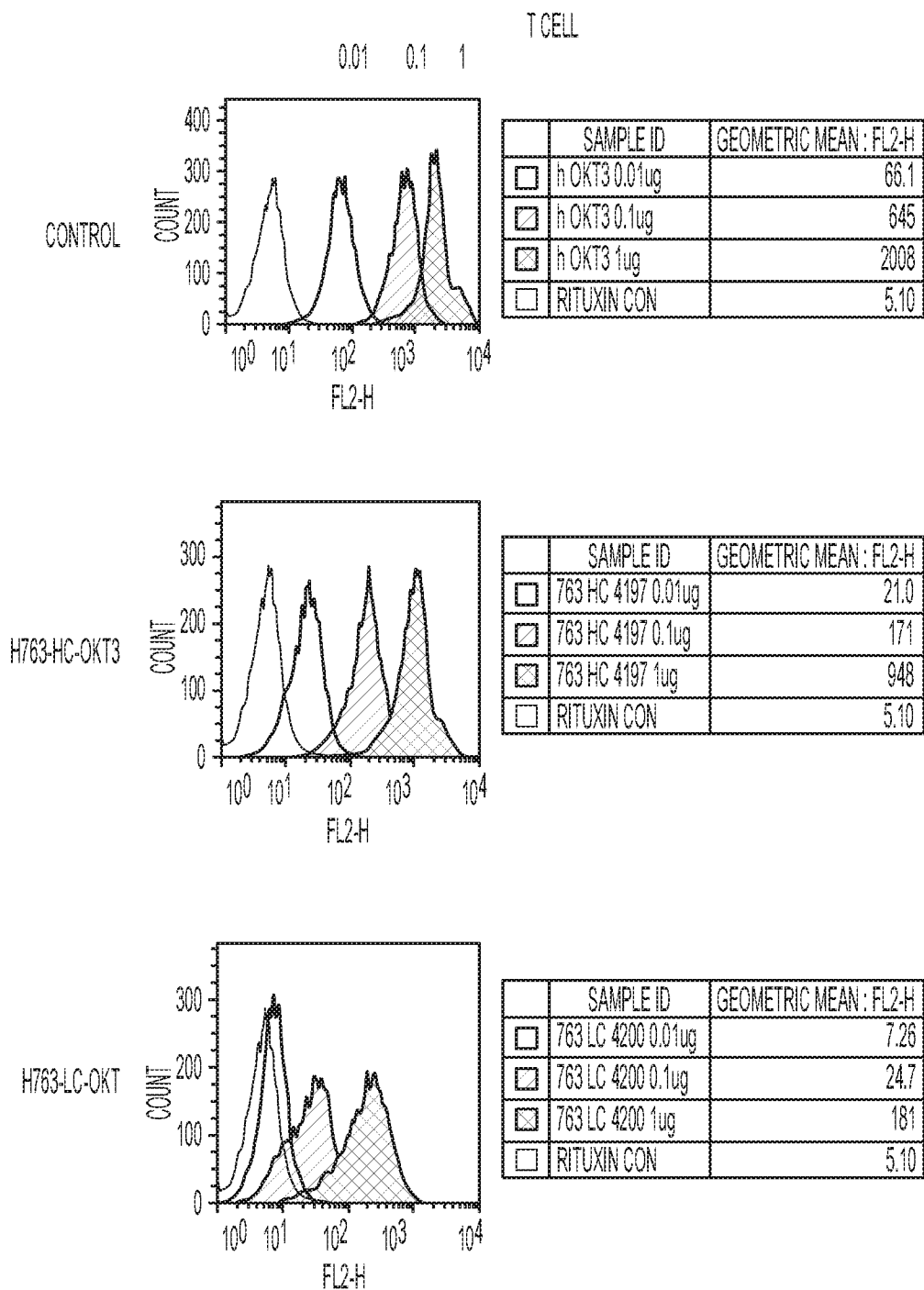


FIG. 8B

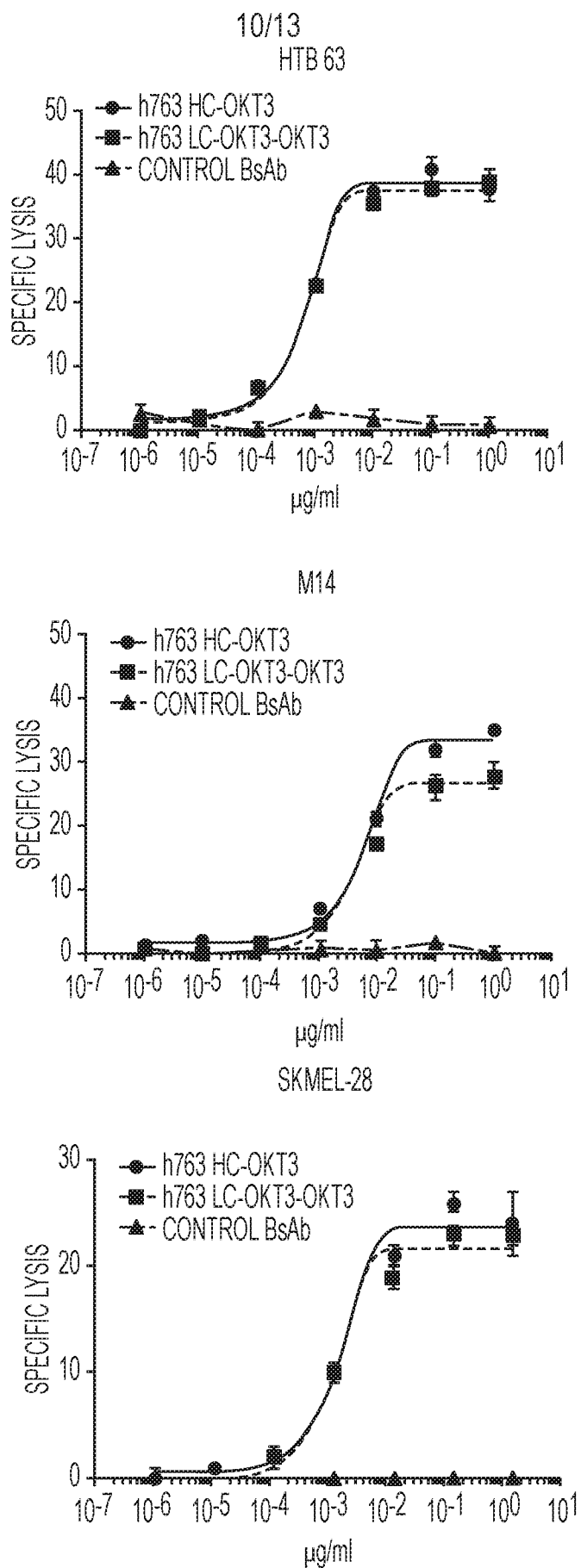


FIG. 9

