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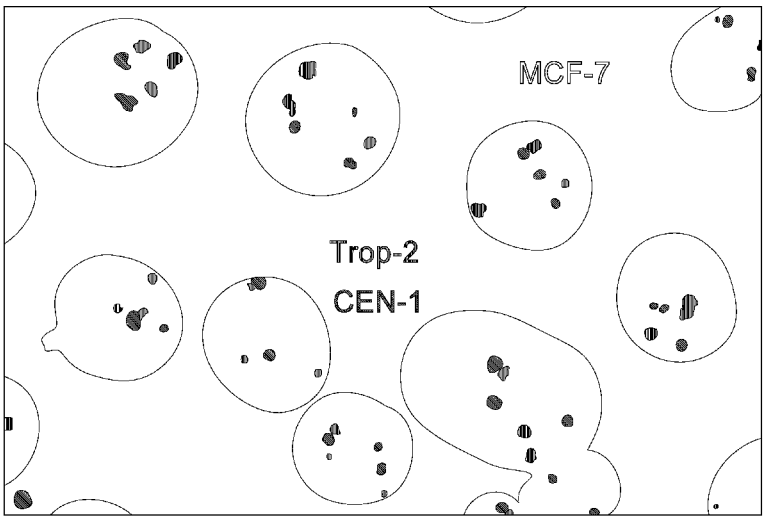
(54) Title: ISOLATION, DETECTION, DIAGNOSIS AND/OR CHARACTERIZATION OF CIRCULATING TROP-2-POSITIVE CANCER CELLS

FIG. 1



(57) Abstract: Described herein are compositions and methods of use of anti-Trop-2 antibodies or antigen-binding fragment thereof to isolate, enrich, detect, diagnose and/or characterize circulating tumor cells (CTCs) from patients with a Trop-2 positive cancer. Preferably, the antibody is an RS7, 162-46.2 or MAB650 antibody. The compositions and methods are of use to detect, diagnose and/or treat metastatic Trop-2$^+$ cancers, such as breast, ovarian, cervical, endometrial, lung, prostate, colon, rectum, stomach, esophageal, bladder, renal, pancreatic, thyroid, epithelial or head-and-neck cancer.

WO 2016/172427 A1

ISOLATION, DETECTION, DIAGNOSIS AND/OR CHARACTERIZATION OF CIRCULATING TROP-2-POSITIVE CANCER CELLS

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ASSIGNEE: IMMUNOMEDICS, INC.

RELATED APPLICATIONS

[001] This application claims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Patent Application 62/151,169, filed 4/22/15, the text of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[002] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on April 21, 2016, is named IMM359WO1_SL.txt and is 44,906 bytes in size.

BACKGROUND OF THE INVENTION

Field of the Invention

[003] This invention relates to methods and compositions for isolating, detecting, diagnosing and/or characterizing Trop-2+ cancer cells, preferably from the circulation. The methods and compositions utilize anti-Trop-2 antibodies, which may be monovalent, bivalent or multivalent. In a preferred embodiment, anti-Trop-2 antibodies are the sole anti-TAA (tumor-associated antigen) capture antibodies utilized in the assay, which does not include use of mixtures of antibodies against TAAs other than Trop-2. In alternative embodiments, the capture antibody may be a bispecific antibody comprising an anti-Trop-2 antibody or fragment and a second antibody or fragment against a different TAA. More preferably, the antibodies are rodent, chimeric, humanized or human antibodies or antigen-binding fragments thereof. Expression of Trop-2 in cancer cells may be assessed using known techniques, including but not limited to binding of anti-Trop-2 antibodies as detected by flow cytometry or immunohistochemistry, and quantitative RT-PCR. Automated systems and devices that have been developed to isolate and/or detect circulating tumor cells (CTCs), including but not limited to the MagSweeper device (Illumina, Inc., San Diego, CA), LIQUIDBIOPSY® system (Cynvenio Biosystems, Inc., Westlake Village, CA), CELLSEARCH® system (Veridex LLC, Raritan, NJ), GILUPI CELLCOLLECTOR™ (GILUPI GmbH, Potsdam, Germany), APOSTREAM® system (Apocell, Houston, TX), ONCOCEE™ microfluidic

platform (BioCept Laboratories, San Diego, CA), VerIFAST System (Casavant et al., 2013, Lab Chip 13:391-6; 2014, Lab Chip 14:99-105) or ISOFLUX™ system (Fluxion, South San Francisco, CA) may be utilized in the practice of the claimed methods. Most preferably, the anti-Trop-2 antibody is a murine, chimeric or humanized RS7 (hRS7) antibody, comprising the light chain CDR sequences CDR1 (KASQDVSIAVA, SEQ ID NO:1); CDR2 (SASYRYT, SEQ ID NO:2); and CDR3 (QQHYITPLT, SEQ ID NO:3) and the heavy chain CDR sequences CDR1 (NYGMN, SEQ ID NO:4); CDR2 (WINTYTGEPTYTDDFKG, SEQ ID NO:5) and CDR3 (GGFGSSYWYFDV, SEQ ID NO:6). However, in alternative embodiments other known anti-Trop-2 antibodies may be utilized, as discussed below. The methods and compositions are applicable for the enrichment, isolation, detection, diagnosis and/or characterization of various metastatic Trop-2-expressing cancers, such as breast (e.g., triple-negative breast cancer), ovarian, cervical, endometrial, lung, prostate, colon, rectum, stomach, esophageal, bladder, renal, pancreatic, thyroid, epithelial, and head-and-neck cancers. Anti-Trop-2 antibodies may be utilized in combination with one or more labeled detection antibodies, or may be directly labeled by conjugation with at least one diagnostic agent. Alternatively, a bispecific antibody may comprise one binding site for Trop-2 and another binding site for a hapten on a targetable construct, typically a small peptide labeled with at least one diagnostic agent. In certain alternative embodiments, detection of Trop-2^+ CTCs may be followed by therapeutic treatment of the Trop-2^+ cancer, using anti-Trop-2 antibodies or fragments thereof. Preferably the antibody or fragment is conjugated to at least one therapeutic agent, such as antibodies, antibody fragments, drugs, toxins, nucleases, hormones, immunomodulators, pro-apoptotic agents, anti-angiogenic agents, boron compounds, photoactive agents or dyes or radioisotopes. More preferably, the therapeutic agent is SN-38 or P2PDOX.

Related Art

[004] Trop-2 (human trophoblast-cell-surface marker) is a cell surface glycoprotein that was originally identified in normal and malignant trophoblast cells (Lipinski et al., 1981, Proc Natl. Acad Sci USA 78:5147-50). Trop-2 is highly expressed in most human carcinomas, particularly in epithelial carcinomas and adenocarcinomas, with reported low to restricted expression in normal tissues (see, e.g., Cubas et al., 2010, Molec Cancer 9:253; Stepan et al., 2011, J Histochem Cytochem 59:701-10; Varughese et al., 2011, Am J Obst Gyn 205:567e-e7). Expression of Trop-2 is associated with metastasis, increased tumor aggressiveness and decreased patient survival (Cubas et al., 2010; Varughese et al., 2011). Pathogenic effects of

Trop-2 have been reported to be mediated, at least in part, by the ERK 1/2 MAPK pathway (Cubas et al., 2010).

[005] It has been proposed that early in tumor progression, cancer cells may be found in low concentration in the circulation (see, e.g., Krishnamurthy et al., 2013, Cancer Medicine 2:226-33; Alix-Panabieres & Pantel, 2013, Clin Chem 50:110-18; Wang et al., Feb. 24, 2015, Int J Clin Oncol, Epub ahead of print). Due to the relatively non-invasive nature of blood sample collection, there has been great interest in the isolation and detection of CTCs, to promote cancer diagnosis at an earlier stage of the disease and as a predictor for tumor progression, disease prognosis and/or responsiveness to drug therapy (see, e.g., Alix-Panabieres & Pantel, 2013, Clin Chem 50:110-18; Winer-Jones et al., 2014, PLoS One 9:e86717; U.S. Patent Appl. Publ. No. 2014/0357659).

[006] Various techniques and apparatus have been developed to isolate and/or detect circulating tumor cells. Several reviews of the field have recently been published (see, e.g., Alix-Panabieres & Pantel, 2013, Clin Chem 50:110-18; Joosse et al., 2014, EMBO Mol Med 7:1-11; Truini et al., 2014, Fron Oncol 4:242). The techniques have involved enrichment and/or isolation of CTCs, generally using capture antibodies against an antigen expressed on tumor cells, and separation with magnetic nanoparticles, microfluidic devices, filtration, magnetic separation, centrifugation, flow cytometry and/or cell sorting devices (e.g., Krishnamurthy et al., 2013, Cancer Medicine 2:226-33; Alix-Panabieres & Pantel, 2013, Clin Chem 50:110-18; Joosse et al., 2014, EMBO Mol Med 7:1-11; Truini et al., 2014, Fron Oncol 4:242; Powell et al., 2012, PLoS ONE 7:e33788; Winer-Jones et al., 2014, PLoS One 9:e86717; Gupta et al., 2012, Biomicrofluidics 6:24133; Saucedo-Zeni et al., 2012, Int J Oncol 41:1241-50; Harb et al., 2013, Transl Oncol 6:528-38). The enriched or isolated CTCs may then be analyzed using a variety of known methods, as discussed further below. Systems or apparatus that have been used for CTC isolation and detection include the CELLSEARCH® system (e.g., Truini et al., 2014, Front Oncol 4:242), MagSweeper device (e.g., Powell et al., 2012, PLoS ONE 7:e33788), LIQUIDBIOPSY® system (Winer-Jones et al., 2014, PLoS One 9:e86717), APOSTREAM® system (e.g., Gupta et al., 2012, Biomicrofluidics 6:24133), GILUPI CELLCOLLECTOR™ (e.g., Saucedo-Zeni et al., 2012, Int J Oncol 41:1241-50), and ISOFLUX™ system (Harb et al., 2013, Transl Oncol 6:528-38).

[007] To date, the only FDA-approved technology for CTC detection involves the CELLSEARCH® platform (Veridex LLC, Raritan, NJ), which utilizes anti-EpCAM antibodies attached to magnetic nanoparticles to capture CTCs. Detection of bound cells

occurs with fluorescent-labeled antibodies against cytokeratin (CK) and CD45. Fluorescently labeled cells bound to magnetic particles are separated out using a strong magnetic field and are counted by digital fluorescence microscopy. The CELLSEARCH® system has received FDA approval for detection of metastatic breast, prostate and colorectal cancers.

[008] Most CTC detection systems have focused on use of anti-EpCAM capture antibodies (see, e.g., Truini et al., 2014, Front Oncol 4:242; Powell et al., 2012, PLoS ONE 7:e33788; Alix-Panabieres & Pantel, 2013, Clin Chem 50:110-18; Lin et al., 2013, Biosens Bioelectron 40:63-67; Wang et al., Feb. 24, 2015, Int J Clin Oncol Epub ahead of print; Magbanua et al., 2015, Clin Cancer Res 21:1098-105; Harb et al., 2013, Transl Oncol 6:528-38). However, not all metastatic tumors express EpCAM (see, e.g., Mikolajcyzyk et al., 2011, J Oncol 2011:252361; Pecot et al., 2011, Cancer Discovery 1:580-86; Gupta et al., 2012, Biomicrofluidics 6:24133). Attempts have been made to utilize alternative schemes for isolating and detecting EpCAM-negative CTCs, such as use of antibody combinations against TAAs. Antibodies against as many as 10 different TAAs have been utilized in an attempt to increase recovery of metastatic circulating tumor cells (e.g., Mikolajcyzyk et al., 2011, J Oncol 2011:252361; Pecot et al., 2011, Cancer Discovery 1:580-86; Krishnamurthy et al., 2013, Cancer Medicine 2:226-33; Winer-Jones et al., 2014, PLoS One 9:e86717).

[009] Drawbacks exist to such approaches, including the complexity of preparing and using large numbers of different antibodies and their attachment to magnetic nanoparticles, microfluidic devices or other separation technologies, as well as potential cross-reactivity against normal cell populations when using a broad spectrum of anti-tumor antibodies. A need exists in the art for improved methods of isolating, detecting, diagnosing and/or characterizing CTCs, using antibodies against a single TAA that is expressed in a broad range of tumors.

SUMMARY

[010] In various embodiments, the present invention concerns enrichment, isolation, detection, diagnosis and/or characterization of Trop-2-positive circulating tumor cells (CTCs) using anti-Trop-2 antibodies and/or antigen-binding fragments thereof. The anti-Trop-2 antibody may be used to enrich and/or isolate tumor cells from the circulation. Bound CTCs may be detected by a variety of known techniques and/or apparatus, as discussed in detail below. Any known method for detecting biomarkers of isolated CTCs may be utilized, such as FISH, FACS, fluorescence microscopy, fluorescent detection, flow cytometry,

immunohistochemistry, microchip-based systems, RT-PCR, ELISA, or any other technique known in the art for detecting the presence of cancer cells.

[011] In a specific embodiment, the anti-Trop-2 antibody may be a murine, chimeric or humanized RS7 antibody (see, e.g., U.S. Patent No. 7,238,785, the Figures and Examples section of which are incorporated herein by reference), comprising the light chain CDR sequences CDR1 (KASQDVSIAVA, SEQ ID NO:1); CDR2 (SASYRYT, SEQ ID NO:2); and CDR3 (QQHYITPLT, SEQ ID NO:3) and the heavy chain CDR sequences CDR1 (NYGMN, SEQ ID NO:4); CDR2 (WINTYTGEPTYTDDFKG, SEQ ID NO:5) and CDR3 (GGFGSSYWYFDV, SEQ ID NO:6). However, as discussed below other anti-Trop-2 antibodies are known and may be used.

[012] The anti-Trop-2 antibody moiety may be a monoclonal antibody, an antigen-binding antibody fragment, a bispecific or multivalent antibody, or other antibody-based molecule. The antibody can be of various isotypes, preferably human IgG1, IgG2, IgG3 or IgG4, more preferably comprising human IgG1 hinge and constant region sequences. The antibody or fragment thereof can be a rodent, chimeric, a humanized, or a human antibody, as well as variations thereof, such as half-IgG4 antibodies (referred to as "unibodies"), as described by van der Neut Kolfschoten et al. (*Science* 2007; 317:1554-1557). More preferably, the antibody or fragment thereof may be designed or selected to comprise human constant region sequences that belong to specific allotypes, such as G1m3, G1m3,1, G1m3,2 or G1m3,1,2. More preferably, the allotype is selected from the group consisting of the nG1m1, G1m3, nG1m1,2 and Km3 allotypes.

[013] Where bispecific antibodies are used to capture CTCs, the antibody may comprises at least one anti-Trop-2 antibody or fragment thereof, and at least one antibody or fragment thereof against a different TAA. Exemplary TAAs may include carbonic anhydrase IX, CCL19, CCL21, CSAp, CD1, CD1a, CD2, CD3, CD4, CD5, CD8, CD11A, CD14, CD15, CD16, CD18, CD19, IGF-1R, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD55, CD59, CD64, CD66a-e, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, CXCR4, CXCR7, CXCL12, HIF-1-α, AFP, PSMA, CEACAM5, CEACAM-6, c-met, B7, ED-B of fibronectin, Factor H, FHL-1, Flt-3, folate receptor, GROB, HMGB-1, hypoxia inducible factor (HIF), insulin-like growth factor-1 (ILGF-1), IFN-γ, IFN-α, IFN-β, IL-2, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, IP-10, MAGE, mCRP, MCP-1, MIP-1A, MIP-1B, MIF,

MUC1, MUC2, MUC3, MUC4, MUC5ac, NCA-95, NCA-90, Ia, EGP-1, EGP-2, HLA-DR, tenascin, Le(y), RANTES, T101, TAC, Tn antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, TNF-α, TRAIL receptor (R1 and R2), VEGFR, EGFR, P1GF, complement factors C3, C3a, C3b, C5a, or C5. Preferably, the TAA is selected from the group consisting of CEACAM5, MUC5ac, CD74, HLA-DR, CSAp, AFP (alpha-fetoprotein), HER2, vimentin, EGFR, IGF-1R, PD-L1 and PD-L2.

[014] Because the detected tumors will be Trop-2-positive, they may be treated with anti-Trop-2 antibodies, such as anti-Trop-2 antibody-drug conjugates (ADCs). An anti-Trop-2 antibody may initially be used to detect and/or quantify expression or gene copy number of Trop-2 in the CTC. Such analysis may be used to predict response to therapeutic anti-Trop-2 antibodies, as well as to monitor response of the tumor(s) to treatment. As discussed below, immunoconjugates of anti-Trop-2 antibodies may include any known therapeutic agent, such as a chemotherapeutic agent. A number of cytotoxic drugs of use for cancer treatment are well-known in the art and any such known drug may be conjugated to the antibody of interest. In a preferred embodiment, the drug conjugated to the antibody is a camptothecin or anthracycline, most preferably SN-38 or a pro-drug form of 2-pyrrolinodoxorubicin (2-PDox) (see, e.g., U.S. Patent Nos. 8,877,202 and 8,750,496, the Figures and Examples section of each incorporated herein by reference). The drug to be conjugated to the anti-Trop-2 antibody or antibody fragment may be selected from the group consisting of an anthracycline, a camptothecin, a tubulin inhibitor, a maytansinoid, a calicheamycin, an auristatin, a nitrogen mustard, an ethylenimine derivative, an alkyl sulfonate, a nitrosourea, a triazene, a folic acid analog, a taxane, a COX-2 inhibitor, a pyrimidine analog, a purine analog, an antibiotic, an enzyme inhibitor, an epipodophyllotoxin, a platinum coordination complex, a vinca alkaloid, a substituted urea, a methyl hydrazine derivative, an adrenocortical suppressant, a hormone antagonist, an antimetabolite, an alkylating agent, an antimitotic, an anti-angiogenic agent, a tyrosine kinase inhibitor, an mTOR inhibitor, a heat shock protein (HSP90) inhibitor, a proteosome inhibitor, an HDAC inhibitor, a pro-apoptotic agent, and a combination thereof.

[015] The anti-Trop-2 antibodies are of use for detection, diagnosis, characterization and/or treatment of Trop-2 expressing cancers, such as breast, ovarian, cervical, endometrial, lung, prostate, colon, rectum, stomach, esophageal, bladder, renal, pancreatic, thyroid, epithelial or head-and-neck cancers. The methods and compositions may be of particular use for detection and/or treatment of metastatic colorectal cancer, triple-negative breast cancer, HER+, ER+, progesterone+ breast cancer, metastatic non-small-cell lung cancer (NSCLC), metastatic

small-cell lung cancer (SCLC), metastatic pancreatic cancer, metastatic renal cell carcinoma, metastatic gastric cancer, metastatic esophageal cancer, metastatic urothelial cancer, or metastatic prostate cancer.

DETAILED DESCRIPTION

Definitions

[016] Unless otherwise specified, "a" or "an" means one or more.

[017] As used herein, “about” means plus or minus 10%. For example, “about 100” would include any number between 90 and 110.

[018] An antibody, as described herein, refers to a full-length (i.e., naturally occurring or formed by normal immunoglobulin gene fragment recombinatorial processes) immunoglobulin molecule (e.g., an IgG antibody) or an immunologically active (i.e., specifically binding) portion of an immunoglobulin molecule, like an antibody fragment.

[019] An antibody fragment is a portion of an antibody such as $F(ab')_2$, Fab', Fab, Fv, sFv and the like. Antibody fragments may also include single domain antibodies and IgG4 half-molecules, as discussed below. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the full-length antibody. The term "antibody fragment" also includes isolated fragments consisting of the variable regions of antibodies, such as the "Fv" fragments consisting of the variable regions of the heavy and light chains and recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("scFv proteins").

[020] A chimeric antibody is a recombinant protein that contains the variable domains including the complementarity determining regions (CDRs) of an antibody derived from one species, preferably a rodent antibody, while the constant domains of the antibody molecule are derived from those of a human antibody. For veterinary applications, the constant domains of the chimeric antibody may be derived from that of other species, such as a cat or dog.

[021] A humanized antibody is a recombinant protein in which the CDRs from an antibody from one species; e.g., a rodent antibody, are transferred from the heavy and light variable chains of the rodent antibody into human heavy and light variable domains (e.g., framework region sequences). The constant domains of the antibody molecule are derived from those of a human antibody. In certain embodiments, a limited number of framework region amino acid residues from the parent (rodent) antibody may be substituted into the human antibody framework region sequences.