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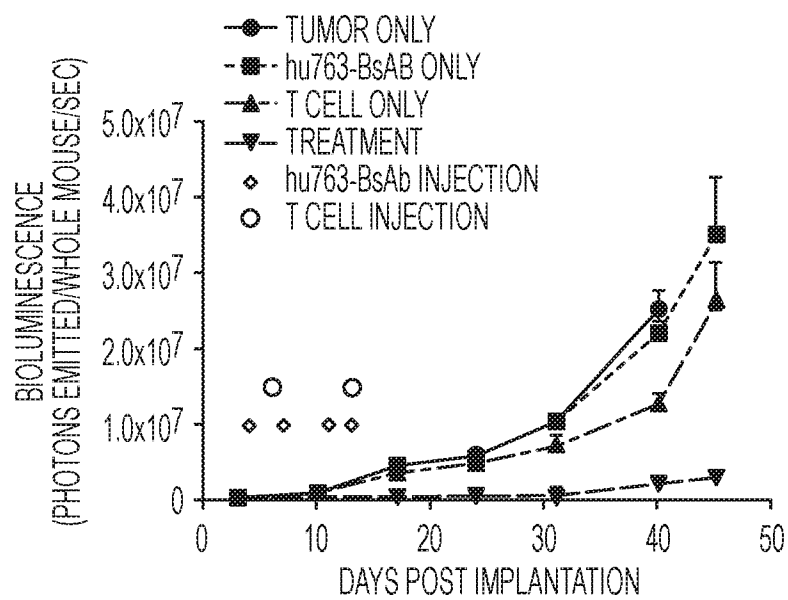