[022] A human antibody is, e.g., an antibody obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous murine heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for particular antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green et al., Nature Genet. 7:13 (1994), Lonberg et al., Nature 368:856 (1994), and Taylor et al., Int. Immun. 6:579 (1994). A fully human antibody also can be constructed by genetic or chromosomal transfection methods, as well as phage display technology, all of which are known in the art. See for example, McCafferty et al., Nature 348:552-553 (1990) for the production of human antibodies and fragments thereof *in vitro*, from immunoglobulin variable domain gene repertoires from unimmunized donors. In this technique, antibody variable domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. In this way, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats, for review, see e.g. Johnson and Chiswell, Current Opinion in Structural Biology 3:5564-571 (1993). Human antibodies may also be generated by *in vitro* activated B cells. See U.S. Pat. Nos. 5,567,610 and 5,229,275, the Examples section of which are incorporated herein by reference.

[023] A "diagnostic agent" is an atom, molecule, or compound that is useful in diagnosing a disease. Useful diagnostic agents include, but are not limited to, radioisotopes, dyes, contrast agents, luminescent agents, chemiluminescent agents, fluorescent compounds or molecules and enhancing agents (e.g., paramagnetic ions). Preferably, the diagnostic agents are selected from the group consisting of radioisotopes, enhancing agents, and fluorescent compounds.

[024] A therapeutic agent is a compound, molecule or atom which is administered separately, concurrently or sequentially with an antibody moiety or conjugated to an antibody moiety, i.e., antibody or antibody fragment, or a subfragment, and is useful in the treatment of a disease. Examples of therapeutic agents include antibodies, antibody fragments, drugs, toxins, nucleases, hormones, immunomodulators, pro-apoptotic agents, anti-angiogenic

agents, boron compounds, photoactive agents or dyes and radioisotopes. Therapeutic agents of use are described in more detail below.

[025] An immunoconjugate is an antibody, antibody fragment or fusion protein conjugated to at least one therapeutic and/or diagnostic agent.

[026] A multispecific antibody is an antibody that can bind 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. Multispecific, multivalent antibodies are constructs that have more than one binding site, and the binding sites are of different specificity.

[027] A bispecific antibody is an antibody that can bind simultaneously to two different targets. Bispecific antibodies (bsAb) and bispecific antibody fragments (bsFab) may have at least one arm that specifically binds to, for example, a tumor-associated antigen and at least one other arm that specifically binds to a targetable conjugate that bears a therapeutic or diagnostic agent. A variety of bispecific fusion proteins can be produced using molecular engineering.

FIGURE LEGENDS

[028] **FIG. 1. Analysis of Trop-2 copy number by FISH.** MCF-7 (Trop-2 positive) cells were analyzed by FISH. Trop-2 copy number was determined using anti-Trop-2 and anti-chromosome-1 specific probes (Empire Genomics, Buffalo, NY).

[029] **FIG. 2. Analysis of Trop-2 copy number by FISH.** A549 (Trop-2 negative) cells were analyzed by FISH. Trop-2 copy number was determined using anti-Trop-2 and anti-chromosome-1 specific probes (Empire Genomics, Buffalo, NY).

[030] **FIG. 3. Analysis of topoisomerase-I copy number by FISH.** MCF-7 cells were analyzed by FISH. Topoisomerase I (TOP1) copy number was determined using anti- TOP1 and anti-chromosome-20 specific probes (ABNOVA®, Taipei, Taiwan).

[031] **FIG. 4. Analysis of topoisomerase-I copy number by FISH.** A549 cells were analyzed by FISH. Topoisomerase I (TOP1) copy number was determined using anti- TOP1 and anti-chromosome-20 specific probes (ABNOVA®, Taipei, Taiwan).

Anti-Trop-2 Antibodies

[032] The subject methods and compositions for CTC isolation and/or detection utilize at least one antibody or fragment thereof that binds to Trop-2, including rodent, chimeric, human or humanized antibodies. In a specific preferred embodiment, the anti-Trop-2 antibody may be a humanized RS7 antibody (see, e.g., U.S. Patent No. 7,238,785,

incorporated herein by reference in its entirety), comprising the light chain CDR sequences CDR1 (KASQDVSIAVA, SEQ ID NO:1); CDR2 (SASYRYT, SEQ ID NO:2); and CDR3 (QQHYITPLT, SEQ ID NO:3) and the heavy chain CDR sequences CDR1 (NYGMN, SEQ ID NO:4); CDR2 (WINTYTGEPTYTDDFKG, SEQ ID NO:5) and CDR3 (GGFGSSYWYFDV, SEQ ID NO:6).

[033] The RS7 antibody was a murine IgG_1 raised against a crude membrane preparation of a human primary squamous cell lung carcinoma. (Stein et al., Cancer Res. 50: 1330, 1990) The RS7 antibody recognizes a 46-48 kDa glycoprotein, characterized as cluster 13. (Stein et al., Int. J. Cancer Supp. 8:98-102, 1994) The antigen was designated as EGP-1 (epithelial glycoprotein-1), but is also referred to as Trop-2.

[034] Trop-2 is a type-I transmembrane protein and has been cloned from both human (Fornaro et al., Int J Cancer 1995; 62:610-8) and mouse cells (Sewedy et al., Int J Cancer 1998; 75:324-30). In addition to its role as a tumor-associated calcium signal transducer (Ripani et al., Int J Cancer 1998;76:671-6), the expression of human Trop-2 was shown to be necessary for tumorigenesis and invasiveness of colon cancer cells, which could be effectively reduced with a polyclonal antibody against the extracellular domain of Trop-2 (Wang et al., Mol Cancer Ther 2008;7:280-5). Trop-2 is highly expressed in the vast majority of human tumors and animal models of cancer (McDougall et al., 2015, Dev Dyn 244:99-109).

[035] The utility of Trop-2 as a marker for solid cancers (Cubas et al., Biochim Biophys Acta 2009;1796:309-14) is attested by further reports that documented the clinical significance of overexpressed Trop-2 in breast (Huang et al., Clin Cancer Res 2005;11:4357-64), colorectal (Ohmachi et al., Clin Cancer Res 2006;12:3057-63; Fang et al., Int J Colorectal Dis 2009;24:875-84), and oral squamous cell (Fong et al., Modern Pathol 2008;21:186-91) carcinomas. The latest evidence that prostate basal cells expressing high levels of Trop-2 are enriched for *in vitro* and *in vivo* stem-like activity is particularly noteworthy (Goldstein et al., Proc Natl Acad Sci USA 2008;105:20882-7).

[036] Flow cytometry and immunohistochemical staining studies have shown that the RS7 MAb detects antigen on a variety of tumor types, with limited binding to normal human tissue (Stein et al., 1990). Trop-2 is expressed primarily by carcinomas such as carcinomas of the lung, stomach, urinary bladder, breast, ovary, uterus, and prostate. Localization and therapy studies using radiolabeled murine RS7 MAb in animal models have demonstrated tumor targeting and therapeutic efficacy (Stein et al., 1990; Stein et al., 1991). Drug-

conjugated RS7 MAb in animal models also have shown targeting and therapeutic efficacy of human cancer xenografts (Cardillo et al., Clinical Cancer Res., 17:3157-69, 2011).

[037] Strong RS7 staining has been demonstrated in tumors from the lung, breast, bladder, ovary, uterus, stomach, and prostate. (Stein et al., Int. J. Cancer 55:938, 1993) The lung cancer cases comprised both squamous cell carcinomas and adenocarcinomas. (Stein et al., Int. J. Cancer 55:938, 1993) Both cell types stained strongly, indicating that the RS7 antibody does not distinguish between histologic classes of non-small-cell carcinoma of the lung.

[038] While the hRS7 antibody is preferred, other anti-Trop-2 antibodies are known and/or publicly available and in alternative embodiments may be utilized in the subject methods and compositions. While humanized or human antibodies are preferred for reduced immunogenicity, in alternative embodiments a chimeric antibody may be of use, while rodent MAbs can be useful for *in-vitro* and *ex-vivo* studies. As discussed below, methods of antibody humanization are well known in the art and may be utilized to convert an available murine or chimeric antibody into a humanized form.

[039] Anti-Trop-2 antibodies are commercially available from a number of sources and include LS-C126418, LS-C178765, LS-C126416, LS-C126417 (LifeSpan BioSciences, Inc., Seattle, WA); 10428-MM01, 10428-MM02, 10428-R001, 10428-R030 (Sino Biological Inc., Beijing, China); MR54 (eBioscience, San Diego, CA); sc-376181, sc-376746, Santa Cruz Biotechnology (Santa Cruz, CA); MM0588-49D6, (Novus Biologicals, Littleton, CO); ab79976, and ab89928 (ABCAM®, Cambridge, MA).

[040] Other anti-Trop-2 antibodies have been disclosed in the patent literature. For example, U.S. Publ. No. 2013/0089872 discloses anti-Trop-2 antibodies K5-70 (Accession No. FERM BP-11251), K5-107 (Accession No. FERM BP-11252), K5-116-2-1 (Accession No. FERM BP-11253), T6-16 (Accession No. FERM BP-11346), and T5-86 (Accession No. FERM BP-11254), deposited with the International Patent Organism Depositary, Tsukuba, Japan. U.S. Patent No. 5,840,854 disclosed the anti-Trop-2 monoclonal antibody BR110 (ATCC No. HB11698). U.S. Patent No. 7,420,040 disclosed an anti-Trop-2 antibody produced by hybridoma cell line AR47A6.4.2, deposited with the IDAC (International Depository Authority of Canada, Winnipeg, Canada) as accession number 141205-05. U.S. Patent No. 7,420,041 disclosed an anti-Trop-2 antibody produced by hybridoma cell line AR52A301.5, deposited with the IDAC as accession number 141205-03. U.S. Publ. No. 2013/0122020 disclosed anti-Trop-2 antibodies 3E9, 6G11, 7E6, 15E2, 18B1. Hybridomas encoding a

representative antibody were deposited with the American Type Culture Collection (ATCC), Accession Nos. PTA-12871 and PTA-12872. U.S. Patent No. 8,715,662 discloses anti-Trop-2 antibodies produced by hybridomas deposited at the AID-ICLC (Genoa, Italy) with deposit numbers PD 08019, PD 08020 and PD 08021. U.S. Patent Application Publ. No. 20120237518 discloses anti-Trop-2 antibodies 77220, KM4097 and KM4590. U.S. Patent No. 8,309,094 (Wyeth) discloses antibodies A1 and A3, identified by sequence listing. The Examples section of each patent or patent application cited above in this paragraph is incorporated herein by reference. For non-patent publications, Lipinski et al. (1981, Proc Natl. Acad Sci USA, 78:5147-50) disclosed anti-Trop-2 antibodies 162-25.3 and 162-46.2. More recently, the Pr1E11 anti-Trop-2 antibody was reported to recognize a unique epitope on Trop-2 (Ikeda et al., Biochem Biophys Res Comm 458:877-82).

[041] Numerous anti-Trop-2 antibodies are known in the art and/or publicly available. As discussed below, methods for preparing antibodies against known antigens were routine in the art. The sequence of the human Trop-2 protein was also known in the art (see, e.g., GenBank Accession No. CAA54801.1). Methods for producing humanized, human or chimeric antibodies were also known. The person of ordinary skill, reading the instant disclosure in light of general knowledge in the art, would have been able to make and use the genus of anti-Trop-2 antibodies.

[042] None of the prior studies discussed above contained any disclosure of the use of anti-Trop-2 antibodies for isolating or detecting Trop-2 positive CTCs. A need exists for compositions and methods for enriching, isolating, detecting, diagnosing and/or characterizing Trop-2 positive CTCs.

Isolation and Detection of Circulating Tumor Cells

[043] The anti-Trop-2 antibodies may be utilized to enrich, isolate, detect and/or diagnose Trop-2 positive CTCs using any known technology for CTC isolation and detection. Numerous systems have been developed and are commercially available for CTC detection. Although the majority were developed using specific anti-EpCAM antibodies, the compositions and methods may be modified to utilize anti-Trop-2 antibodies instead. Thus, isolation and detection of Trop-2 positive CTCs may be performed using any such known system, or more traditional methods of cell isolation and detection. Non-limiting examples of such known techniques are discussed below.

[044] The present invention may be used with an affinity-based enrichment step, as well as methods without an enrichment steps, such as MAINTRAC® (Pachmann et al. 2005, Breast

Cancer Res, 7: R975). Methods that use a magnetic device for affinity-based enrichment, include the CELLSEARCH® system (Veridex), the LIQUIDBIOPSY® platform (Cynvenio Biosystems) and the MagSweeper device (Talasaz et al, PNAS, 2009, 106: 3970). Methods that do not use a magnetic device for affinity-based enrichment, include a variety of fabricated microfluidic devices, such as CTC-chips (Stott et al. 2010, Sci Transl Med, 2: 25ra23), HB-chips (Stott et al, 2010, PNAS, 107: 18392), NanoVelcro chips (Lu et al., 2013, Methods, 64: 144), GEDI microdevice (Kirby et al., 2012, PLoS ONE, 7: e35976), and Biocept's ONCOCEE™ technology (Pecot et al., 2011, Cancer Discov, 1: 580).

[045] Use of the FDA-approved CELLSEARCH® system for CTC detection in non-small cell and small cell lung cancer patients is discussed in Truini et al. (2014, Front Oncol 4:242). A 7.5 ml sample of peripheral blood is mixed with magnetic iron nanoparticles coated with an nanti-EpCAM antibody. A strong magnetic field is used to separate EpCAM positive from EpCAM- negative cells. Detection of bound CTCs was performed using fluorescently labeled anti-CK and anti-CD45 antibodies, along with DAPI (4',6'diamidino-2-phynlindole) fluorescent labeling of cell nuclei. CTCs were identified by fluorescent detection as CK positive, CD45 negative and DAPI positive.

[046] The VerIFAST system was used for diagnosis and pharmacodynamic analysis of circulating tumor cells (CTCs) in non-small cell lung cancer (NSCLC) (Casavant et al., 2013, Lab Chip 13:391-6; 2014, Lab Chip 14:99-105). The VerIFAST platform utilizes the relative dominance of surface tension over gravity in the microscale to load immiscible phases side by side. This pins aqueous and oil fields in adjacent chambers to create a virtual filter between two aqueous wells (Casavant et al., 2013, Lab Chip 13:391-6). Using paramagnetic particles (PMPs) with attached antibody or other targeting moieties, specific cell populations can be targeted and isolated from complex backgrounds through a simple traverse of the oil barrier. In the NSCLC example, streptavidin was conjugated to DYNABEADS® FLOWCOMP™ PMPs (Life Technologies, USA) and cells were captured using biotinylated anti-EpCAM antibody. A handheld magnet was used to transfer CTCs bound to PMPs between aqueous chambers. Collected CTCs were released with PMP release buffer (DYNABEADS®) and stained for EpCAM, EGFR or transcription termination factor (TTF-1).

[047] The VerIFAST platform integrates a microporous membrane into an aqueous chamber to enable multiple fluid transfers without the need for cell transfer or centrifugation. With physical characteristic scales enabling high precision relative to macroscale techniques, such

microfluidic techniques are well adapted to capture and assess CTCs with minimal sample loss. The VerIFAST platform effectively captured CTCs from blood of NSCLC patients.

[048] The GILUPI CELLCOLLECTOR™ (Saucedo-Zeni et al., 2012, Int J Oncol 41:1241-50) is based on a functionalized medical Seldinger guidewire (FSMW) coated with chimeric anti-EpCAM antibody. The guidewire was functionalized with a polycarboxylate hydrogel layer that was activated with EDC and NHS, allowing covalent bonding of antibody. The antibody-coated FSMW was inserted in the cubital veins of breast cancer or NSCLC lung cancer patients through a standard venous cannula for 30 minutes. Following binding of cells to the guidewire, CTCs were identified by immunocytochemical staining of EpCAM and/or cytokeratins and nuclear staining. Fluorescent labeling was analyzed with an Axio Imager.A1m microscope (Zeiss, Jena, Germany) equipped with an AxioCam digital camera system and AxioVision 4.6 software. The FSMW system was capable of enriching EpCAM-positive CTCs from 22 of 24 patients tested, including those with early stage cancer in which distant metasteses had not yet been diagnosed. No CTCs were detected in healthy volunteers. An advantage of the FSMW system is that it is not limited by the volume of *ex vivo* blood samples that may be processed using alternative methodologies, such as the CELLSEARCH® system. Estimated blood volume in contact with the FSMW during the 30 minute exposure was 1.5 to 3 liters.

[049] The MagSweeper device (e.g., Powell et al., 2012, PLoS ONE 7:e33788) is another system utilized antibody-coated magnetic particles for CTC detection. Nine milliliters of whole blood was mixed *ex vivo* for 1 hr at RT with 4.5 μM DYNABEADS® (Invitrogen, Life Technologies, Grand Island, NY) coated with the BerEP4 anti-EpCAM antibody. After dilution with PBS, cells bound to DYNABEADS® were captured by a sweeping magnetic device (MagSweeper, see Figure 1 of Powel et al., 2012). Two cycles of capture-wash-relase were performed, using a controlled shear force that released non-specifically bound leukocytes and RBCs. Captured cells were released into fresh buffer and examined using an Axio Observer A1 inverted microscope (Zeiss). Single CTCs were manually aspirated and stored frozen, prior to analysis of expression of 87 genes by chip based high-throughput qRT-PCR.

[050] Gupta et al. (2012, Biomicrofluidics 6:24133) discussed use of the APOSTREAM™ dielectrophoretic device for CTC collection and analysis. A microfluidic flow chamber is used with dielectrophoretic (DEP) technology to capture CTCs (see Figure 1, Gupta et al., 2012). The system may be operated in continuous mode for flow-through isolation and

enrichment of CTCs from peripheral blood. DEP sorts cells with distinct biophysical characteristics by exploiting the frequency-dependent dielectric properties of different cell types, arising from differences in morphologic properties and electrical conductivity. These differences result in differential frequency-dependent migration of CTCs and normal cells in the microfluidic chamber. At an AC frequency in the range of 45-85 kHz, cancer cells experience a positive (attractive) DEP force, which causes them to migrate towards the electrode plane and away from the hydrodynamic flow through the chamber. At the same frequency, normal cells experience a negative (repulsive) DEP force, which moves them into the hydrodynamic flow velocity profile and out of the chamber. A collection port is used to remove separated CTCs for further analysis. For the initial optimization study, cultured cancer cells were spiked into normal blood mononuclear cells and were recovered with over a 70% efficiency. Although the APOSTREAM™ system disclosed by Gupta does not use capture antibodies, the subject anti-Trop-2 antibodies may potentially be utilized to increase the efficiency of CTC separation and/or for post-separation characterization of the isolated CTCs.

[051] Winer-Jones et al. (2014, PLoS One 9:e86717) discussed use of the LIQUIDBIOPSY® system for isolation and characterization of CTCs. The LIQUIDBIOPSY® system uses high throughput sheath flow microfluidics through a flow cell, combined with anti-EpCAM antibodies as a capture agent. Biotinylated anti-EpCAM was attached to streptavidin-coated IMAG™ beads (BD, Franklin Lakes, NJ) and mixed with blood samples, containing spiked tumor cells labeled with CFSE or FITC. Normal nucleated cells were labeled with DAPI. After antibody binding, the blood samples were processed on the CTC flow cell, attached to a glass slide. An external magnetic field is used to capture magnetic-bead bound CTCs on the glass surface, separating them from the laminar flow containing normal cells. Captured cells were counted using an Eclipse E80i fluorescent microscope (Nikon Instruments, Melville, NY).

[052] The person of ordinary skill will realize that any of these systems, or any other known system for CTC enrichment and/or isolation, may be used with the subject anti-Trop-2 antibodies for enrichment, isolation, detection and/or characterization of CTCs. Where an anti-Trop-2 capture antibody is utilized, the bound CTCs may be detected and/or characterized using labeled antibodies against a different Trop-2 epitope, or against other known tumor-associated antigens, including but not limited to carbonic anhydrase IX, CCL19, CCL21, CSAp, CD1, CD1a, CD2, CD3, CD4, CD5, CD8, CD11A, CD14, CD15,

CD16, CD18, CD19, IGF-1R, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD55, CD59, CD64, CD66a-e, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, CXCR4, CXCR7, CXCL12, HIF-1-α, AFP, PSMA, CEACAM5, CEACAM-6, c-met, B7, ED-B of fibronectin, Factor H, FHL-1, Flt-3, folate receptor, GROB, HMGB-1, hypoxia inducible factor (HIF), insulin-like growth factor-1 (ILGF-1), IFN-γ, IFN-α, IFN-β, IL-2, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, IP-10, MAGE, mCRP, MCP-1, MIP-1A, MIP-1B, MIF, MUC1, MUC2, MUC3, MUC4, MUC5ac, NCA-95, NCA-90, Ia, EGP-1, EGP-2, HLA-DR, tenascin, Le(y), RANTES, T101, TAC, Tn antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, TNF-α, TRAIL receptor (R1 and R2), VEGFR, EGFR, P1GF, complement factors C3, C3a, C3b, C5a, and C5.