FIG. 10A

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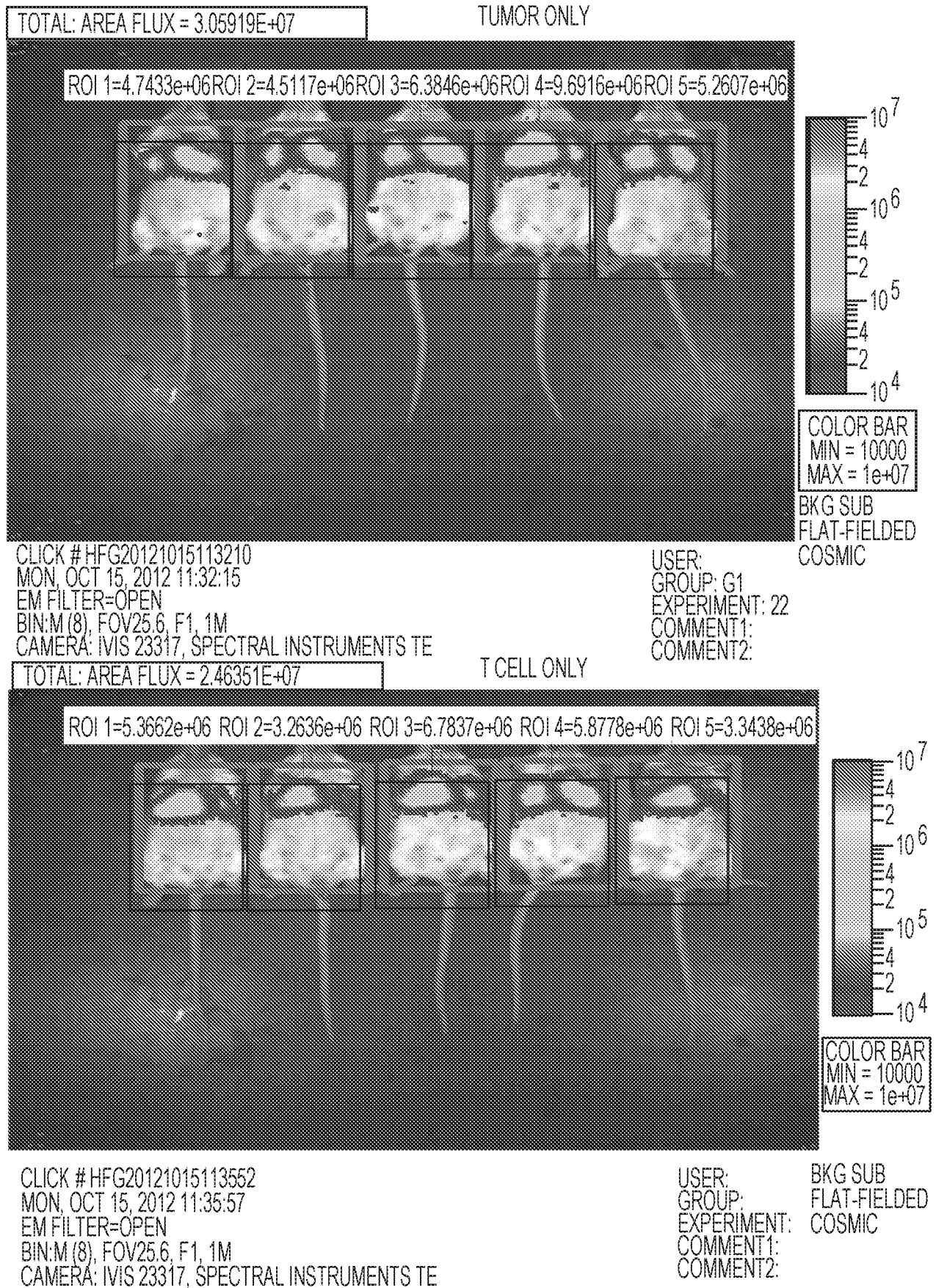