Antibody Preparation

[053] Techniques for preparing monoclonal antibodies against virtually any target antigen, such as Trop-2, are well known in the art. *See*, for example, Köhler and Milstein, *Nature 256*: 495 (1975), and Coligan *et al.* (eds.), CURRENT PROTOCOLS IN IMMUNOLOGY, VOL. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991). Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

[054] MAbs can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A or Protein-G Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3. Also, see Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in METHODS IN MOLECULAR BIOLOGY, VOL. 10, pages 79-104 (The Humana Press, Inc. 1992).

[055] After the initial raising of antibodies to the immunogen, the antibodies can be sequenced and subsequently prepared by recombinant techniques. Humanization and chimerization of murine antibodies and antibody fragments are well known to those skilled in the art, as discussed below.

Chimeric Antibodies

[056] A chimeric antibody is a recombinant protein in which the variable regions of a human antibody have been replaced by the variable regions of, for example, a mouse antibody, including the complementarity-determining regions (CDRs) of the mouse antibody. Chimeric antibodies exhibit decreased immunogenicity and increased stability when administered to a subject. General techniques for cloning murine immunoglobulin variable domains are disclosed, for example, in Orlandi *et al., Proc. Nat'l Acad. Sci. USA 6*: 3833 (1989). Techniques for constructing chimeric antibodies are well known to those of skill in the art. As an example, Leung *et al., Hybridoma 13:*469 (1994), produced an LL2 chimera by combining DNA sequences encoding the V_κ and V_H domains of murine LL2, an anti-CD22 monoclonal antibody, with respective human κ and IgG_1 constant region domains.

Humanized Antibodies

[057] Techniques for producing humanized MAbs are well known in the art (see, e.g., Jones *et al., Nature 321*: 522 (1986), Riechmann *et al., Nature 332*: 323 (1988), Verhoeyen *et al., Science 239*: 1534 (1988), Carter *et al., Proc. Nat'l Acad. Sci. USA 89*: 4285 (1992), Sandhu, *Crit. Rev. Biotech. 12*: 437 (1992), and Singer *et al., J. Immun. 150*: 2844 (1993)). A chimeric or murine monoclonal antibody may be humanized by transferring the mouse CDRs from the heavy and light variable chains of the mouse immunoglobulin into the corresponding variable domains of a human antibody. The mouse framework regions (FR) in the chimeric monoclonal antibody are also replaced with human FR sequences. As simply transferring mouse CDRs into human FRs often results in a reduction or even loss of antibody affinity, additional modification might be required in order to restore the original affinity of the murine antibody. This can be accomplished by the replacement of one or more human residues in the FR regions with their murine counterparts to obtain an antibody that possesses good binding affinity to its epitope. See, for example, Tempest *et al.*, *Biotechnology* 9:266 (1991) and Verhoeyen *et al.*, *Science* 239: 1534 (1988). Preferred residues for substitution include FR residues that are located within 1, 2, or 3 Angstroms of a CDR residue side chain, that are located adjacent to a CDR sequence, or that are predicted to interact with a CDR residue.

Human Antibodies

[058] Methods for producing fully human antibodies using either combinatorial approaches or transgenic animals transformed with human immunoglobulin loci are known in the art (*e.g.,* Mancini et al., 2004, *New Microbiol.* 27:315-28; Conrad and Scheller, 2005, *Comb. Chem. High Throughput Screen.* 8:117-26; Brekke and Loset, 2003, *Curr. Opin. Pharmacol.* 3:544-50). A fully human antibody also can be constructed by genetic or chromosomal

transfection methods, as well as phage display technology, all of which are known in the art. See for example, McCafferty *et al., Nature* 348:552-553 (1990). Where antibodies are to be utilized *in vivo*, for example in tumor therapy following detection of a Trop-2 positive cancer, such fully human antibodies are expected to exhibit even fewer side effects than chimeric or humanized antibodies and to function *in vivo* as essentially endogenous human antibodies.

[059] In one alternative, the phage display technique may be used to generate human antibodies (*e.g.*, Dantas-Barbosa et al., 2005, *Genet. Mol. Res.* 4:126-40). Human antibodies may be generated from normal humans or from humans that exhibit a particular disease state, such as cancer (Dantas-Barbosa et al., 2005). The advantage to constructing human antibodies from a diseased individual is that the circulating antibody repertoire may be biased towards antibodies against disease-associated antigens.

[060] In one non-limiting example of this methodology, Dantas-Barbosa et al. (2005) constructed a phage display library of human Fab antibody fragments from osteosarcoma patients. Generally, total RNA was obtained from circulating blood lymphocytes (*Id.*). Recombinant Fab were cloned from the μ, γ and κ chain antibody repertoires and inserted into a phage display library (*Id.*). RNAs were converted to cDNAs and used to make Fab cDNA libraries using specific primers against the heavy and light chain immunoglobulin sequences (Marks et al., 1991, *J. Mol. Biol.* 222:581-97). Library construction was performed according to Andris-Widhopf et al. (2000, In: *Phage Display Laboratory Manual*, Barbas et al. (eds), 1st edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY pp. 9.1 to 9.22). The final Fab fragments were digested with restriction endonucleases and inserted into the bacteriophage genome to make the phage display library. Such libraries may be screened by standard phage display methods, as known in the art. Phage display can be performed in a variety of formats, for their review, see e.g. Johnson and Chiswell, *Current Opinion in Structural Biology* 3:5564-571 (1993).

[061] Human antibodies may also be generated by *in vitro* activated B-cells. See U.S. Patent Nos. 5,567,610 and 5,229,275, incorporated herein by reference in their entirety. The skilled artisan will realize that these techniques are exemplary and any known method for making and screening human antibodies or antibody fragments may be utilized.

[062] In another alternative, transgenic animals that have been genetically engineered to produce human antibodies may be used to generate antibodies against essentially any immunogenic target, using standard immunization protocols. Methods for obtaining human antibodies from transgenic mice are disclosed by Green *et al., Nature Genet.* 7:13 (1994),

Lonberg *et al., Nature 368*:856 (1994), and Taylor *et al., Int. Immun. 6*:579 (1994). A non-limiting example of such a system is the XenoMouse® (*e.g.*, Green et al., 1999, *J. Immunol. Methods* 231:11-23, incorporated herein by reference) from Abgenix (Fremont, CA). In the XenoMouse® and similar animals, the mouse antibody genes have been inactivated and replaced by functional human antibody genes, while the remainder of the mouse immune system remains intact.

[063] The XenoMouse® was transformed with germline-configured YACs (yeast artificial chromosomes) that contained portions of the human IgH and Igkappa loci, including the majority of the variable region sequences, along with accessory genes and regulatory sequences. The human variable region repertoire may be used to generate antibody producing B-cells, which may be processed into hybridomas by known techniques. A XenoMouse® immunized with a target antigen will produce human antibodies by the normal immune response, which may be harvested and/or produced by standard techniques discussed above. A variety of strains of XenoMouse® are available, each of which is capable of producing a different class of antibody. Transgenically produced human antibodies have been shown to have therapeutic potential, while retaining the pharmacokinetic properties of normal human antibodies (Green et al., 1999). The skilled artisan will realize that the claimed compositions and methods are not limited to use of the XenoMouse® system but may utilize any transgenic animal that has been genetically engineered to produce human antibodies.

Known Antibodies and Target Antigens

[064] As discussed above, in certain alternative embodiments the anti-Trop-2 antibodies are of use for treating Trop-2-expressing cancers, following detection of circulating Trop-2-positive tumor cells. In some embodiments, the target cancer may express one or more additional tumor-associated antigens (TAAs) that may be targeted for tumor therapy. Particular antibodies that may be of use for therapy of cancer include, but are not limited to, LL1 (anti-CD74), LL2 or RFB4 (anti-CD22), veltuzumab (hA20, anti-CD20), rituxumab (anti-CD20), obinutuzumab (GA101, anti-CD20), lambrolizumab (anti-PD-1 receptor), nivolumab (anti-PD-1 receptor), ipilimumab (anti-CTLA-4), RS7 (anti-epithelial glycoprotein-1 (EGP-1, also known as Trop-2)), PAM4 or KC4 (both anti-mucin), MN-14 (anti-carcinoembryonic antigen (CEA, also known as CD66e or CEACAM5), MN-15 or MN-3 (anti-CEACAM6), Mu-9 (anti-colon-specific antigen-p), Immu 31 (an anti-alpha-fetoprotein), R1 (anti-IGF-1R), A19 (anti-CD19), TAG-72 (e.g., CC49), Tn, J591 or HuJ591 (anti-PSMA (prostate-specific membrane antigen)), AB-PG1-XG1-026 (anti-PSMA dimer),

D2/B (anti-PSMA), G250 (an anti-carbonic anhydrase IX MAb), L243 (anti-HLA-DR) alemtuzumab (anti-CD52), bevacizumab (anti-VEGF), cetuximab (anti-EGFR), gemtuzumab (anti-CD33), ibritumomab tiuxetan (anti-CD20); panitumumab (anti-EGFR); tositumomab (anti-CD20); PAM4 (aka clivatuzumab, anti-mucin) and trastuzumab (anti-ErbB2). Such antibodies are known in the art (e.g., U.S. Patent Nos. 5,686,072; 5,874,540; 6,107,090; 6,183,744; 6,306,393; 6,653,104; 6,730.300; 6,899,864; 6,926,893; 6,962,702; 7,074,403; 7,230,084; 7,238,785; 7,238,786; 7,256,004; 7,282,567; 7,300,655; 7,312,318; 7,585,491; 7,612,180; 7,642,239; and U.S. Patent Application Publ. No. 20050271671; 20060193865; 20060210475; 20070087001; the Examples section of each incorporated herein by reference.) Specific known antibodies of use include hPAM4 (U.S. Patent No. 7,282,567), hA20 (U.S. Patent No. 7,251,164), hA19 (U.S. Patent No. 7,109,304), hIMMU-31 (U.S. Patent No. 7,300,655), hLL1 (U.S. Patent No. 7,312,318), hLL2 (U.S. Patent No. 7,074,403), hMu-9 (U.S. Patent No. 7,387,773), hL243 (U.S. Patent No. 7,612,180), hMN-14 (U.S. Patent No. 6,676,924), hMN-15 (U.S. Patent No. 7,541,440), hR1 (U.S. Patent Application 12/772,645), hRS7 (U.S. Patent No. 7,238,785), hMN-3 (U.S. Patent No. 7,541,440), AB-PG1-XG1-026 (U.S. Patent Application 11/983,372, deposited as ATCC PTA-4405 and PTA-4406) and D2/B (WO 2009/130575) the text of each recited patent or application is incorporated herein by reference with respect to the Figures and Examples sections.

[065] Alternative antibodies of use include, but are not limited to, abciximab (anti-glycoprotein IIb/IIIa), alemtuzumab (anti-CD52), bevacizumab (anti-VEGF), cetuximab (anti-EGFR), gemtuzumab (anti-CD33), ibritumomab (anti-CD20), panitumumab (anti-EGFR), rituximab (anti-CD20), tositumomab (anti-CD20), trastuzumab (anti-ErbB2), lambrolizumab (anti-PD-1 receptor), nivolumab (anti-PD-1 receptor), ipilimumab (anti-CTLA-4), abagovomab (anti-CA-125), adecatumumab (anti-EpCAM), atlizumab (anti-IL-6 receptor), benralizumab (anti-CD125), obinutuzumab (GA101, anti-CD20), CC49 (anti-TAG-72), AB-PG1-XG1-026 (anti-PSMA, U.S. Patent Application 11/983,372, deposited as ATCC PTA-4405 and PTA-4406), D2/B (anti-PSMA, WO 2009/130575), tocilizumab (anti-IL-6 receptor), basiliximab (anti-CD25), daclizumab (anti-CD25), efalizumab (anti-CD11a), GA101 (anti-CD20; Glycart Roche), muromonab-CD3 (anti-CD3 receptor), natalizumab (anti-α4 integrin), omalizumab (anti-IgE); anti-TNF-α antibodies such as CDP571 (Ofei et al., 2011, Diabetes 45:881-85), MTNFAI, M2TNFAI, M3TNFAI, M3TNFABI, M302B, M303 (Thermo Scientific, Rockford, IL), infliximab (Centocor, Malvern, PA), certolizumab

pegol (UCB, Brussels, Belgium), anti-CD40L (UCB, Brussels, Belgium), adalimumab (Abbott, Abbott Park, IL), and Benlysta (Human Genome Sciences).

[066] Other useful tumor-associated antigens that may be targeted include carbonic anhydrase IX, B7, CCL19, CCL21, CSAp, HER-2/*neu*, BrE3, CD1, CD1a, CD2, CD3, CD4, CD5, CD8, CD11A, CD14, CD15, CD16, CD18, CD19, CD20 (e.g., C2B8, hA20, 1F5 MAbs), CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD44, CD45, CD46, CD47, CD52, CD54, CD55, CD59, CD64, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, CEACAM5, CEACAM6, CTLA-4, alpha-fetoprotein (AFP), VEGF (e.g., AVASTIN®, fibronectin splice variant), ED-B fibronectin (e.g., L19), EGP-1 (Trop-2), EGP-2 (e.g., 17-1A), EGF receptor (ErbB1) (e.g., ERBITUX®), ErbB2, ErbB3, Factor H, FHL-1, Flt-3, folate receptor, Ga 733,GRO-β, HMGB-1, hypoxia inducible factor (HIF), HM1.24, HER-2/*neu,* histone H2B, histone H3, histone H4, insulin-like growth factor (ILGF), IFN-γ, IFN-α, IFN-β, IFN-λ, IL-2R, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-2, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, IP-10, IGF-1R, Ia, HM1.24, gangliosides, HCG, the HLA-DR antigen to which L243 binds, CD66 antigens, i.e., CD66a-d or a combination thereof, MAGE, mCRP, MCP-1, MIP-1A, MIP-1B, macrophage migration-inhibitory factor (MIF), MUC1, MUC2, MUC3, MUC4, MUC5ac, placental growth factor (PlGF), PSA (prostate-specific antigen), PSMA, PAM4 antigen, PD-1 receptor, PD-L1, NCA-95, NCA-90, A3, A33, Ep-CAM, KS-1, Le(y), mesothelin, S100, tenascin, TAC, Tn antigen, Thomas-Friedenreich antigens, tumor necrosis antigens, tumor angiogenesis antigens, TNF-α, TRAIL receptor (R1 and R2), Trop-2, VEGFR, RANTES, T101, as well as cancer stem cell antigens, complement factors C3, C3a, C3b, C5a, C5, and an oncogene product.

[067] Cancer stem cells, which are ascribed to be more therapy-resistant precursor malignant cell populations (Hill and Perris, *J. Natl. Cancer Inst.* 2007; 99:1435-40), have antigens that can be targeted in certain cancer types, such as CD133 in prostate cancer (Maitland et al., *Ernst Schering Found. Sympos. Proc.* 2006; 5:155-79), non-small-cell lung cancer (Donnenberg et al., *J. Control Release* 2007; 122(3):385-91), and glioblastoma (Beier et al., *Cancer Res.* 2007; 67(9):4010-5), and CD44 in colorectal cancer (Dalerba er al., *Proc. Natl. Acad. Sci. USA* 2007; 104(24)10158-63), pancreatic cancer (Li et al., *Cancer Res.* 2007; 67(3):1030-7), and in head and neck squamous cell carcinoma (Prince et al., *Proc. Natl. Acad. Sci. USA* 2007; 104(3)973-8). Another useful target for breast cancer therapy is the LIV-1 antigen described by Taylor et al. (*Biochem. J.* 2003; 375:51-9).

[068] Checkpoint-inhibitor antibodies have been used in cancer therapy. Immune checkpoints refer to inhibitory pathways in the immune system that are responsible for maintaining self-tolerance and modulating the degree of immune system response to minimize peripheral tissue damage. However, tumor cells can also activate immune system checkpoints to decrease the effectiveness of immune response against tumor tissues. Exemplary checkpoint inhibitor antibodies against cytotoxic T-lymphocyte antigen 4 (CTLA4, also known as CD152), programmed cell death protein 1 (PD1, also known as CD279), programmed cell death 1 ligand 1 (PD-L1, also known as CD274) and programmed cell death 1 ligand 2 (PD-L2) (Latchman et al., 2001, Nat Immunol 2:261-8), may be used in combination with one or more other agents to enhance the effectiveness of immune response against disease cells, tissues or pathogens. Exemplary anti-PD1 antibodies include lambrolizumab (MK-3475, MERCK), nivolumab (BMS-936558, BRISTOL-MYERS SQUIBB), AMP-224 (MERCK), and pidilizumab (CT-011, CURETECH LTD.). Anti-PD1 antibodies are commercially available, for example from ABCAM® (AB137132), BIOLEGEND® (EH12.2H7, RMP1-14) and AFFYMETRIX EBIOSCIENCE (J105, J116, MIH4). Exemplary anti-PD-L1 antibodies include MDX-1105 (MEDAREX), MEDI4736 (MEDIMMUNE) MPDL3280A (GENENTECH) and BMS-936559 (BRISTOL-MYERS SQUIBB). Anti-PD-L1 antibodies are also commercially available, for example from AFFYMETRIX EBIOSCIENCE (MIH1). Exemplary anti-CTLA4 antibodies include ipilimumab (Bristol-Myers Squibb) and tremelimumab (PFIZER). Anti-PD1 antibodies are commercially available, for example from ABCAM® (AB134090), SINO BIOLOGICAL INC. (11159-H03H, 11159-H08H), and THERMO SCIENTIFIC PIERCE (PA5-29572, PA5-23967, PA5-26465, MA1-12205, MA1-35914). Ipilimumab has recently received FDA approval for treatment of metastatic melanoma (Wada et al., 2013, J Transl Med 11:89).

[069] Macrophage migration inhibitory factor (MIF) is an important regulator of innate and adaptive immunity and apoptosis. It has been reported that CD74 is the endogenous receptor for MIF (Leng et al., 2003, *J Exp Med* 197:1467-76). The therapeutic effect of antagonistic anti-CD74 antibodies on MIF-mediated intracellular pathways may be of use for treatment of a broad range of disease states, such as cancers of the bladder, prostate, breast, lung, and colon (e.g., Meyer-Siegler et al., 2004, *BMC Cancer* 12:34; Shachar & Haran, 2011, *Leuk Lymphoma* 52:1446-54). Milatuzumab (hLL1) is an exemplary anti-CD74 antibody of therapeutic use for treatment of MIF-mediated diseases.

[070] Various other antibodies of use are known in the art (e.g., U.S. Patent Nos. 5,686,072; 5,874,540; 6,107,090; 6,183,744; 6,306,393; 6,653,104; 6,730.300; 6,899,864; 6,926,893; 6,962,702; 7,074,403; 7,230,084; 7,238,785; 7,238,786; 7,256,004; 7,282,567; 7,300,655; 7,312,318; 7,585,491; 7,612,180; 7,642,239 and U.S. Patent Application Publ. No. 20060193865; each incorporated herein by reference.)

[071] Antibodies of use may be commercially obtained from a wide variety of known sources. For example, a variety of antibody secreting hybridoma lines are available from the American Type Culture Collection (ATCC, Manassas, VA). A large number of antibodies against various disease targets, including tumor-associated antigens, have been deposited at the ATCC and/or have published variable region sequences and are available for use in the claimed methods and compositions. See, e.g., U.S. Patent Nos. 7,312,318; 7,282,567; 7,151,164; 7,074,403; 7,060,802; 7,056,509; 7,049,060; 7,045,132; 7,041,803; 7,041,802; 7,041,293; 7,038,018; 7,037,498; 7,012,133; 7,001,598; 6,998,468; 6,994,976; 6,994,852; 6,989,241; 6,974,863; 6,965,018; 6,964,854; 6,962,981; 6,962,813; 6,956,107; 6,951,924; 6,949,244; 6,946,129; 6,943,020; 6,939,547; 6,921,645; 6,921,645; 6,921,533; 6,919,433; 6,919,078; 6,916,475; 6,905,681; 6,899,879; 6,893,625; 6,887,468; 6,887,466; 6,884,594; 6,881,405; 6,878,812; 6,875,580; 6,872,568; 6,867,006; 6,864,062; 6,861,511; 6,861,227; 6,861,226; 6,838,282; 6,835,549; 6,835,370; 6,824,780; 6,824,778; 6,812,206; 6,793,924; 6,783,758; 6,770,450; 6,767,711; 6,764,688; 6,764,681; 6,764,679; 6,743,898; 6,733,981; 6,730,307; 6,720,155; 6,716,966; 6,709,653; 6,693,176; 6,692,908; 6,689,607; 6,689,362; 6,689,355; 6,682,737; 6,682,736; 6,682,734; 6,673,344; 6,653,104; 6,652,852; 6,635,482; 6,630,144; 6,610,833; 6,610,294; 6,605,441; 6,605,279; 6,596,852; 6,592,868; 6,576,745; 6,572;856; 6,566,076; 6,562,618; 6,545,130; 6,544,749; 6,534,058; 6,528,625; 6,528,269; 6,521,227; 6,518,404; 6,511,665; 6,491,915; 6,488,930; 6,482,598; 6,482,408; 6,479,247; 6,468,531; 6,468,529; 6,465,173; 6,461,823; 6,458,356; 6,455,044; 6,455,040; 6,451,310; 6,444,206; 6,441,143; 6,432,404; 6,432,402; 6,419,928; 6,413,726; 6,406,694; 6,403,770; 6,403,091; 6,395,276; 6,395,274; 6,387,350; 6,383,759; 6,383,484; 6,376,654; 6,372,215; 6,359,126; 6,355,481; 6,355,444; 6,355,245; 6,355,244; 6,346,246; 6,344,198; 6,340,571; 6,340,459; 6,331,175; 6,306,393; 6,254,868; 6,187,287; 6,183,744; 6,129,914; 6,120,767; 6,096,289; 6,077,499; 5,922,302; 5,874,540; 5,814,440; 5,798,229; 5,789,554; 5,776,456; 5,736,119; 5,716,595; 5,677,136; 5,587,459; 5,443,953; 5,525,338. These are exemplary only and a wide variety of other antibodies and their hybridomas are known in the art. The skilled artisan will realize that antibody sequences or antibody-secreting hybridomas against almost

any disease-associated antigen may be obtained by a simple search of the ATCC, NCBI and/or USPTO databases for antibodies against a selected disease-associated target of interest. The antigen binding domains of the cloned antibodies may be amplified, excised, ligated into an expression vector, transfected into an adapted host cell and used for protein production, using standard techniques well known in the art.

Antibody Allotypes

[072] Immunogenicity of therapeutic antibodies is associated with increased risk of infusion reactions and decreased duration of therapeutic response (Baert et al., 2003, N Engl J Med 348:602-08). The extent to which therapeutic antibodies induce an immune response in the host may be determined in part by the allotype of the antibody (Stickler et al., 2011, Genes and Immunity 12:213-21). Antibody allotype is related to amino acid sequence variations at specific locations in the constant region sequences of the antibody. The allotypes of IgG antibodies containing a heavy chain γ-type constant region are designated as Gm allotypes (1976, J Immunol 117:1056-59).

[073] For the common IgG1 human antibodies, the most prevalent allotype is G1m1 (Stickler et al., 2011, Genes and Immunity 12:213-21). However, the G1m3 allotype also occurs frequently in Caucasians (Stickler et al., 2011). It has been reported that G1m1 antibodies contain allotypic sequences that tend to induce an immune response when administered to non-G1m1 (nG1m1) recipients, such as G1m3 patients (Stickler et al., 2011). Non-G1m1 allotype antibodies are not as immunogenic when administered to G1m1 patients (Stickler et al., 2011).

[074] The human G1m1 allotype comprises the amino acids aspartic acid at Kabat position 356 and leucine at Kabat position 358 in the CH3 sequence of the heavy chain IgG1. The nG1m1 allotype comprises the amino acids glutamic acid at Kabat position 356 and methionine at Kabat position 358. Both G1ml and nG1ml allotypes comprise a glutamic acid residue at Kabat position 357 and the allotypes are sometimes referred to as DEL and EEM allotypes. A non-limiting example of the heavy chain constant region sequences for G1m1 and nG1m1 allotype antibodies is shown below for the exemplary antibodies rituximab (SEQ ID NO:7) and veltuzumab (SEQ ID NO:8).

Rituximab heavy chain variable region sequence (SEQ ID NO:7)

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP
AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK**KA**EPKSCDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY

VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSR**D**E**L**TKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH
YTQKSLSLSPGK

Veltuzumab heavy chain variable region (SEQ ID NO:8)

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP
AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK**RV**EPKSCDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSR**E**E**M**TKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH
YTQKSLSLSPGK

[075] Jefferis and Lefranc (2009, mAbs 1:1-7) reviewed sequence variations characteristic of IgG allotypes and their effect on immunogenicity. They reported that the G1m3 allotype is characterized by an arginine residue at Kabat position 214, compared to a lysine residue at Kabat 214 in the G1m17 allotype. The nG1m1,2 allotype was characterized by glutamic acid at Kabat position 356, methionine at Kabat position 358 and alanine at Kabat position 431. The G1m1,2 allotype was characterized by aspartic acid at Kabat position 356, leucine at Kabat position 358 and glycine at Kabat position 431. In addition to heavy chain constant region sequence variants, Jefferis and Lefranc (2009) reported allotypic variants in the kappa light chain constant region, with the Km1 allotype characterized by valine at Kabat position 153 and leucine at Kabat position 191, the Km1,2 allotype by alanine at Kabat position 153 and leucine at Kabat position 191, and the Km3 allotype characterized by alanine at Kabat position 153 and valine at Kabat position 191.

[076] With regard to therapeutic antibodies, veltuzumab and rituximab are, respectively, humanized and chimeric IgG1 antibodies against CD20, of use for therapy of a wide variety of hematological malignancies and/or autoimmune diseases. **Table 1** compares the allotype sequences of rituximab vs. veltuzumab. As shown in **Table 1**, rituximab (G1m17,1) is a DEL allotype IgG1, with an additional sequence variation at Kabat position 214 (heavy chain CH1) of lysine in rituximab vs. arginine in veltuzumab. It has been reported that veltuzumab is less immunogenic in subjects than rituximab (*see, e.g.,* Morchhauser et al., 2009, J Clin Oncol 27:3346-53; Goldenberg et al., 2009, Blood 113:1062-70; Robak & Robak, 2011, BioDrugs 25:13-25), an effect that has been attributed to the difference between humanized

and chimeric antibodies. However, the difference in allotypes between the EEM and DEL allotypes likely also accounts for the lower immunogenicity of veltuzumab.

Table 1. Allotypes of Rituximab vs. Veltuzumab

		Heavy chain position and associated allotypes					
	Complete allotype	*214*	*(allotype)*	*356/358*	*(allotype)*	*431*	*(allotype)*
Rituximab	*G1m17,1*	*K*	*17*	*D/L*	*1*	*A*	*-*
Veltuzumab	*G1m3*	*R*	*3*	*E/M*	*-*	*A*	*-*

[077] In order to reduce the immunogenicity of therapeutic antibodies in individuals of nG1m1 genotype, it is desirable to select the allotype of the antibody to correspond to the G1m3 allotype, characterized by arginine at Kabat 214, and the nG1m1,2 null-allotype, characterized by glutamic acid at Kabat position 356, methionine at Kabat position 358 and alanine at Kabat position 431. Surprisingly, it was found that repeated subcutaneous administration of G1m3 antibodies over a long period of time did not result in a significant immune response. In alternative embodiments, the human IgG4 heavy chain in common with the G1m3 allotype has arginine at Kabat 214, glutamic acid at Kabat 356, methionine at Kabat 359 and alanine at Kabat 431. Since immunogenicity appears to relate at least in part to the residues at those locations, use of the human IgG4 heavy chain constant region sequence for therapeutic antibodies is also a preferred embodiment. Combinations of G1m3 IgG1 antibodies with IgG4 antibodies may also be of use for therapeutic administration.

Nanobodies

[078] Nanobodies are single-domain antibodies of about 12-15 kDa in size (about 110 amino acids in length). Nanobodies can selectively bind to target antigens, like full-size antibodies, and have similar affinities for antigens. However, because of their much smaller size, they may be capable of better penetration into solid tumors. The smaller size also contributes to the stability of the nanobody, which is more resistant to pH and temperature extremes than full size antibodies (Van Der Linden et al., 1999, Biochim Biophys Act 1431:37-46). Single-domain antibodies were originally developed following the discovery that camelids (camels, alpacas, llamas) possess fully functional antibodies without light chains (e.g., Hamsen et al., 2007, Appl Microbiol Biotechnol 77:13-22). The heavy-chain antibodies consist of a single variable domain (V_{HH}) and two constant domains (C_H2 and C_H3). Like antibodies, nanobodies may be developed and used as multivalent and/or bispecific constructs. Humanized forms of nanobodies are in commercial development that are targeted to a variety of target antigens, such as IL-6R, vWF, TNF, RSV, RANKL, IL-17A

& F and IgE (e.g., ABLYNX®, Ghent, Belgium), with potential clinical use in cancer and other disorders (e.g., Saerens et al., 2008, Curr Opin Pharmacol 8:600-8; Muyldermans, 2013, Ann Rev Biochem 82:775-97; Ibanez et al., 2011, J Infect Dis 203:1063-72).

[079] The plasma half-life of nanobodies is shorter than that of full-size antibodies, with elimination primarily by the renal route. Because they lack an Fc region, they do not exhibit complement dependent cytotoxicity.

[080] Nanobodies may be produced by immunization of camels, llamas, alpacas or sharks with target antigen, following by isolation of mRNA, cloning into libraries and screening for antigen binding. Nanobody sequences may be humanized by standard techniques (e.g., Jones et al., 1986, Nature 321: 522, Riechmann et al., 1988, Nature 332: 323, Verhoeyen et al., 1988, Science 239: 1534, Carter et al., 1992, Proc. Nat'l Acad. Sci. USA 89: 4285, Sandhu, 1992, Crit. Rev. Biotech. 12: 437, Singer et al., 1993, J. Immun. 150: 2844). Humanization is relatively straight-forward because of the high homology between camelid and human FR sequences.

[081] In various embodiments, the subject antibodies may comprise nanobodies for targeted delivery of conjugated diagnostic agent(s) to targeted cancer cells. Nanobodies of use are disclosed, for example, in U.S. Patent Nos. 7,807,162; 7,939,277; 8,188,223; 8,217,140; 8,372,398; 8,557,965; 8,623,361 and 8,629,244, the Examples section of each incorporated herein by reference.)

Antibody Fragments

[082] Antibody fragments are antigen binding portions of an antibody, such as $F(ab')_2$, Fab', $F(ab)_2$, Fab, Fv, sFv, scFv and the like. Antibody fragments which recognize specific epitopes can be generated by known techniques. $F(ab')_2$ fragments, for example, can be produced by pepsin digestion of the antibody molecule. These and other methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647 and references contained therein. Also, see Nisonoff et al., Arch Biochem. Biophys. 89: 230 (1960); Porter, Biochem. J. 73: 119 (1959), Edelman et al., in METHODS IN ENZYMOLOGY VOL. 1, page 422 (Academic Press 1967), and Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4. Alternatively, Fab' expression libraries can be constructed (Huse et al., 1989, Science, 246:1274-1281) to allow rapid and easy identification of monoclonal Fab' fragments with the desired specificity.

[083] A single chain Fv molecule (scFv) comprises a VL domain and a VH domain. The VL and VH domains associate to form a target binding site. These two domains are further covalently linked by a peptide linker (L). A scFv molecule is denoted as either VL-L-VH if

the VL domain is the N-terminal part of the scFv molecule, or as VH-L-VL if the VH domain is the N-terminal part of the scFv molecule. Methods for making scFv molecules and designing suitable peptide linkers are described in U.S. Pat. No. 4,704,692, U.S. Pat. No. 4,946,778, R. Raag and M. Whitlow, "Single Chain Fvs." FASEB Vol 9:73-80 (1995) and R. E. Bird and B. W. Walker, Single Chain Antibody Variable Regions, TIBTECH, Vol 9: 132-137 (1991).

[084] Other antibody fragments, for example single domain antibody fragments, are known in the art and may be used in the claimed constructs. Single domain antibodies (VHH) may be obtained, for example, from camels, alpacas or llamas by standard immunization techniques. (See, e.g., Muyldermans et al., TIBS 26:230-235, 2001; Yau et al., J Immunol Methods 281:161-75, 2003; Maass et al., J Immunol Methods 324:13-25, 2007). The VHH may have potent antigen-binding capacity and can interact with novel epitopes that are inaccessible to conventional VH-VL pairs. (Muyldermans et al., 2001). Alpaca serum IgG contains about 50% camelid heavy chain only IgG antibodies (HCAbs) (Maass et al., 2007). Alpacas may be immunized with known antigens, such as TNF-α, and VHHs can be isolated that bind to and neutralize the target antigen (Maass et al., 2007). PCR primers that amplify virtually all alpaca VHH coding sequences have been identified and may be used to construct alpaca VHH phage display libraries, which can be used for antibody fragment isolation by standard biopanning techniques well known in the art (Maass et al., 2007).

[085] An antibody fragment can also be prepared by proteolytic hydrolysis of a full-length antibody or by expression in E. coli or another host of the DNA coding for the fragment. An antibody fragment can be obtained by pepsin or papain digestion of full-length antibodies by conventional methods. For example, an antibody fragment can be produced by enzymatic cleavage of antibodies with pepsin to provide an approximate 100 kD fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce an approximate 50 Kd Fab' monovalent fragment. Alternatively, an enzymatic cleavage using papain produces two monovalent Fab fragments and an Fc fragment directly.

[086] Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

General techniques for antibody cloning and production

[087] Various techniques, such as production of chimeric or humanized antibodies, may involve procedures of antibody cloning and construction. The antigen-binding Vκ (variable light chain) and V_H (variable heavy chain) sequences for an antibody of interest may be obtained by a variety of molecular cloning procedures, such as RT-PCR, 5'-RACE, and cDNA library screening. The V genes of a MAb from a cell that expresses a murine MAb can be cloned by PCR amplification and sequenced. To confirm their authenticity, the cloned V_L and V_H genes can be expressed in cell culture as a chimeric Ab as described by Orlandi *et al.*, (*Proc. Natl. Acad. Sci.*, USA, 86: 3833 (1989)). Based on the V gene sequences, a humanized MAb can then be designed and constructed as described by Leung et al. (*Mol. Immunol.*, 32: 1413 (1995)).

[088] cDNA can be prepared from any known hybridoma line or transfected cell line producing a murine MAb by general molecular cloning techniques (Sambrook et al., Molecular Cloning, A laboratory manual, 2nd Ed (1989)). The Vκ sequence for the MAb may be amplified using the primers VK1BACK and VK1FOR (Orlandi *et al.*, 1989) or the extended primer set described by Leung et al. (*BioTechniques*, 15: 286 (1993)). The V_H sequences can be amplified using the primer pair VH1BACK/VH1FOR (Orlandi *et al.*, 1989) or the primers annealing to the constant region of murine IgG described by Leung et al. (Hybridoma, 13:469 (1994)). Humanized V genes can be constructed by a combination of long oligonucleotide template syntheses and PCR amplification as described by Leung et al. (*Mol. Immunol.*, 32: 1413 (1995)).

[089] PCR products for Vκ can be subcloned into a staging vector, such as a pBR327-based staging vector, VKpBR, that contains an Ig promoter, a signal peptide sequence and convenient restriction sites. PCR products for V_H can be subcloned into a similar staging vector, such as the pBluescript-based VHpBS. Expression cassettes containing the Vκ and V_H sequences together with the promoter and signal peptide sequences can be excised from VKpBR and VHpBS and ligated into appropriate expression vectors, such as pKh and pG1g, respectively (Leung et al., Hybridoma, 13:469 (1994)). The expression vectors can be co-transfected into an appropriate cell and supernatant fluids monitored for production of a chimeric, humanized or human MAb. Alternatively, the Vκ and V_H expression cassettes can be excised and subcloned into a single expression vector, such as pdHL2, as described by Gillies *et al.* (*J. Immunol. Methods* 125:191 (1989) and also shown in Losman et al., *Cancer*, 80:2660 (1997)).

[090] In an alternative embodiment, expression vectors may be transfected into host cells that have been pre-adapted for transfection, growth and expression in serum-free medium. Exemplary cell lines that may be used include the Sp/EEE, Sp/ESF and Sp/ESF-X cell lines (see, e.g., U.S. Patent Nos. 7,531,327; 7,537,930 and 7,608,425; the Examples section of each of which is incorporated herein by reference). These exemplary cell lines are based on the Sp2/0 myeloma cell line, transfected with a mutant Bcl-EEE gene, exposed to methotrexate to amplify transfected gene sequences and pre-adapted to serum-free cell line for protein expression.

Bispecific and Multispecific Antibodies

[091] In certain alternative embodiments, the anti-Trop-2 antibody or fragment thereof may be co-administered with, for example, a hapten-binding antibody or fragment thereof, such as an anti-HSG or anti-In-DTPA antibody. Such bispecific antibodies may be of use in pretargeting techniques for administration of diagnostic and/or therapeutic agents to Trop-2 positive tumors *in vivo*. In other embodiments, bispecific or multispecific antibodies may be utilized directly for anti-cancer therapy.

[092] Numerous methods to produce bispecific or multispecific antibodies are known, as disclosed, for example, in U.S. Patent No. 7,405,320, the Examples section of which is incorporated herein by reference. Bispecific antibodies can be produced by the quadroma method, which involves the fusion of two different hybridomas, each producing a monoclonal antibody recognizing a different antigenic site (Milstein and Cuello, Nature, 1983; 305:537-540).

[093] Another method for producing bispecific antibodies uses heterobifunctional cross-linkers to chemically tether two different monoclonal antibodies (Staerz, et al. Nature. 1985; 314:628-631; Perez, et al. Nature. 1985; 316:354-356). Bispecific antibodies can also be produced by reduction of each of two parental monoclonal antibodies to the respective half molecules, which are then mixed and allowed to reoxidize to obtain the hybrid structure (Staerz and Bevan. Proc Natl Acad Sci U S A. 1986; 83:1453-1457). Other methods include improving the efficiency of generating hybrid hybridomas by gene transfer of distinct selectable markers via retrovirus-derived shuttle vectors into respective parental hybridomas, which are fused subsequently (DeMonte, et al. Proc Natl Acad Sci U S A. 1990, 87:2941-2945); or transfection of a hybridoma cell line with expression plasmids containing the heavy and light chain genes of a different antibody.

[094] Cognate V_H and V_L domains can be joined with a peptide linker of appropriate composition and length (usually consisting of more than 12 amino acid residues) to form a single-chain Fv (scFv), as discussed above. Reduction of the peptide linker length to less than 12 amino acid residues prevents pairing of V_H and V_L domains on the same chain and forces pairing of V_H and V_L domains with complementary domains on other chains, resulting in the formation of functional multimers. Polypeptide chains of V_H and V_L domains that are joined with linkers between 3 and 12 amino acid residues form predominantly dimers (termed diabodies). With linkers between 0 and 2 amino acid residues, trimers (termed triabody) and tetramers (termed tetrabody) are favored, but the exact patterns of oligomerization appear to depend on the composition as well as the orientation of V-domains (V_H-linker-V_L or V_L-linker-V_H), in addition to the linker length.

[095] These techniques for producing multispecific or bispecific antibodies exhibit various difficulties in terms of low yield, necessity for purification, low stability or the labor-intensiveness of the technique. More recently, a technique known as "DOCK-AND-LOCK®" (DNL®), discussed in more detail below, has been utilized to produce combinations of virtually any desired antibodies, antibody fragments and other effector molecules. Any of the techniques known in the art for making bispecific or multispecific antibodies may be utilized in the practice of the presently claimed methods.

DOCK-AND-LOCK® (DNL®)

[096] Bispecific or multispecific antibodies or other constructs may be produced using the DOCK-AND-LOCK® technology (see, e.g., U.S. Patent Nos. 7,550,143; 7,521,056; 7,534,866; 7,527,787 and 7,666,400, the Examples section of each incorporated herein by reference). Generally, the technique takes advantage of the specific and high-affinity binding interactions that occur between a dimerization and docking domain (DDD) sequence of the regulatory (R) subunits of cAMP-dependent protein kinase (PKA) and an anchor domain (AD) sequence derived from any of a variety of AKAP proteins (Baillie *et al.*, FEBS Letters. 2005; 579: 3264. Wong and Scott, Nat. Rev. Mol. Cell Biol. 2004; 5: 959). The DDD and AD peptides may be attached to any protein, peptide or other molecule, preferably as a fusion protein comprising the AD or DDD sequence. Because the DDD sequences spontaneously dimerize and bind to the AD sequence, the technique allows the formation of complexes between any selected molecules that may be attached to DDD or AD sequences.

[097] Although the standard DNL ® complex comprises a trimer with two DDD-linked molecules attached to one AD-linked molecule, variations in complex structure allow the

formation of dimers, trimers, tetramers, pentamers, hexamers and other multimers. In some embodiments, the DNL® complex may comprise two or more antibodies, antibody fragments or fusion proteins which bind to the same antigenic determinant or to two or more different antigens. The DNL® complex may also comprise one or more other effectors, such as proteins, peptides, immunomodulators, cytokines, interleukins, interferons, binding proteins, peptide ligands, carrier proteins, toxins, ribonucleases such as onconase, inhibitory oligonucleotides such as siRNA, antigens or xenoantigens, polymers such as PEG, enzymes, therapeutic agents, hormones, cytotoxic agents, anti-angiogenic agents, pro-apoptotic agents or any other molecule or aggregate.