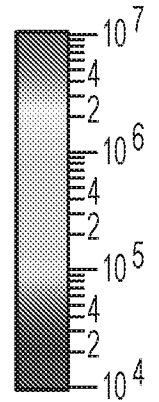
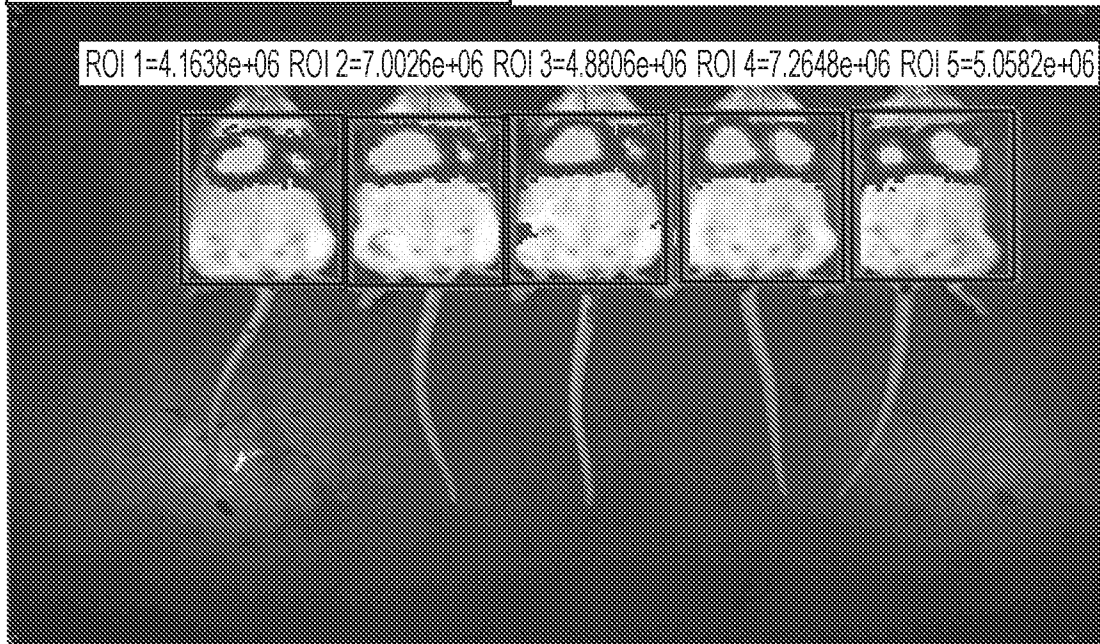
FIG. 10B

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Hu763-BsAB ONLY

TOTAL: AREA FLUX = 2.83701E+07

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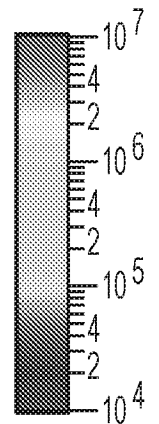
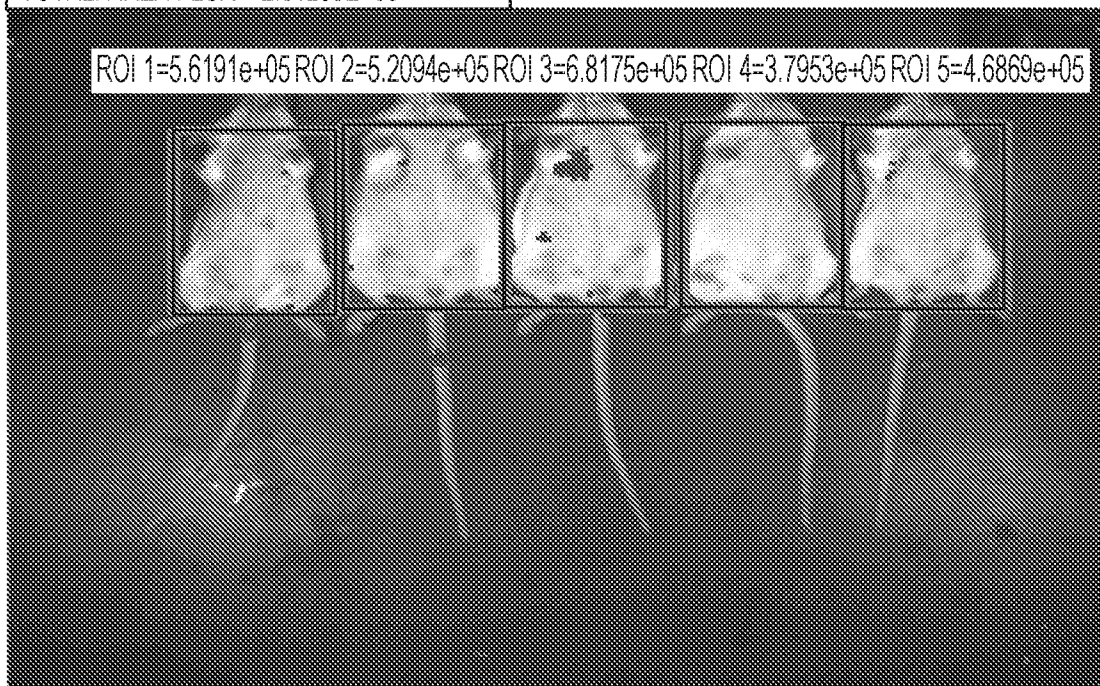
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EXPERIMENT: 22 BKG SUB
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COMMENT2: COSMIC