[098] PKA, which plays a central role in one of the best studied signal transduction pathways triggered by the binding of the second messenger cAMP to the R subunits, was first isolated from rabbit skeletal muscle in 1968 (Walsh *et al.*, J. Biol. Chem. 1968;243:3763). The structure of the holoenzyme consists of two catalytic subunits held in an inactive form by the R subunits (Taylor, J. Biol. Chem. 1989;264:8443). Isozymes of PKA are found with two types of R subunits (RI and RII), and each type has α and β isoforms (Scott, Pharmacol. Ther. 1991;50:123). Thus, the four isoforms of PKA regulatory subunits are RIα, RIβ, RIIα and RIIβ. The R subunits have been isolated only as stable dimers and the dimerization domain has been shown to consist of the first 44 amino-terminal residues of RIIα (Newlon *et al.*, Nat. Struct. Biol. 1999; 6:222). As discussed below, similar portions of the amino acid sequences of other regulatory subunits are involved in dimerization and docking, each located near the N-terminal end of the regulatory subunit. Binding of cAMP to the R subunits leads to the release of active catalytic subunits for a broad spectrum of serine/threonine kinase activities, which are oriented toward selected substrates through the compartmentalization of PKA via its docking with AKAPs (Scott *et al.*, J. Biol. Chem. 1990;265;21561)

[099] Since the first AKAP, microtubule-associated protein-2, was characterized in 1984 (Lohmann *et al.*, Proc. Natl. Acad. Sci USA. 1984; 81:6723), more than 50 AKAPs that localize to various sub-cellular sites, including plasma membrane, actin cytoskeleton, nucleus, mitochondria, and endoplasmic reticulum, have been identified with diverse structures in species ranging from yeast to humans (Wong and Scott, Nat. Rev. Mol. Cell Biol. 2004;5:959). The AD of AKAPs for PKA is an amphipathic helix of 14-18 residues (Carr *et al.*, J. Biol. Chem. 1991;266:14188). The amino acid sequences of the AD are quite varied among individual AKAPs, with the binding affinities reported for RII dimers ranging from 2 to 90 nM (Alto *et al.*, Proc. Natl. Acad. Sci. USA. 2003;100:4445). AKAPs will only

bind to dimeric R subunits. For human RIIα, the AD binds to a hydrophobic surface formed by the 23 amino-terminal residues (Colledge and Scott, Trends Cell Biol. 1999; 6:216). Thus, the dimerization domain and AKAP binding domain of human RIIα are both located within the same N-terminal 44 amino acid sequence (Newlon *et al.*, Nat. Struct. Biol. 1999;6:222; Newlon *et al.*, EMBO J. 2001;20:1651), which is termed the DDD herein.

[0100] We have developed a platform technology to utilize the DDD of human PKA regulatory subunits and the AD of AKAP as an excellent pair of linker modules for docking any two entities, referred to hereafter as **A** and **B**, into a noncovalent complex, which could be further locked into a DNL® complex through the introduction of cysteine residues into both the DDD and AD at strategic positions to facilitate the formation of disulfide bonds. The general methodology of the approach is as follows. Entity **A** is constructed by linking a DDD sequence to a precursor of **A,** resulting in a first component hereafter referred to as **a**. Because the DDD sequence would effect the spontaneous formation of a dimer, **A** would thus be composed of $\mathbf{a}_2$. Entity **B** is constructed by linking an AD sequence to a precursor of **B,** resulting in a second component hereafter referred to as **b**. The dimeric motif of DDD contained in $\mathbf{a}_2$ will create a docking site for binding to the AD sequence contained in **b,** thus facilitating a ready association of $\mathbf{a}_2$ and **b** to form a binary, trimeric complex composed of $\mathbf{a}_2\mathbf{b}$. This binding event is made irreversible with a subsequent reaction to covalently secure the two entities via disulfide bridges, which occurs very efficiently based on the principle of effective local concentration because the initial binding interactions should bring the reactive thiol groups placed onto both the DDD and AD into proximity (Chmura *et al.*, Proc. Natl. Acad. Sci. USA. 2001;98:8480) to ligate site-specifically. Using various combinations of linkers, adaptor modules and precursors, a wide variety of DNL® constructs of different stoichiometry may be produced and used (see, e.g., U.S. Nos. 7,550,143; 7,521,056; 7,534,866; 7,527,787 and 7,666,400.)

[0101] By attaching the DDD and AD away from the functional groups of the two precursors, such site-specific ligations are also expected to preserve the original activities of the two precursors. This approach is modular in nature and potentially can be applied to link, site-specifically and covalently, a wide range of substances, including peptides, proteins, antibodies, antibody fragments, and other effector moieties with a wide range of activities. Utilizing the fusion protein method of constructing AD and DDD conjugated effectors described below, virtually any protein or peptide may be incorporated into a DNL® construct. However, the technique is not limiting and other methods of conjugation may be utilized.

[0102] A variety of methods are known for making fusion proteins, including nucleic acid synthesis, hybridization and/or amplification to produce a synthetic double-stranded nucleic acid encoding a fusion protein of interest. Such double-stranded nucleic acids may be inserted into expression vectors for fusion protein production by standard molecular biology techniques (see, e.g. Sambrook et al., Molecular Cloning, A laboratory manual, 2nd Ed, 1989). In such preferred embodiments, the AD and/or DDD moiety may be attached to either the N-terminal or C-terminal end of an effector protein or peptide. However, the skilled artisan will realize that the site of attachment of an AD or DDD moiety to an effector moiety may vary, depending on the chemical nature of the effector moiety and the part(s) of the effector moiety involved in its physiological activity. Site-specific attachment of a variety of effector moieties may be performed using techniques known in the art, such as the use of bivalent cross-linking reagents and/or other chemical conjugation techniques.

Structure-Function Relationships in AD and DDD Moieties

[0103] For different types of DNL® constructs, different AD or DDD sequences may be utilized. Exemplary DDD and AD sequences are provided below.

DDD1

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:9)

DDD2

CGHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:10)

AD1

QIEYLAKQIVDNAIQQA (SEQ ID NO:11)

AD2

CGQIEYLAKQIVDNAIQQAGC (SEQ ID NO:12)

[0104] The skilled artisan will realize that DDD1 and DDD2 are based on the DDD sequence of the human RIIα isoform of protein kinase A. However, in alternative embodiments, the DDD and AD moieties may be based on the DDD sequence of the human RIα form of protein kinase A and a corresponding AKAP sequence, as exemplified in DDD3, DDD3C and AD3 below.

DDD3

SLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK (SEQ ID NO:13)

DDD3C

MSCGGSLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK (SEQ ID NO:14)

AD3

CGFEELAWKIAKMIWSDVFQQGC (SEQ ID NO:15)

[0105] In other alternative embodiments, other sequence variants of AD and/or DDD moieties may be utilized in construction of the DNL® complexes. For example, there are only four variants of human PKA DDD sequences, corresponding to the DDD moieties of PKA RIα, RIIα, RIβ and RIIβ. The RIIα DDD sequence is the basis of DDD1 and DDD2 disclosed above. The four human PKA DDD sequences are shown below. The DDD sequence represents residues 1-44 of RIIα, 1-44 of RIIβ, 12-61 of RIα and 13-66 of RIβ. (Note that the sequence of DDD1 is modified slightly from the human PKA RIIα DDD moiety.)

PKA RIα

SLRECELYVQKHNIQALLKDVSIVQLCTARPERPMAFLREYFEKLEKEEAK (SEQ ID NO:16)

PKA RIβ

SLKGCELYVQLHGIQQVLKDCIVHLCISKPERPMKFLREHFEKLEKEENRQILA (SEQ ID NO:17)

PKA RIIα

SHIQIPPGLTELLQGYTVEVGQQPPDLVDFAVEYFTRLREARRQ (SEQ ID NO:18)

PKA RIIβ

SIEIPAGLTELLQGFTVEVLRHQPADLLEFALQHFTRLQQENER (SEQ ID NO:19)

[0106] The structure-function relationships of the AD and DDD domains have been the subject of investigation. (See, e.g., Burns-Hamuro et al., 2005, Protein Sci 14:2982-92; Carr et al., 2001, J Biol Chem 276:17332-38; Alto et al., 2003, Proc Natl Acad Sci USA 100:4445-50; Hundsrucker et al., 2006, Biochem J 396:297-306; Stokka et al., 2006, Biochem J

400:493-99; Gold et al., 2006, Mol Cell 24:383-95; Kinderman et al., 2006, Mol Cell 24:397-408, the entire text of each of which is incorporated herein by reference.)

[0107] For example, Kinderman et al. (2006, Mol Cell 24:397-408) examined the crystal structure of the AD-DDD binding interaction and concluded that the human DDD sequence contained a number of conserved amino acid residues that were important in either dimer formation or AKAP binding, underlined in SEQ ID NO:9 below. (See Figure 1 of Kinderman et al., 2006, incorporated herein by reference.) The skilled artisan will realize that in designing sequence variants of the DDD sequence, one would desirably avoid changing any of the underlined residues, while conservative amino acid substitutions might be made for residues that are less critical for dimerization and AKAP binding.

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:9)

[0108] As discussed in more detail below, conservative amino acid substitutions have been characterized for each of the twenty common L-amino acids. Thus, based on the data of Kinderman (2006) and conservative amino acid substitutions, potential alternative DDD sequences based on SEQ ID NO:9 are shown in **Table 2**. In devising **Table 2**, only highly conservative amino acid substitutions were considered. For example, charged residues were only substituted for residues of the same charge, residues with small side chains were substituted with residues of similar size, hydroxyl side chains were only substituted with other hydroxyls, etc. Because of the unique effect of proline on amino acid secondary structure, no other residues were substituted for proline. A limited number of such potential alternative DDD moiety sequences are shown in SEQ ID NO:20 to SEQ ID NO:39 below. The skilled artisan will realize that an almost unlimited number of alternative species within the genus of DDD moieties can be constructed by standard techniques, for example using a commercial peptide synthesizer or well known site-directed mutagenesis techniques. The effect of the amino acid substitutions on AD moiety binding may also be readily determined by standard binding assays, for example as disclosed in Alto et al. (2003, Proc Natl Acad Sci USA 100:4445-50).

Table 2. Conservative Amino Acid Substitutions in DDD1 (SEQ ID NO:9). Consensus sequence disclosed as SEQ ID NO: 94.

S	**H**	**I**	**Q**	**I**	**P**	**P**	**G**	**L**	**T**	**E**	**L**	**L**	**Q**	**G**	**Y**	**T**	**V**	**E**	**V**	**L**	**R**
T	K R		N				A		S	D			N	A		S		D			K

Q	Q	P	P	D	L	V	E	F	A	V	E	Y	F	T	R	L	R	E	A	R	A
N	N			E			D		L I V		D			S	K		K	D	L I V	K	L I V

THIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:20)

SKIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:21)

SRIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:22)

SHINIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:23)

SHIQIPPALTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:24)

SHIQIPPGLSELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:25)

SHIQIPPGLTDLLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:26)

SHIQIPPGLTELLNGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:27)

SHIQIPPGLTELLQAYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:28)

SHIQIPPGLTELLQGYSVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:29)

SHIQIPPGLTELLQGYTVDVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:30)

SHIQIPPGLTELLQGYTVEVLKQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:31)

SHIQIPPGLTELLQGYTVEVLRNQPPDLVEFAVEYFTRLREARA (SEQ ID NO:32)

SHIQIPPGLTELLQGYTVEVLRQNPPDLVEFAVEYFTRLREARA (SEQ ID NO:33)

SHIQIPPGLTELLQGYTVEVLRQQPPELVEFAVEYFTRLREARA (SEQ ID NO:34)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVDFAVEYFTRLREARA (SEQ ID NO:35)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFLVEYFTRLREARA (SEQ ID NO:36)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFIVEYFTRLREARA (SEQ ID NO:37)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFVVEYFTRLREARA (SEQ ID NO:38)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVDYFTRLREARA (SEQ ID NO:39)

[0109] Alto et al. (2003, Proc Natl Acad Sci USA 100:4445-50) performed a bioinformatic analysis of the AD sequence of various AKAP proteins to design an RII selective AD sequence called AKAP-IS (SEQ ID NO:11), with a binding constant for DDD of 0.4 nM. The AKAP-IS sequence was designed as a peptide antagonist of AKAP binding to PKA. Residues

in the AKAP-IS sequence where substitutions tended to decrease binding to DDD are underlined in SEQ ID NO:11 below. The skilled artisan will realize that in designing sequence variants of the AD sequence, one would desirably avoid changing any of the underlined residues, while conservative amino acid substitutions might be made for residues that are less critical for DDD binding. **Table 3** shows potential conservative amino acid substitutions in the sequence of AKAP-IS (AD1, SEQ ID NO:19), similar to that shown for DDD1 (SEQ ID NO:16) in **Table 2** above.

[0110] A limited number of such potential alternative AD moiety sequences are shown in SEQ ID NO:40 to SEQ ID NO:57 below. Again, a very large number of species within the genus of possible AD moiety sequences could be made, tested and used by the skilled artisan, based on the data of Alto et al. (2003). It is noted that Figure 2 of Alto (2003) shows an even large number of potential amino acid substitutions that may be made, while retaining binding activity to DDD moieties, based on actual binding experiments.

AKAP-IS

QIEYLAKQIVDNAIQQA (SEQ ID NO:11)

Table 3. Conservative Amino Acid Substitutions in AD1 (SEQ ID NO:11). Consensus sequence disclosed as SEQ ID NO: 95.

Q	**I**	**E**	**Y**	**L**	**A**	**K**	**Q**	**I**	**V**	**D**	**N**	**A**	**I**	**Q**	**Q**	**A**
N	L V	D	F T S	I V		R	N			E	Q			N	N	L I V

NIEYLAKQIVDNAIQQA (SEQ ID NO:40)
QLEYLAKQIVDNAIQQA (SEQ ID NO:41)
QVEYLAKQIVDNAIQQA (SEQ ID NO:42)
QIDYLAKQIVDNAIQQA (SEQ ID NO:43)
QIEFLAKQIVDNAIQQA (SEQ ID NO:44)
QIETLAKQIVDNAIQQA (SEQ ID NO:45)
QIESLAKQIVDNAIQQA (SEQ ID NO:46)
QIEYIAKQIVDNAIQQA (SEQ ID NO:47)
QIEYVAKQIVDNAIQQA (SEQ ID NO:48)

QIEYLARQIVDNAIQQA (SEQ ID NO:49)

QIEYLAKNIVDNAIQQA (SEQ ID NO:50)

QIEYLAKQIVENAIQQA (SEQ ID NO:51)

QIEYLAKQIVDQAIQQA (SEQ ID NO:52)

QIEYLAKQIVDNAINQA (SEQ ID NO:53)

QIEYLAKQIVDNAIQNA (SEQ ID NO:54)

QIEYLAKQIVDNAIQQL (SEQ ID NO:55)

QIEYLAKQIVDNAIQQI (SEQ ID NO:56)

QIEYLAKQIVDNAIQQV (SEQ ID NO:57)

[0111] Gold et al. (2006, Mol Cell 24:383-95) utilized crystallography and peptide screening to develop a SuperAKAP-IS sequence (SEQ ID NO:58), exhibiting a five order of magnitude higher selectivity for the RII isoform of PKA compared with the RI isoform. Underlined residues indicate the positions of amino acid substitutions, relative to the AKAP-IS sequence, which increased binding to the DDD moiety of RIIα. In this sequence, the N-terminal Q residue is numbered as residue number 4 and the C-terminal A residue is residue number 20. Residues where substitutions could be made to affect the affinity for RIIα were residues 8, 11, 15, 16, 18, 19 and 20 (Gold et al., 2006). It is contemplated that in certain alternative embodiments, the SuperAKAP-IS sequence may be substituted for the AKAP-IS AD moiety sequence to prepare DNL® constructs. Other alternative sequences that might be substituted for the AKAP-IS AD sequence are shown in SEQ ID NO:59-61. Substitutions relative to the AKAP-IS sequence are underlined. It is anticipated that, as with the AD2 sequence shown in SEQ ID NO:12, the AD moiety may also include the additional N-terminal residues cysteine and glycine and C-terminal residues glycine and cysteine.

SuperAKAP-IS

QIEY<u>V</u>AKQIVD<u>Y</u>AI<u>H</u>QA (SEQ ID NO:58)

Alternative AKAP sequences

QIEY<u>K</u>AKQIVD<u>H</u>AI<u>H</u>QA (SEQ ID NO:59)

QIEY<u>H</u>AKQIVD<u>H</u>AI<u>H</u>QA (SEQ ID NO:60)

QIEY<u>V</u>AKQIVD<u>H</u>AI<u>H</u>QA (SEQ ID NO:61)

[0112] Figure 2 of Gold et al. disclosed additional DDD-binding sequences from a variety of AKAP proteins, shown below.

RII-Specific AKAPs

AKAP-KL

PLEYQAGLLVQNAIQQAI (SEQ ID NO:62)

AKAP79

LLIETASSLVKNAIQLSI (SEQ ID NO:63)

AKAP-Lbc

LIEEAASRIVDAVIEQVK (SEQ ID NO:64)

RI-Specific AKAPs

AKAPce

ALYQFADRFSELVISEAL (SEQ ID NO:65)

RIAD

LEQVANQLADQIIKEAT (SEQ ID NO:66)

PV38

FEELAWKIAKMIWSDVF (SEQ ID NO:67)

Dual-Specificity AKAPs

AKAP7

ELVRLSKRLVENAVLKAV (SEQ ID NO:68)

MAP2D

TAEEVSARIVQVVTAEAV (SEQ ID NO:69)

DAKAP1

QIKQAAFQLISQVILEAT (SEQ ID NO:70)

DAKAP2

LAWKIAKMIVSDVMQQ (SEQ ID NO:71)

[0113] Stokka et al. (2006, Biochem J 400:493-99) also developed peptide competitors of AKAP binding to PKA, shown in SEQ ID NO:72-74. The peptide antagonists were designated as Ht31 (SEQ ID NO:72), RIAD (SEQ ID NO:73) and PV-38 (SEQ ID NO:74). The Ht-31 peptide exhibited a greater affinity for the RII isoform of PKA, while the RIAD and PV-38 showed higher affinity for RI.

Ht31

DLIEEAASRIVDAVIEQVKAAGAY (SEQ ID NO:72)

RIAD

LEQYANQLADQIIKEATE (SEQ ID NO:73)

PV-38

FEELAWKIAKMIWSDVFQQC (SEQ ID NO:74)

[0114] Hundsrucker et al. (2006, Biochem J 396:297-306) developed still other peptide competitors for AKAP binding to PKA, with a binding constant as low as 0.4 nM to the DDD of the RII form of PKA. The sequences of various AKAP antagonistic peptides are provided in Table 1 of Hundsrucker et al., reproduced in **Table 4** below. AKAPIS represents a synthetic RII subunit-binding peptide. All other peptides are derived from the RII-binding domains of the indicated AKAPs.

Table 4. AKAP Peptide sequences

	Peptide Sequence
AKAPIS	QIEYLAKQIVDNAIQQA (SEQ ID NO:11)
AKAPIS-P	QIEYLAKQIPDNAIQQA (SEQ ID NO:75)
Ht31	KGADLIEEAASRIVDAVIEQVKAAG (SEQ ID NO:76)
Ht31-P	KGADLIEEAASRIPDAPIEQVKAAG (SEQ ID NO:77)
AKAP7δ-wt-pep	PEDAELVRLSKRLVENAVLKAVQQY (SEQ ID NO:78)
AKAP7δ-L304T-pep	PEDAELVRTSKRLVENAVLKAVQQY (SEQ ID NO:79)
AKAP7δ-L308D-pep	PEDAELVRLSKRDVENAVLKAVQQY (SEQ ID NO:80)
AKAP7δ-P-pep	PEDAELVRLSKRLPENAVLKAVQQY (SEQ ID NO:81)
AKAP7δ-PP-pep	PEDAELVRLSKRLPENAPLKAVQQY (SEQ ID NO:82)
AKAP7δ-L314E-pep	PEDAELVRLSKRLVENAVEKAVQQY (SEQ ID NO:83)
AKAP1-pep	EEGLDRNEEIKRAAFQIISQVISEA (SEQ ID NO:84)
AKAP2-pep	LVDDPLEYQAGLLVQNAIQQAIAEQ (SEQ ID NO:85)
AKAP5-pep	QYETLLIETASSLVKNAIQLSIEQL (SEQ ID NO:86)
AKAP9-pep	LEKQYQEQLEEEVAKVIVSMSIAFA (SEQ ID NO:87)
AKAP10-pep	NTDEAQEELAWKIAKMIVSDIMQQA (SEQ ID NO:88)
AKAP11-pep	VNLDKKAVLAEKIVAEAIEKAEREL (SEQ ID NO:89)
AKAP12-pep	NGILELETKSSKLVQNIIQTAVDQF (SEQ ID NO:90)
AKAP14-pep	TQDKNYEDELTQVALALVEDVINYA (SEQ ID NO:91)
Rab32-pep	ETSAKDNINIEEAARFLVEKILVNH (SEQ ID NO:92)

[0115] Residues that were highly conserved among the AD domains of different AKAP proteins are indicated below by underlining with reference to the AKAP IS sequence (SEQ ID NO:11). The residues are the same as observed by Alto et al. (2003), with the addition of the C-terminal alanine residue. (See FIG. 4 of Hundsrucker et al. (2006), incorporated herein by reference.) The sequences of peptide antagonists with particularly high affinities for the

RII DDD sequence were those of AKAP-IS, AKAP7δ-wt-pep, AKAP7δ-L304T-pep and AKAP7δ-L308D-pep.

AKAP-IS

QIEYLAKQIVDNAIQQA (SEQ ID NO:11)

[0116] Carr et al. (2001, J Biol Chem 276:17332-38) examined the degree of sequence homology between different AKAP-binding DDD sequences from human and non-human proteins and identified residues in the DDD sequences that appeared to be the most highly conserved among different DDD moieties. These are indicated below by underlining with reference to the human PKA RIIα DDD sequence of SEQ ID NO:9. Residues that were particularly conserved are further indicated by italics. The residues overlap with, but are not identical to those suggested by Kinderman et al. (2006) to be important for binding to AKAP proteins. The skilled artisan will realize that in designing sequence variants of DDD, it would be most preferred to avoid changing the most conserved residues (italicized), and it would be preferred to also avoid changing the conserved residues (underlined), while conservative amino acid substitutions may be considered for residues that are neither underlined nor italicized..

SHIQ*IP*P*GL*TE*LL*Q*G*Y*T*V*EVLR*Q*Q*P*P*DL*VE*FA*VE*YF*TR*L*REA*R*A (SEQ ID NO:9)

[0117] A modified set of conservative amino acid substitutions for the DDD1 (SEQ ID NO:9) sequence, based on the data of Carr et al. (2001) is shown in **Table 5**. Even with this reduced set of substituted sequences, there are over 65,000 possible alternative DDD moiety sequences that may be produced, tested and used by the skilled artisan without undue experimentation. The skilled artisan could readily derive such alternative DDD amino acid sequences as disclosed above for **Table 2** and **Table 3.**

Table 5. Conservative Amino Acid Substitutions in DDD1 (SEQ ID NO:9). Consensus sequence disclosed as SEQ ID NO: 96.

S	**H**	**I**	**Q**	***I***	***P***	**P**	***G***	***L***	**T**	**E**	***L***	***L***	**Q**	***G***	***Y***	***T***	**V**	***E***	***V***	***L***	***R***
T			N						S								I L A				

Q	*Q*	*P*	P	*D*	*L*	V	E	*F*	*A*	V	E	*Y*	*F*	T	R	*L*	R	E	A	*R*	A
N										I L A	D			S	K		K		L I V		L I V

[0118] The skilled artisan will realize that these and other amino acid substitutions in the DDD or AD amino acid sequences may be utilized to produce alternative species within the genus of AD or DDD moieties, using techniques that are standard in the field and only routine experimentation.

Alternative DNL® Structures

[0119] In certain alternative embodiments, DNL® constructs may be formed using alternatively constructed antibodies or antibody fragments, in which an AD moiety may be attached at the C-terminal end of the kappa light chain (C_k), instead of the C-terminal end of the Fc on the heavy chain. The alternatively formed DNL® constructs may be prepared as disclosed in Provisional U.S. Patent Application Serial Nos. 61/654,310, filed June 1, 2012, 61/662,086, filed June 20, 2012, 61/673,553, filed July 19, 2012, and 61/682,531, filed August 13, 2012, the entire text of each incorporated herein by reference. The light chain conjugated DNL® constructs exhibit enhanced Fc-effector function activity *in vitro* and improved pharmacokinetics, stability and anti-lymphoma activity *in vivo* (Rossi et al., 2013, Bioconjug Chem 24:63-71).

[0120] C_k-conjugated DNL® constructs may be prepared as disclosed in Provisional U.S. Patent Application Serial Nos. 61/654,310, 61/662,086, 61/673,553, and 61/682,531. Briefly, C_k-AD2-IgG, was generated by recombinant engineering, whereby the AD2 peptide was fused to the C-terminal end of the kappa light chain. Because the natural C-terminus of C_K is a cysteine residue, which forms a disulfide bridge to C_H1, a 16-amino acid residue "hinge" linker was used to space the AD2 from the C_K-V_H1 disulfide bridge. The mammalian expression vectors for C_k-AD2-IgG-veltuzumab and C_k-AD2-IgG-epratuzumab were constructed using the pdHL2 vector, which was used previously for expression of the homologous C_H3-AD2-IgG modules. A 2208-bp nucleotide sequence was synthesized comprising the pdHL2 vector sequence ranging from the *Bam HI* restriction site within the V_K/C_K intron to the *Xho I* restriction site 3' of the C_k intron, with the insertion of the coding sequence for the hinge linker (EFPKPSTPPGSSGGAP, SEQ ID NO:93) and AD2, in frame at the 3'end of the coding sequence for C_K. This synthetic sequence was inserted into the IgG-pdHL2 expression vectors for veltuzumab and epratuzumab via *Bam HI* and *Xho I*

restriction sites. Generation of production clones with SpESFX-10 were performed as described for the C_H3-AD2-IgG modules. C_k-AD2-IgG-veltuzumab and C_k-AD2-IgG-epratuzumab were produced by stably-transfected production clones in batch roller bottle culture, and purified from the supernatant fluid in a single step using MabSelect (GE Healthcare) Protein A affinity chromatography.

[0121] Following the same DNL® process described previously for 22-(20)-(20) (Rossi et al., 2009, *Blood* 113:6161-71), C_k-AD2-IgG-epratuzumab was conjugated with C_H1-DDD2-Fab-veltuzumab, a Fab-based module derived from veltuzumab, to generate the bsHexAb 22*-(20)-(20), where the 22* indicates the C_k-AD2 module of epratuzumab and each (20) symbolizes a stabilized dimer of veltuzumab Fab. The properties of 22*-(20)-(20) were compared with those of 22-(20)-(20), the homologous Fc-bsHexAb comprising C_H3-AD2-IgG-epratuzumab, which has similar composition and molecular size, but a different architecture.

[0122] Following the same DNL® process described previously for 20-2b (Rossi et al., 2009, *Blood* 114:3864-71), C_k-AD2-IgG-veltuzumab, was conjugated with IFNα2b-DDD2, a module of IFNα2b with a DDD2 peptide fused at its C-terminal end, to generate 20*-2b, which comprises veltuzumab with a dimeric IFNα2b fused to each light chain. The properties of 20*-2b were compared with those of 20-2b, which is the homologous Fc-IgG-IFNα.

[0123] Each of the bsHexAbs and IgG-IFNα were isolated from the DNL® reaction mixture by MabSelect affinity chromatography. The two C_k-derived prototypes, an anti-CD22/CD20 bispecific hexavalent antibody, comprising epratuzumab (anti-CD22) and four Fabs of veltuzumab (anti-CD20), and a CD20-targeting immunocytokine, comprising veltuzumab and four molecules of interferon-α2b, displayed enhanced Fc-effector functions *in vitro*, as well as improved pharmacokinetics, stability and anti-lymphoma activity *in vivo*, compared to their Fc-derived counterparts.

Amino Acid Substitutions

[0124] In alternative embodiments, the disclosed methods and compositions may involve production and use of proteins or peptides with one or more substituted amino acid residues. For example, the DDD and/or AD sequences used to make DNL® constructs may be modified as discussed above.

[0125] The skilled artisan will be aware that, in general, amino acid substitutions typically involve the replacement of an amino acid with another amino acid of relatively similar properties (i.e., conservative amino acid substitutions). The properties of the various amino

acids and effect of amino acid substitution on protein structure and function have been the subject of extensive study and knowledge in the art.

[0126] For example, the hydropathic index of amino acids may be considered (Kyte & Doolittle, 1982, J. Mol. Biol., 157:105-132). The relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5). In making conservative substitutions, the use of amino acids whose hydropathic indices are within ± 2 is preferred, within ± 1 are more preferred, and within ± 0.5 are even more preferred.

[0127] Amino acid substitution may also take into account the hydrophilicity of the amino acid residue (e.g., U.S. Pat. No. 4,554,101). Hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0); glutamate (+3.0); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 .+-.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). Replacement of amino acids with others of similar hydrophilicity is preferred.

[0128] Other considerations include the size of the amino acid side chain. For example, it would generally not be preferred to replace an amino acid with a compact side chain, such as glycine or serine, with an amino acid with a bulky side chain, e.g., tryptophan or tyrosine. The effect of various amino acid residues on protein secondary structure is also a consideration. Through empirical study, the effect of different amino acid residues on the tendency of protein domains to adopt an alpha-helical, beta-sheet or reverse turn secondary structure has been determined and is known in the art (see, e.g., Chou & Fasman, 1974, Biochemistry, 13:222-245; 1978, Ann. Rev. Biochem., 47: 251-276; 1979, Biophys. J., 26:367-384).

[0129] Based on such considerations and extensive empirical study, tables of conservative amino acid substitutions have been constructed and are known in the art. For example: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine;

and valine, leucine and isoleucine. Alternatively: Ala (A) leu, ile, val; Arg (R) gln, asn, lys; Asn (N) his, asp, lys, arg, gln; Asp (D) asn, glu; Cys (C) ala, ser; Gln (Q) glu, asn; Glu (E) gln, asp; Gly (G) ala; His (H) asn, gln, lys, arg; Ile (I) val, met, ala, phe, leu; Leu (L) val, met, ala, phe, ile; Lys (K) gln, asn, arg; Met (M) phe, ile, leu; Phe (F) leu, val, ile, ala, tyr; Pro (P) ala; Ser (S), thr; Thr (T) ser; Trp (W) phe, tyr; Tyr (Y) trp, phe, thr, ser; Val (V) ile, leu, met, phe, ala.

[0130] Other considerations for amino acid substitutions include whether or not the residue is located in the interior of a protein or is solvent exposed. For interior residues, conservative substitutions would include: Asp and Asn; Ser and Thr; Ser and Ala; Thr and Ala; Ala and Gly; Ile and Val; Val and Leu; Leu and Ile; Leu and Met; Phe and Tyr; Tyr and Trp. (See, e.g., PROWL website at rockefeller.edu) For solvent exposed residues, conservative substitutions would include: Asp and Asn; Asp and Glu; Glu and Gln; Glu and Ala; Gly and Asn; Ala and Pro; Ala and Gly; Ala and Ser; Ala and Lys; Ser and Thr; Lys and Arg; Val and Leu; Leu and Ile; Ile and Val; Phe and Tyr. (Id.) Various matrices have been constructed to assist in selection of amino acid substitutions, such as the PAM250 scoring matrix, Dayhoff matrix, Grantham matrix, McLachlan matrix, Doolittle matrix, Henikoff matrix, Miyata matrix, Fitch matrix, Jones matrix, Rao matrix, Levin matrix and Risler matrix (*Idem.*)

[0131] In determining amino acid substitutions, one may also consider the existence of intermolecular or intramolecular bonds, such as formation of ionic bonds (salt bridges) between positively charged residues (e.g., His, Arg, Lys) and negatively charged residues (e.g., Asp, Glu) or disulfide bonds between nearby cysteine residues.

[0132] Methods of substituting any amino acid for any other amino acid in an encoded protein sequence are well known and a matter of routine experimentation for the skilled artisan, for example by the technique of site-directed mutagenesis or by synthesis and assembly of oligonucleotides encoding an amino acid substitution and splicing into an expression vector construct.

Pre-Targeting

[0133] Bispecific or multispecific antibodies may be of use in pretargeting techniques. In this case, one or more diagnostic and/or therapeutic agents may be conjugated to a targetable construct that comprises one or more haptens. The hapten is recognized by at least one arm of a bispecific or multispecific antibody that also binds to a tumor-associated antigen or other disease-associated antigen. In this case, the therapeutic agent binds indirectly to the

antibodies, via the binding of the targetable construct. This process is referred to as pretargeting.

[0134] Pre-targeting is a multistep process originally developed to resolve the slow blood clearance of directly targeting antibodies, which contributes to undesirable toxicity to normal tissues such as bone marrow. With pre-targeting, a therapeutic agent is attached to a small delivery molecule (targetable construct) that is cleared within minutes from the blood. A pre-targeting bispecific or multispecific antibody, which has binding sites for the targetable construct as well as a target antigen, is administered first, free antibody is allowed to clear from circulation and then the targetable construct is administered.

[0135] Pre-targeting methods are disclosed, for example, in Goodwin et al., U.S. Pat. No. 4,863,713; Goodwin et al., J. Nucl. Med. 29:226, 1988; Hnatowich et al., J. Nucl. Med. 28:1294, 1987; Oehr et al., J. Nucl. Med. 29:728, 1988; Klibanov et al., J. Nucl. Med. 29:1951, 1988; Sinitsyn et al., J. Nucl. Med. 30:66, 1989; Kalofonos et al., J. Nucl. Med. 31:1791, 1990; Schechter et al., Int. J. Cancer 48:167, 1991; Paganelli et al., Cancer Res. 51:5960, 1991; Paganelli et al., Nucl. Med. Commun. 12:211, 1991; U.S. Pat. No. 5,256,395; Stickney et al., Cancer Res. 51:6650, 1991; Yuan et al., Cancer Res. 51:3119, 1991; U.S. Pat. Nos. 6,077,499; 7,011,812; 7,300,644; 7,074,405; 6,962,702; 7,387,772; 7,052,872; 7,138,103; 6,090,381; 6,472,511; 6,962,702; and 6,962,702, each incorporated herein by reference.

[0136] A pre-targeting method of diagnosing or treating a disease or disorder in a subject may be provided by: (1) administering to the subject a bispecific antibody or antibody fragment; (2) optionally administering to the subject a clearing composition, and allowing the composition to clear the antibody from circulation; and (3) administering to the subject the targetable construct, containing one or more chelated or chemically bound therapeutic or diagnostic agents.

Targetable Constructs

[0137] In certain embodiments, targetable construct peptides labeled with one or more therapeutic or diagnostic agents for use in pre-targeting may be selected to bind to a bispecific antibody with one or more binding sites for a targetable construct peptide and one or more binding sites for a target antigen associated with a disease or condition. Bispecific antibodies may be used in a pretargeting technique wherein the antibody may be administered first to a subject. Sufficient time may be allowed for the bispecific antibody to bind to a target antigen and for unbound antibody to clear from circulation. Then a targetable construct, such

as a labeled peptide, may be administered to the subject and allowed to bind to the bispecific antibody and localize at the diseased cell or tissue.

[0138] Such targetable constructs can be of diverse structure and are selected not only for the availability of an antibody or fragment that binds with high affinity to the targetable construct, but also for rapid *in vivo* clearance when used within the pre-targeting method and bispecific antibodies (bsAb) or multispecific antibodies. Hydrophobic agents are best at eliciting strong immune responses, whereas hydrophilic agents are preferred for rapid *in vivo* clearance. Thus, a balance between hydrophobic and hydrophilic character is established. This may be accomplished, in part, by using hydrophilic chelating agents to offset the inherent hydrophobicity of many organic moieties. Also, sub-units of the targetable construct may be chosen which have opposite solution properties, for example, peptides, which contain amino acids, some of which are hydrophobic and some of which are hydrophilic.

[0139] Peptides having as few as two amino acid residues, preferably two to ten residues, may be used and may also be coupled to other moieties, such as chelating agents. The linker should be a low molecular weight conjugate, preferably having a molecular weight of less than 50,000 daltons, and advantageously less than about 20,000 daltons, 10,000 daltons or 5,000 daltons. More usually, the targetable construct peptide will have four or more residues and one or more haptens for binding, e.g., to a bispecific antibody. Exemplary haptens may include In-DTPA (indium-diethylene triamine pentaacetic acid) or HSG (histamine succinyl glycine). The targetable construct may also comprise one or more chelating moieties, such as DOTA (1,4,7,10-tetraazacyclododecane1,4,7,10-tetraacetic acid), NOTA (1,4,7-triaza-cyclononane-1,4,7-triacetic acid), TETA (*p*-bromoacetamido-benzyl-tetraethylaminetetraacetic acid), NETA ([2-(4,7-biscarboxymethyl[1,4,7]triazacyclononan-1-yl-ethyl]-2-carbonylmethyl-amino]acetic acid) or other known chelating moieties. Chelating moieties may be used, for example, to bind to a therapeutic and or diagnostic radionuclide, paramagnetic ion or contrast agent.

[0140] The targetable construct may also comprise unnatural amino acids, e.g., D-amino acids, in the backbone structure to increase the stability of the peptide *in vivo*. In alternative embodiments, other backbone structures such as those constructed from non-natural amino acids or peptoids may be used.

[0141] The peptides used as targetable constructs are conveniently synthesized on an automated peptide synthesizer using a solid-phase support and standard techniques of repetitive orthogonal deprotection and coupling. Free amino groups in the peptide, that are to

be used later for conjugation of chelating moieties or other agents, are advantageously blocked with standard protecting groups such as a Boc group, while N-terminal residues may be acetylated to increase serum stability. Such protecting groups are well known to the skilled artisan. See Greene and Wuts Protective Groups in Organic Synthesis, 1999 (John Wiley and Sons, N.Y.). When the peptides are prepared for later use within the bispecific antibody system, they are advantageously cleaved from the resins to generate the corresponding C-terminal amides, in order to inhibit *in vivo* carboxypeptidase activity.

[0142] Where pretargeting with bispecific antibodies is used, the antibody will contain a first binding site for an antigen produced by or associated with a target tissue and a second binding site for a hapten on the targetable construct. Exemplary haptens include, but are not limited to, HSG and In-DTPA. Antibodies raised to the HSG hapten are known (e.g. 679 antibody) and can be easily incorporated into the appropriate bispecific antibody (see, e.g., U.S. Patent Nos. 6,962,702; 7,138,103 and 7,300,644, incorporated herein by reference with respect to the Examples sections). However, other haptens and antibodies that bind to them are known in the art and may be used, such as In-DTPA and the 734 antibody (e.g., U.S. Patent No.7,534,431, the Examples section incorporated herein by reference).

Immunoconjugates

[0143] Various embodiments may involve use of immunoconjugates, comprising an anti-Trop-2 antibody or antigen-binding fragment thereof attached to one or more diagnostic or therapeutic agents. In some embodiments, a drug or other agent may be attached to an antibody or fragment thereof via a carrier moiety. Carrier moieties may be attached, for example to reduced SH groups and/or to carbohydrate side chains. A carrier moiety can be attached at the hinge region of a reduced antibody component via disulfide bond formation. Alternatively, such agents can be attached using a heterobifunctional cross-linker, such as *N*-succinyl 3-(2-pyridyldithio)propionate (SPDP). Yu *et al.*, *Int. J. Cancer 56*: 244 (1994). General techniques for such conjugation are well-known in the art. See, for example, Wong, CHEMISTRY OF PROTEIN CONJUGATION AND CROSS-LINKING (CRC Press 1991); Upeslacis *et al.*, "Modification of Antibodies by Chemical Methods," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, Birch *et al.* (eds.), pages 187-230 (Wiley-Liss, Inc. 1995); Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter *et al.* (eds.), pages 60-84 (Cambridge University

Press 1995). Alternatively, the carrier moiety can be conjugated via a carbohydrate moiety in the Fc region of the antibody.

[0144] Methods for conjugating functional groups to antibodies via an antibody carbohydrate moiety are well-known to those of skill in the art. See, for example, Shih *et al., Int. J. Cancer 41*: 832 (1988); Shih *et al., Int. J. Cancer 46*: 1101 (1990); and Shih *et al.*, U.S. Patent No. 5,057,313, the Examples section of which is incorporated herein by reference. The general method involves reacting an antibody having an oxidized carbohydrate portion with a carrier polymer that has at least one free amine function. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate.

[0145] The Fc region may be absent if the antibody component is an antibody fragment. However, it is possible to introduce a carbohydrate moiety into the light chain variable region of a full length antibody or antibody fragment. See, for example, Leung *et al., J. Immunol. 154*: 5919 (1995); U.S. Patent Nos. 5,443,953 and 6,254,868, the Examples section of which is incorporated herein by reference. The engineered carbohydrate moiety is used to attach the therapeutic or diagnostic agent.