COLOR BAR
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MAX = 1e+07

TOTAL: AREA FLUX = 2.61283E+06

TREATMENT

ROI 1=5.6191e+05 ROI 2=5.2094e+05 ROI 3=6.8175e+05 ROI 4=3.7953e+05 ROI 5=4.6869e+05



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DAY 24

FIG. 10B continued

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/060465

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 16/30 (2016.01)

CPC - C07K 16/30 (2016.02)

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)

IPC(8) - A61K 39/395; A61P 35/00; C07K 16/30, 16/46; C12N 15/13 (2016.01)

CPC - A61K 39/0011, 39/39558, 47/48569, 2039/505; C07K 16/30, 16/468, 2317/24, 2317/31, 2317/41, 2317/56, 2317/70, 231 (2016.02)

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

USPC - 424/130.1, 133.1, 155.1; 435/375; 530/387.3; 536/23.53 (keyword delimited)

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

PatBase, Google Patents, Google Scholar, PubMed.

Search terms used: bispecific antibody CSPG4 chondroitin sulfate proteoglycan 4 763.74 CD3 OKT3

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2013/0259873 A1 (FERRONE et al) 03 October 2013 (03.10.2013) entire document	1, 2, 7, 10, 12, 42, 43, 45
Y		11, 13, 14, 26-32, 34, 36, 37, 40, 44, 46-56, 58
Y	US 2013/0183288 A1 (REFF et al) 18 July 2013 (18.07.2013) entire document	11
Y	WO 2013/102123 A2 (NOVELMED THERAPEUTICS, INC) 04 July 2013 (04.07.2013) entire document	13, 14
Y	WO 2014/138306 A1 (BAYLOR COLLEGE OF MEDICINE et al) 12 September 2014 (12.09.2014) entire document	26-32, 34, 36, 37, 40, 44, 46-59
Y	ORCUTT et al. "A modular IgG-scFv bispecific antibody topology," Protein Eng Des Sel. 17 December 2009 (17.12.2009), Vol. 23, Pgs. 221-228. entire document	28, 31, 32, 34, 49-51, 54-59
Y	WO 2013/173820 A2 (SCOTT & WHITE HEALTHCARE) 21 November 2013 (21.11.2013) entire document	37
A	WO 2014/165644 A2 (THE GENERAL HOSPITAL CORPORATION) 09 October 2014 (09.10.2014) entire document	1-18, 26-37, 40, 42-59
A	US 2014/0242079 A1 (ROCHE GLYCART AG) 28 August 2014 (28.08.2014) entire document	1-18, 26-37, 40, 42-59
A	US 2014/0004124 A1 (UNIVERSITY OF PITTSBURGH - OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION) 02 January 2014 (02.01.2014) entire document	1-18, 26-37, 40, 42-59



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

15 February 2016

Date of mailing of the international search report

23 FEB 2016

Name and mailing address of the ISA/

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/US2015/060465

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:

SEQ ID NOs: 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 were searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/060465

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.:
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:

3. ☒ Claims Nos.: 19-25, 38, 39, 41, 60-63
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.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/060465

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2013/0259865 A1 (UNIVERSITY OF PITTSBURGH - OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION) 03 October 2013 (03.10.2013) entire document	1-18, 26-37, 40, 42-59
A	COLOMA et al. "Design and production of novel tetravalent bispecific antibodies," Nat Biotechnol. February 1997, Vol. 15, Pgs. 159-63. entire document	1-18, 26-37, 40, 42-59