[0146] An alternative method for attaching carrier moieties to a targeting molecule involves use of click chemistry reactions. The click chemistry approach was originally conceived as a method to rapidly generate complex substances by joining small subunits together in a modular fashion. (See, e.g., Kolb et al., 2004, Angew Chem Int Ed 40:3004-31; Evans, 2007, Aust J Chem 60:384-95.) Various forms of click chemistry reaction are known in the art, such as the Huisgen 1,3-dipolar cycloaddition copper catalyzed reaction (Tornoe et al., 2002, J Organic Chem 67:3057-64), which is often referred to as the "click reaction." Other alternatives include cycloaddition reactions such as the Diels-Alder, nucleophilic substitution reactions (especially to small strained rings like epoxy and aziridine compounds), carbonyl chemistry formation of urea compounds and reactions involving carbon-carbon double bonds, such as alkynes in thiol-yne reactions.

[0147] The azide alkyne Huisgen cycloaddition reaction uses a copper catalyst in the presence of a reducing agent to catalyze the reaction of a terminal alkyne group attached to a first molecule. In the presence of a second molecule comprising an azide moiety, the azide reacts with the activated alkyne to form a 1,4-disubstituted 1,2,3-triazole. The copper catalyzed reaction occurs at room temperature and is sufficiently specific that purification of the reaction product is often not required. (Rostovstev et al., 2002, Angew Chem Int Ed

41:2596; Tornoe et al., 2002, J Org Chem 67:3057.) The azide and alkyne functional groups are largely inert towards biomolecules in aqueous medium, allowing the reaction to occur in complex solutions. The triazole formed is chemically stable and is not subject to enzymatic cleavage, making the click chemistry product highly stable in biological systems. Although the copper catalyst is toxic to living cells, the copper-based click chemistry reaction may be used *in vitro* for immunoconjugate formation.

[0148] A copper-free click reaction has been proposed for covalent modification of biomolecules. (See, e.g., Agard et al., 2004, J Am Chem Soc 126:15046-47.) The copper-free reaction uses ring strain in place of the copper catalyst to promote a [3 + 2] azide-alkyne cycloaddition reaction (*Id.*) For example, cyclooctyne is an 8-carbon ring structure comprising an internal alkyne bond. The closed ring structure induces a substantial bond angle deformation of the acetylene, which is highly reactive with azide groups to form a triazole. Thus, cyclooctyne derivatives may be used for copper-free click reactions (*Id.*)

[0149] Another type of copper-free click reaction was reported by Ning et al. (2010, Angew Chem Int Ed 49:3065-68), involving strain-promoted alkyne-nitrone cycloaddition. To address the slow rate of the original cyclooctyne reaction, electron-withdrawing groups are attached adjacent to the triple bond (*Id.*) Examples of such substituted cyclooctynes include difluorinated cyclooctynes, 4-dibenzocyclooctynol and azacyclooctyne (*Id.*) An alternative copper-free reaction involved strain-promoted alkyne-nitrone cycloaddition to give N-alkylated isoxazolines (*Id.*) The reaction was reported to have exceptionally fast reaction kinetics and was used in a one-pot three-step protocol for site-specific modification of peptides and proteins (*Id.*) Nitrones were prepared by the condensation of appropriate aldehydes with *N*-methylhydroxylamine and the cycloaddition reaction took place in a mixture of acetonitrile and water (*Id.*) These and other known click chemistry reactions may be used to attach carrier moieties to antibodies *in vitro*.

[0150] Agard et al. (2004, J Am Chem Soc 126:15046-47) demonstrated that a recombinant glycoprotein expressed in CHO cells in the presence of peracetylated *N*-azidoacetylmannosamine resulted in the bioincorporation of the corresponding *N*-azidoacetyl sialic acid in the carbohydrates of the glycoprotein. The azido-derivatized glycoprotein reacted specifically with a biotinylated cyclooctyne to form a biotinylated glycoprotein, while control glycoprotein without the azido moiety remained unlabeled (*Id.*) Laughlin et al. (2008, Science 320:664-667) used a similar technique to metabolically label cell-surface glycans in zebrafish embryos incubated with peracetylated *N*-azidoacetylgalactosamine. The azido-

derivatized glycans reacted with difluorinated cyclooctyne (DIFO) reagents to allow visualization of glycans *in vivo*.

[0151] The Diels-Alder reaction has also been used for *in vivo* labeling of molecules. Rossin et al. (2010, Angew Chem Int Ed 49:3375-78) reported a 52% yield *in vivo* between a tumor-localized anti-TAG72 (CC49) antibody carrying a *trans*-cyclooctene (TCO) reactive moiety and an ^{111}In-labeled tetrazine DOTA derivative. The TCO-labeled CC49 antibody was administered to mice bearing colon cancer xenografts, followed 1 day later by injection of ^{111}In-labeled tetrazine probe (*Id.*) The reaction of radiolabeled probe with tumor localized antibody resulted in pronounced radioactivity localization in the tumor, as demonstrated by SPECT imaging of live mice three hours after injection of radiolabeled probe, with a tumor-to-muscle ratio of 13:1 (*Id.*) The results confirmed the *in vivo* chemical reaction of the TCO and tetrazine-labeled molecules.

[0152] Antibody labeling techniques using biological incorporation of labeling moieties are further disclosed in U.S. Patent No. 6,953,675 (the Examples section of which is incorporated herein by reference). Such "landscaped" antibodies were prepared to have reactive ketone groups on glycosylated sites. The method involved expressing cells transfected with an expression vector encoding an antibody with one or more N-glycosylation sites in the CH1 or Vκ domain in culture medium comprising a ketone derivative of a saccharide or saccharide precursor. Ketone-derivatized saccharides or precursors included N-levulinoyl mannosamine and N-levulinoyl fucose. The landscaped antibodies were subsequently reacted with agents comprising a ketone-reactive moiety, such as hydrazide, hydrazine, hydroxylamino or thiosemicarbazide groups, to form a labeled targeting molecule. Exemplary agents attached to the landscaped antibodies included chelating agents like DTPA, large drug molecules such as doxorubicin-dextran, and acyl-hydrazide containing peptides. The landscaping technique is not limited to producing antibodies comprising ketone moieties, but may be used instead to introduce a click chemistry reactive group, such as a nitrone, an azide or a cyclooctyne, onto an antibody or other biological molecule.

[0153] Modifications of click chemistry reactions are suitable for use *in vitro* or *in vivo*. Reactive targeting molecule may be formed either by either chemical conjugation or by biological incorporation. The targeting molecule, such as an antibody or antibody fragment, may be activated with an azido moiety, a substituted cyclooctyne or alkyne group, or a nitrone moiety. Where the targeting molecule comprises an azido or nitrone group, the corresponding targetable construct will comprise a substituted cyclooctyne or alkyne group,

and vice versa. Such activated molecules may be made by metabolic incorporation in living cells, as discussed above.

[0154] Alternatively, methods of chemical conjugation of such moieties to biomolecules are well known in the art, and any such known method may be utilized. General methods of immunoconjugate formation are disclosed, for example, in U.S. Patent Nos. 4,699,784; 4,824,659; 5,525,338; 5,677,427; 5,697,902; 5,716,595; 6,071,490; 6,187,284; 6,306,393; 6,548,275; 6,653,104; 6,962,702; 7,033,572; 7,147,856; and 7,259,240, the Examples section of each incorporated herein by reference.

Diagnostic Agents

[0155] Diagnostic agents may comprise any detectable agent that may be used to label a detection antibody, or to directly label a CTC, and are preferably selected from the group consisting of a radionuclide, a radiological contrast agent, a paramagnetic ion, a metal, a fluorescent label, a chemiluminescent label, an ultrasound contrast agent and a photoactive agent. Such diagnostic agents are well known and any such known diagnostic agent may be used. Non-limiting examples of diagnostic agents may include a radionuclide such as ^{110}In, ^{111}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{90}Y, ^{89}Zr, ^{94m}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}$Gd, ^{32}P, ^{11}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, ^{83}Sr, or other gamma-, beta-, or positron-emitters. Paramagnetic ions of use may include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III). Metal contrast agents may include lanthanum (III), gold (III), lead (II) or bismuth (III). Ultrasound contrast agents may comprise liposomes, such as gas filled liposomes. Radiopaque diagnostic agents may be selected from compounds, barium compounds, gallium compounds, and thallium compounds.

[0156] In certain embodiments, the fluorescent probe may be a DYLIGHT® dye (Thermo Fisher Scientific, Rockford, IL). The DYLIGHT® dye series are highly polar (hydrophilic), compatible with aqueous buffers, photostable and exhibit high fluorescence intensity. They remain highly fluorescent over a wide pH range and are preferred for various applications. However, the skilled artisan will realize that a variety of fluorescent dyes are known and/or are commercially available and may be utilized. Other fluorescent agents include, but are not limited to, dansyl chloride, rhodamine isothiocyanate, Alexa 350, Alexa 430, AMCA, aminoacridine, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, 5-carboxy-4',5'-dichloro-2',7'-dimethoxy fluorescein, 5-

carboxy-2',4',5',7'-tetrachlorofluorescein, 5-carboxyfluorescein, 5-carboxyrhodamine, 6-carboxyrhodamine, 6-carboxytetramethyl amino, Cascade Blue, Cy2, Cy3, Cy5,6-FAM, dansyl chloride, fluorescein, HEX, 6-JOE, NBD (7-nitrobenz-2-oxa-1,3-diazole), Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, phthalic acid, terephthalic acid, isophthalic acid, cresyl fast violet, cresyl blue violet, brilliant cresyl blue, para-aminobenzoic acid, erythrosine, phthalocyanines, azomethines, cyanines, xanthines, succinylfluoresceins, rare earth metal cryptates, europium trisbipyridine diamine, a europium cryptate or chelate, diamine, dicyanins, La Jolla blue dye, allopycocyanin, allococyanin B, phycocyanin C, phycocyanin R, thiamine, phycoerythrocyanin, phycoerythrin R, REG, Rhodamine Green, rhodamine isothiocyanate, Rhodamine Red, ROX, TAMRA, TET, TRIT (tetramethyl rhodamine isothiol), Tetramethylrhodamine, and Texas Red. (See, e.g., U.S. Pat. Nos. 5,800,992; 6,319,668.) These and other luminescent labels may be obtained from commercial sources such as Molecular Probes (Eugene, Oreg.), and EMD Biosciences (San Diego, Calif.).

[0157] Chemiluminescent labels of use may include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt or an oxalate ester.

Therapeutic Agents

[0158] A wide variety of therapeutic reagents can be administered concurrently or sequentially with an anti-Trop-2 or other anti-TAA antibody. Alternatively, such agents may be conjugated to antibodies, for example, drugs, toxins, oligonucleotides, immunomodulators, hormones, hormone antagonists, enzymes, enzyme inhibitors, radionuclides, angiogenesis inhibitors, etc. Therapeutic agents include, for example, cytotoxic drugs such as vinca alkaloids, anthracyclines such as doxorubicin, 2-PDox or pro-2-PDox, gemcitabine, epipodophyllotoxins, taxanes, antimetabolites, alkylating agents, antibiotics, SN-38, COX-2 inhibitors, antimitotics, anti-angiogenic and pro-apoptotic agents, particularly doxorubicin, methotrexate, taxol, CPT-11, camptothecans, proteosome inhibitors, mTOR inhibitors, HDAC inhibitors, tyrosine kinase inhibitors, and others. Other useful anti-cancer cytotoxic drugs include nitrogen mustards, alkyl sulfonates, nitrosoureas, triazenes, folic acid analogs, COX-2 inhibitors, antimetabolites, pyrimidine analogs, purine analogs, platinum coordination complexes, mTOR inhibitors, tyrosine kinase inhibitors, proteosome inhibitors, HDAC inhibitors, camptothecins, hormones, and the like. Suitable cytotoxic agents are described in REMINGTON'S PHARMACEUTICAL SCIENCES, 19th Ed. (Mack Publishing Co. 1995), and in GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS OF

THERAPEUTICS, 7th Ed. (MacMillan Publishing Co. 1985), as well as revised editions of these publications. Other suitable cytotoxic agents, such as experimental drugs, are known to those of skill in the art. In a preferred embodiment, conjugates of camptothecins and related compounds, such as SN-38, may be conjugated to anti-Trop-2 or other anti-TAA antibodies. In another preferred embodiment, gemcitabine is administered to the subject in conjunction with SN-38-hRS7 and/or ^{90}Y-hPAM4.

[0159] A toxin can be of animal, plant or microbial origin. Toxins of use include ricin, abrin, ribonuclease (RNase), DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, onconase, gelonin, diphtheria toxin, *Pseudomonas* exotoxin, and *Pseudomonas* endotoxin. See, for example, Pastan et al., Cell 47:641 (1986), Goldenberg, CA--A Cancer Journal for Clinicians 44:43 (1994), Sharkey and Goldenberg, CA--A Cancer Journal for Clinicians 56:226 (2006). Additional toxins suitable for use are known to those of skill in the art and are disclosed in U.S. Pat. No. 6,077,499, the Examples section of which is incorporated herein by reference.

[0160] As used herein, the term "immunomodulator" includes a cytokine, a lymphokine, a monokine, a stem cell growth factor, a lymphotoxin, a hematopoietic factor, a colony stimulating factor (CSF), an interferon (IFN), parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), hepatic growth factor, prostaglandin, fibroblast growth factor, prolactin, placental lactogen, OB protein, a transforming growth factor (TGF), TGF-α, TGF-β, insulin-like growth factor (ILGF), erythropoietin, thrombopoietin, tumor necrosis factor (TNF), TNF- α, TNF-β, a mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, integrin, interleukin (IL), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon- α, interferon- β, interferon-γ, interferon-λ, S1 factor, IL-1, IL-1cc, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18 IL-21 and IL-25, LIF, kit-ligand, FLT-3, angiostatin, thrombospondin, endostatin, lymphotoxin, and the like.

[0161] Particularly useful therapeutic radionuclides include, but are not limited to ^{111}In, ^{177}Lu, ^{212}Bi, ^{213}Bi, ^{211}At, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{32}P, ^{33}P, ^{47}Sc, ^{111}Ag, ^{67}Ga, ^{142}Pr, ^{153}Sm, ^{161}Tb, ^{166}Dy, ^{166}Ho, ^{186}Re, ^{188}Re, ^{189}Re, ^{212}Pb, ^{223}Ra, ^{225}Ac, ^{59}Fe, ^{75}Se, ^{77}As, ^{89}Sr, ^{99}Mo, ^{105}Rh, ^{109}Pd, ^{143}Pr, ^{149}Pm, ^{169}Er, ^{194}Ir, ^{198}Au, ^{199}Au, ^{227}Th, and ^{211}Pb. The therapeutic radionuclide preferably has a decay energy in the range of 20 to 6,000 keV, preferably in the

ranges 60 to 200 keV for an Auger emitter, 100-2,500 keV for a beta emitter, and 4,000-6,000 keV for an alpha emitter. Maximum decay energies of useful beta-particle-emitting nuclides are preferably 20-5,000 keV, more preferably 100-4,000 keV, and most preferably 500-2,500 keV. Also preferred are radionuclides that substantially decay with Auger-emitting particles. For example, Co-58, Ga-67, Br-80m, Tc-99m, Rh-103m, Pt-109, In-111, Sb-119, I-125, Ho-161, Os-189m and Ir-192. Decay energies of useful beta-particle-emitting nuclides are preferably <1,000 keV, more preferably <100 keV, and most preferably <70 keV. Also preferred are radionuclides that substantially decay with generation of alpha-particles. Such radionuclides include, but are not limited to: Dy-152, At-211, Bi-212, Ra-223, Rn-219, Po-215, Bi-211, Ac-225, Fr-221, At-217, Bi-213, Fm-255 and Th-227. Decay energies of useful alpha-particle-emitting radionuclides are preferably 2,000-10,000 keV, more preferably 3,000-8,000 keV, and most preferably 4,000-7,000 keV.

[0162] For example, ^{90}Y, which emits an energetic beta particle, can be coupled to an antibody, antibody fragment or fusion protein, using diethylenetriaminepentaacetic acid (DTPA), or more preferably using DOTA. Methods of conjugating ^{90}Y to antibodies or targetable constructs are known in the art and any such known methods may be used. (See, e.g., U.S. Patent No. 7,259,249, the Examples section of which is incorporated herein by reference. See also Lindén et al., Clin Cancer Res. 11:5215-22, 2005; Sharkey et al., J Nucl Med. 46:620-33, 2005; Sharkey et al., J Nucl Med. 44:2000-18, 2003.)

[0163] Additional potential therapeutic radioisotopes include ^{11}C, ^{13}N, ^{15}O, ^{75}Br, ^{198}Au, ^{224}Ac, ^{126}I, ^{133}I, ^{77}Br, ^{113m}In, ^{95}Ru, ^{97}Ru, ^{103}Ru, ^{105}Ru, ^{107}Hg, ^{203}Hg, ^{121m}Te, ^{122m}Te, ^{125m}Te, ^{165}Tm, ^{167}Tm, ^{168}Tm, ^{197}Pt, ^{109}Pd, ^{105}Rh, ^{142}Pr, ^{143}Pr, ^{161}Tb, ^{166}Ho, ^{199}Au, ^{57}Co, ^{58}Co, ^{51}Cr, ^{59}Fe, ^{75}Se, ^{201}Tl, ^{225}Ac, ^{76}Br, ^{169}Yb, and the like.

[0164] In another embodiment, a radiosensitizer can be used in combination with a naked or conjugated antibody or antibody fragment. For example, the radiosensitizer can be used in combination with a radiolabeled antibody or antibody fragment. The addition of the radiosensitizer can result in enhanced efficacy when compared to treatment with the radiolabeled antibody or antibody fragment alone. Radiosensitizers are described in D. M. Goldenberg (ed.), CANCER THERAPY WITH RADIOLABELED ANTIBODIES, CRC Press (1995). Other typical radionsensitizers of interest for use with this technology include gemcitabine, 5-fluorouracil, and cisplatin, and have been used in combination with external irradiation in the therapy of diverse cancers.

[0165] Antibodies or fragments thereof that have a boron addend-loaded carrier for thermal neutron activation therapy will normally be affected in similar ways. However, it will be advantageous to wait until non-targeted immunoconjugate clears before neutron irradiation is performed. Clearance can be accelerated using an anti-idiotypic antibody that binds to the anti-cancer antibody. See U.S. Pat. No. 4,624,846 for a description of this general principle. For example, boron addends such as carboranes, can be attached to antibodies. Carboranes can be prepared with carboxyl functions on pendant side chains, as is well-known in the art. Attachment of carboranes to a carrier, such as aminodextran, can be achieved by activation of the carboxyl groups of the carboranes and condensation with amines on the carrier. The intermediate conjugate is then conjugated to the antibody. After administration of the antibody conjugate, a boron addend is activated by thermal neutron irradiation and converted to radioactive atoms which decay by alpha-emission to produce highly toxic, short-range effects.

Formulation and Administration

[0166] Where therapeutic antibodies are to be administered *in vivo*, suitable routes of administration may include, without limitation, oral, parenteral, rectal, transmucosal, intestinal administration, intramedullary, intrathecal, direct intraventricular, intravenous, intravitreal, intracavitary, intraperitoneal, or intratumoral injections. The preferred routes of administration are parenteral, more preferably intravenous. Alternatively, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a solid or hematological tumor.

[0167] Antibodies can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the antibody is combined in a mixture with a pharmaceutically suitable excipient. Sterile phosphate-buffered saline is one example of a pharmaceutically suitable excipient. Other suitable excipients are well-known to those in the art. See, for example, Ansel *et al.*, PHARMACEUTICAL DOSAGE FORMS AND DRUG DELIVERY SYSTEMS, 5th Edition (Lea & Febiger 1990), and Gennaro (ed.), REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition (Mack Publishing Company 1990), and revised editions thereof.

[0168] In a preferred embodiment, the antibody is formulated in Good's biological buffer (pH 6-7), using a buffer selected from the group consisting of N-(2-acetamido)-2-aminoethanesulfonic acid (ACES); N-(2-acetamido)iminodiacetic acid (ADA); N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES); 4-(2-hydroxyethyl)piperazine-1-

ethanesulfonic acid (HEPES); 2-(N-morpholino)ethanesulfonic acid (MES); 3-(N-morpholino)propanesulfonic acid (MOPS); 3-(N-morpholinyl)-2-hydroxypropanesulfonic acid (MOPSO); and piperazine-N,N'-bis(2-ethanesulfonic acid) [Pipes]. More preferred buffers are MES or MOPS, preferably in the concentration range of 20 to 100 mM, more preferably about 25 mM. Most preferred is 25 mM MES, pH 6.5. The formulation may further comprise 25 mM trehalose and 0.01% v/v polysorbate 80 as excipients, with the final buffer concentration modified to 22.25 mM as a result of added excipients. The preferred method of storage is as a lyophilized formulation of the conjugates, stored in the temperature range of -20 °C to 2 °C, with the most preferred storage at 2 °C to 8 °C.

[0169] The antibody can be formulated for intravenous administration via, for example, bolus injection, slow infusion or continuous infusion. Preferably, the antibody of the present invention is infused over a period of less than about 4 hours, and more preferably, over a period of less than about 3 hours. For example, the first 25-50 mg could be infused within 30 minutes, preferably even 15 min, and the remainder infused over the next 2-3 hrs. Formulations for injection can be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

[0170] Additional pharmaceutical methods may be employed to control the duration of action of the therapeutic conjugate. Control release preparations can be prepared through the use of polymers to complex or adsorb the antibody. For example, biocompatible polymers include matrices of poly(ethylene-co-vinyl acetate) and matrices of a polyanhydride copolymer of a stearic acid dimer and sebacic acid. Sherwood *et al.*, *Bio/Technology* 10: 1446 (1992). The rate of release of an antibody from such a matrix depends upon the molecular weight of the antibody , the amount of antibody within the matrix, and the size of dispersed particles. Saltzman *et al.*, *Biophys. J.* 55: 163 (1989); Sherwood *et al.*, *supra*. Other solid dosage forms are described in Ansel *et al.*, PHARMACEUTICAL DOSAGE FORMS AND DRUG DELIVERY SYSTEMS, 5th Edition (Lea & Febiger 1990), and Gennaro (ed.), REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition (Mack Publishing Company 1990), and revised editions thereof.

[0171] Generally, the dosage of an administered antibody for humans will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. It may be desirable to provide the recipient with a dosage of antibody that is in the range of from about 0.3 mg/kg to 5 mg/kg as a single intravenous infusion, although a lower or higher dosage also may be administered as circumstances dictate. A dosage of 0.3-5 mg/kg for a 70 kg patient, for example, is 21-350 mg, or 12-206 mg/m^2 for a 1.7-m patient. The dosage may be repeated as needed, for example, once per week for 2-10 weeks, once per week for 8 weeks, or once per week for 4 weeks. It may also be given less frequently, such as every other week for several months, or monthly or quarterly for many months, as needed in a maintenance therapy. Preferred dosages may include, but are not limited to, 0.3 mg/kg, 0.5 mg/kg, 0.7 mg/kg, 1.0 mg/kg, 1.2 mg/kg, 1.5 mg/kg, 2.0 mg/kg, 2.5 mg/kg, 3.0 mg/kg, 3.5 mg/kg, 4.0 mg/kg, 4.5 mg/kg, and 5.0 mg/kg. More preferred dosages are 0.6 mg/kg for weekly administration and 1.2 mg/kg for less frequent dosing. Any amount in the range of 0.3 to 5 mg/kg may be used. The dosage is preferably administered multiple times, once a week. A minimum dosage schedule of 4 weeks, more preferably 8 weeks, more preferably 16 weeks or longer may be used, with the dose frequency dependent on toxic side-effects and recovery therefrom, mostly related to hematological toxicities. The schedule of administration may comprise administration once or twice a week, on a cycle selected from the group consisting of: (i) weekly; (ii) every other week; (iii) one week of therapy followed by two, three or four weeks off; (iv) two weeks of therapy followed by one, two, three or four weeks off; (v) three weeks of therapy followed by one, two, three, four or five week off; (vi) four weeks of therapy followed by one, two, three, four or five week off; (vii) five weeks of therapy followed by one, two, three, four or five week off; and (viii) monthly. The cycle may be repeated 2, 4, 6, 8, 10, or 12 times or more.

[0172] Alternatively, an antibody may be administered as one dosage every 2 or 3 weeks, repeated for a total of at least 3 dosages. Or, twice per week for 4-6 weeks. The dosage may be administered once every other week or even less frequently, so the patient can recover from any drug-related toxicities. Alternatively, the dosage schedule may be decreased, namely every 2 or 3 weeks for 2-3 months. The dosing schedule can optionally be repeated at other intervals and dosage may be given through various parenteral routes, with appropriate adjustment of the dose and schedule.

[0173] The methods and compositions described and claimed herein may be used to treat malignant or premalignant conditions and to prevent progression to a neoplastic or malignant

state, including but not limited to those disorders described above. Such uses are indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, Basic Pathology, 2d Ed., W. B. Saunders Co., Philadelphia, pp. 68-79 (1976)).

[0174] Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia. It is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplasia characteristically occurs where there exists chronic irritation or inflammation. In preferred embodiments, the method of the invention is used to inhibit growth, progression, and/or metastasis of cancers, in particular those listed above.

Kits

[0175] Various embodiments may concern kits containing components suitable for detecting Trop-2 positive CTCs in a patient. Exemplary kits may contain at least one anti-Trop-2 antibody as described herein. In certain embodiments, the antibody may be conjugated to at least one diagnostic agent. In alternative embodiments, a second antibody that binds to a Trop-2 positive CTC may be included. The second antibody may bind to a different epitope of Trop-2 or to a different TAA, and may be labeled with at least one diagnostic agent. In certain embodiments, an anti-Trop-2 antibody or antigen binding fragment thereof may be provided in the form of a prefilled syringe or vial containing a sterile, liquid formulation or lyophilized preparation of antibody (e.g., Kivitz et al., Clin. Ther. 2006, 28:1619-29).

[0176] The kit components may be packaged together or separated into two or more containers. In some embodiments, the containers may be vials that contain sterile, lyophilized formulations of a composition that are suitable for reconstitution. A kit may also contain one or more buffers suitable for reconstitution and/or dilution of other reagents. Other containers that may be used include, but are not limited to, a pouch, tray, box, tube, or the like. Kit components may be packaged and maintained sterilely within the containers. Another component that can be included is instructions for use of the kit.

EXAMPLES

[0177] The examples below are illustrative of embodiments of the current invention and are not limiting to the scope of the claims.

Example 1. Cell Binding Assay of Anti-Trop-2 Antibodies

[0178] Two different murine monoclonal antibodies against human Trop-2 were obtained. The first, 162-46.2, was purified from a hybridoma (ATCC, HB-187) grown up in roller-bottles. A second antibody, MAB650, was purchased from R&D Systems (Minneapolis, MN). For a comparison of binding, the Trop-2-positive human gastric carcinoma, NCI-N87, was used as the target. Cells ($1.5x10^5$/well) were plated into 96-well plates the day before the binding assay. The following morning, a dose/response curve was generated with 162-46.2, MAB650, and murine RS7 (0.03 to 66 nM) (not shown). These primary antibodies were incubated with the cells for 1.5 h at 4°C. Wells were washed and an anti-mouse-HRP secondary antibody was added to all the wells for 1 h at 4°C. Wells are washed again followed by the addition of a luminescence substrate. Plates were read using Envision plate reader and values are reported as relative luminescent units.

[0179] All three antibodies had similar K_D-values of 0.57 nM for RS7, 0.52 nM for 162-46.2 and 0.49 nM for MAB650 (not shown). However, when comparing the maximum binding (B_{max}) of 162-46.2 and MAB650 to RS7 they were reduced by 25% and 50%, respectively (B_{Max} 11,250 for RS7, 8,471 for 162-46.2 and 6,018 for MAB650) indicating different binding properties in comparison to RS7 (not shown).

Example 2. Collection and storage of blood samples.

[0180] Ten mL blood samples are drawn from each of 10 healthy donors and 20 patients with metastatic breast cancer and dispensed into a CELLSAVE™ Preservative tube (Jassen Diagnostics LLC, Raritan, NJ). The samples are stored at RT and processed within 72 h of blood collection (Allard et al., 2004, Clin Cancer Res, 10: 6897). Alternatively, 10 mL of blood samples are drawn into a CYTOCHEX® Blood collection tube (Streck, Omaha, NE), maintained at RT, and processed within 7 days (Ng et al., 2012 J Immunol Methods, 385: 79). Blood can also be drawn into 10 mL K2EDTA VACUTAINER® (BD, Waltham, MA), fixed with the LIQUIDBIOPSY® fixative (Cynvenio Biosystems, Westlake Village, CA)) within 4 h of collection, stored at room temperature, and processed within 96 h of fixation.

Example 3. Spiking of cancer cells in blood samples from healthy donors.

[0181] SK-BR-3 and BxPC-3 cells, both expressing high levels of Trop-2, are cultured in their designated medium, and harvested using trypsin. The viability and cell number of the resulting cell suspensions are assessed by Guava EASYCYTE™ flow cytometer. The cell suspensions are used only when their viability exceeds 90%. The number of cells spiked into normal serum is from 1 to 100 per mL. Cancer cells that express moderate levels of Trop-2,

for example, MCF-7, LoVo, and LS 174 T, low levels of Trop-2, for example, HT-29, or are negative for Trop-2, for example, A549 and H460, can also be used to spike blood samples.

Example 4. Isolation of epithelial cancer cells from spiked blood samples with the use of a magnetic device.

[0182] Blood samples spiked with epithelial cancer cells are incubated with biotinylated tri-Fab hRS7 (biotin-E1/3, prepared by the DNL® technique described above), and ferrofluids coated with streptavidin (FF-SV) to immunomagnetically enrich epithelial cells. Briefly, 7.5 mL of a blood sample containing a known number of spiked BxPC-3 or SK-BR-3 are mixed with 6 mL of buffer, and centrifuged at 800 x *g* for 10 min. After removing the plasma and buffer layer, biotin-E1/3 and FF-SV are added and incubated for 1 h. Subsequently, unlabeled cells are removed from labeled cells following magnetic separation. Cells labeled with biotin-E1/3 are then detached from FF-SV with a further wash and centrifugation, and are analyzed by flow cytometry after labeling with DAPI, PE-anti-CK18, and APC-anti-CD45. Nucleated cells lacking CD45 and expressing cytokeratin (CK8, CK18, CK19) are generally defined as CTCs (Swaby & Cristofanilli, 2011, BMC Medicine, 9: 43).

Example 5. Isolation of epithelial cancer cells from spiked blood samples without the use of a magnetic device.

[0183] Blood samples spiked with epithelial cancer cells are incubated with biotin-E1/3 in a microvortex-generating herringbone-chip (HP-Chip) chemically modified with avidin as described by Stott et al (2010, PNAS, 107: 18392), or more preferably, are incubated with biotin-E1/3 for 1 h before adding to NanoVelcro chips functionalized with streptavidin as described by Lu et al. (2013, Methods, 64: 144). After rinsing away the unbound cells, the bound cells are analyzed for CTCs as described in **Example 3**.

Example 6. Detection of epithelial cancer cells from spiked blood samples without prior enrichment.

[0184] Red blood cells in blood samples spiked with BxPC-3 are lysed with ammonium chloride, and centrifuged. The cell pellets are collected and incubated with FITC-labeled E1/3. The live cells in suspension are then applied to a poly-lysine-treated slide and analyzed with a laser scanning cytometer (Pachmann et al., 2005, Breast Cancer Res, 7: R975). Alternatively, the cell pellets collected after lysis of red blood cells are incubated with a cocktail comprising biotinylated E1/3 and one or more of other biotinylated DNL® conjugates in the presence of FITC-labeled avidin. The live cells in suspension are then analyzed by laser scanning cytometer.

Example 7. Detection of epithelial cancer cells from spiked blood samples using a bispecific construct targeting both Trop-2 and EGFR

[0185] Blood samples spiked with BxPC-3 cells, which express high levels of both Trop-2 and EGFR, are incubated with a biotinylated bispecific Tri-Fab, designated (E1)-225, and ferrofluids coated with streptavidin (FF-SV), as described in Example 4. (E1)-225 is generated by conjugating C_H1-DDD2-Fab-hRS7 to C_H1-AD2-Fab-c225, thus providing bivalent and monovalent binding to Trop-2 and EGFR, respectively. When compared with the enrichment using only monospecific hRS7 or c225 (cetuximab), the bispecific (E1)-225 is able to capture more BxPC-3 spiked into the blood samples, with less contamination of CD45-positive white blood cells.

Example 8. Detection of Trop-2$^+$ CTCs using LIQUIDBIOPSY® system

[0186] A LIQUIDBIOPSY® instrument (Cat. No. A28188), LIQUIDBIOPSY® Blood Collection Kit (Cat. No. A28171) and LIQUIDBIOPSY® Reagents and Consumables Kits (Cat. Nos. A28186, A28187) are obtained from Life Technologies, ThermoFisher (Grand Island, NY). The LIQUIDBIOPSY® kits includes a stabilization protocol for whole-blood samples, allowing unrefrigerated shipping of samples (96-hour window), as well as buffers, reagents, vials, elution tubes, and flow cells to process blood samples.

[0187] The humanized RS7 (hRS7) monoclonal antibody (sacituzumab) is biotinylated using the protocols and reagents provided with the Reagents and Consumables kit. Biotinylated hRS7 (sacituzumab) is used in place of the anti-EpCAM biotinylated antibody provided with the Reagents and Consumables Kit. Alternatively, anti-TROP-2 Biotinylated Antibody (Cat. No. BAF650, R&D Systems, Minneapolis, MN) is used in place of anti-EpCAM.

[0188] Circulating tumor cells from the blood of patients with solid tumors are isolated using the anti-TROP-2 biotinylated antibody and the instrument and reagents discussed above, according to the manufacturer's instructions. Isolated tumor cells are released from the slide, and confirmed by flow cytometry after labeling with DAPI, PE-anti-CK18, and APC-anti-CD45, as described in Example 3. Released cells from a second blood specimen are cultured, and a colony of live cells is obtained, which are isolated and analyzed by FISH for the copy number of Trop-2 and chromosome-1 using specific probes available from Empire Genomics (Buffalo, NY). **FIG. 1** and **FIG. 2** are representative results obtained in MCF-7 (Trop-2-positive) and A549 (Trop-2-negative) cells, showing 3 and 2 copies of the Trop-2 gene, respectively. In addition, the copy number of topoisomerase-I (TOP1) and chromosome-20 are also determined using specific probes provided by Abnova (Taipei, Taiwan) and

documented. **FIG. 3** and **FIG. 4** are representative results obtained in MCF-7 and A549 cells, showing 7 and 3 copies of the TOP1 gene, respectively. The simultaneous detection and quantitation of copy numbers of Trop-2 and TOP1 allow the determination of cancer cells that also express TOP1, which would indicate which patient tumors may be particularly responsive or resistant to a TOP1-inhibitor therapy, such as with irinotecan. This is particularly useful when using sacituzumab govitecan (IMMU-132), which targets Trop-2-expressing cancer cells and delivers SN-38 selectively to such cells. Recovery of tumor cells from blood samples is compared using anti-Trop-2 hRS7 antibody versus the anti-EpCAM antibody provided with the kit. Surprisingly, recovery of CTCs is higher with the anti-Trop-2 antibody than the anti-EpCAM antibody.

Example 9. Isolation of Trop-2$^+$ CTCs with IMAG™ magnetic particles

[0189] Purified mouse anti-human Trop-2 antibody is prepared from clone 162-46, purchased from BD Pharmingen (San Jose, CA). The anti-Trop-2 antibody is biotinylated as described in **Example 7**. IMAG™ magnetic particles (Streptavidin Particles Plus – DM) and a BD IMAG™ Cell Separation Magnet are purchased from BD Biosciences (San Jose, CA). Ten ml plastic whole blood tubes spray-coated with K2EDTA (Cat. No. 366643) are also purchased from BD.

[0190] For separation and analysis of CTCs, ten mL blood samples are drawn from patients with lung cancer and stored in K2EDTA tubes. Mononuclear cells are obtained by density gradient centrifugation using Ficoll-Hypaque solution. Protocols for positive selection of CTCs from Ficoll-Hypaque are as disclosed in BD Technical Data Sheet Streptavidin Particles Plus - DM Material Number: 557812. After the final wash step on the BD IMAG™ magnet, the released cells are resuspended in buffer.

[0191] The cells are stained with fluorescently labeled anti-cytokeratin, fluorescently labeled affinity purified goat anti-TROP-2, DAPI and/or anti-CD45. Subsequent immunofluorescence images are taken of the captured cells, followed by comprehensive computer aided analysis based on fluorescence intensities and cell morphology.

Example 10. Detection of Trop-2$^+$ CTCs and Treatment of Metastatic Trop-2 Expressing Cancer

[0192] A CELLSEARCH® system and Circulating Tumor Cell Kit are obtained Veridex LLC (Raritan, NJ). A 7.5 ml blood sample is collected from a 65 year-old male with suspected NSCLC and stored in a CellSave tube (Veridex LLC). The anti-Trop-2 hRS7 antibody is substituted for the anti-EpCAM antibody provided with the CELLSEARCH® kit.

The blood sample is mixed with magnetic nanoparticles conjugated to anti-Trop-2 antibody. Cells are stained with fluorescently labeled anti-CD45 and anti-CK antibodies and cell nuclei are fluorescently labeled with DAPI nuclear dye. A strong magnetic field is generated in the CELLSEARCH® system and used to separate cells bound to the magnetic nanoparticles, which are then analyzed by FISH to determine Trop-2 copy number, as described in Example 7 above. The results show the presence of circulating Trop-2^+ tumor cells, with 4 copies of Trop-2 per cell. The presence of high copy numbers of Trop-2 in the CTCs indicates that the patient is a good candidate for therapy with anti-Trop-2 antibodies.

[0193] Further clinical workup shows the presence of stage IIIB NSCLC (squamous cell carcinoma). Initial treatment of caboplatin/etoposide (3 mo) in concert with 7000 cGy XRT results in a response lasting 10 mo. The patient is then started on Tarceva maintenance therapy, which he continues until he was considered for IMMU-132 (hRS7-CL2A-SN-38) trial, in addition to undergoing a lumbar laminectomy. He receives the first dose of IMMU-132 after 5 months of Tarceva, presenting at the time with a 5.6-cm lesion in the right lung with abundant pleural effusion. He completes his 6^{th} dose two months later when the first CT shows the primary target lesion reduced to 3.2 cm. Periodic assays for Trop-2^+ CTCs show a substantial reduction in CTC number following treatment with IMMU-132.

[0194] This Example shows the feasibility of selecting for patients who are responsive to therapy with IMMU-132 or another therapeutic anti-Trop-2 antibody, by assaying for Trop-2^+ CTCs in the individual patient's blood and/or determinining Trop-2 copy number in CTCs. The Example further demonstrates the feasibility of monitoring relative levels of Trop-2^+ CTCs as an indicator of the efficacy of anti-Trop-2 based therapies. Preferably, patients who show a positive response, including but not limited to a complete response (CR), partial response (PR) and/or stable disease (SD) will show a decrease in levels of Trop-2^+ CTCs of at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99%. Where the treatment is highly efficacious and results in complete response, a decrease of 100% in Trop-2^+ CTCs may be observed.

Example 11. Isolation and Detection of Trop-2^+ CTCs with a VerIFAST System

[0195] A VerIFAST system as disclosed in Casavant et al. (2013, Lab Chip 13:391-6; 2014, Lab Chip 14:99-105) is used to detect Trop-2+ CTCs in TNBC. 7.5 ml blood samples are collected from a series of patients with suspected TNBC or control normal individuals and stored in CellSave tubes (Veridex LLC). Biotinylated anti-Trop-2 hRS7 antibody is prepared as disclosed in Casavant et al. (2013, Lab Chip 13:391-6). The blood samples are mixed with

biotinylated anti-Trop-2 antibody and streptavidin-conjugated PMPs (Casavant et al., 2013, Lab Chip 13:391-6) and CTCs are separated with the VerIFAST platform and a handheld magnet. Cells are stained for tumor markers and cell nuclei are fluorescently labeled with DAPI nuclear dye. The results show the presence of circulating Trop-2^{+} tumor cells in blood samples from individuals with TNBC, but not control normal individuals.

Example 12. Clinical Trials With IMMU-132 Anti-Trop-2 ADC Comprising hRS7 Antibody Conjugated to SN-38

Summary

[0196] The present Example reports results from a phase I clinical trial and ongoing phase II extension with IMMU-132 (sacituzumab govitecan), an antibody-drug conjugate (ADC) of the internalizing, humanized, hRS7 anti-Trop-2 antibody conjugated by a pH-sensitive linker to SN-38 (mean drug-antibody ratio = 7.6). Trop-2 is a type I transmembrane, calcium-transducing, protein expressed at high density (~1 x 10^5), frequency, and specificity by many human carcinomas, with limited normal tissue expression. Preclinical studies in nude mice bearing Capan-1 human pancreatic tumor xenografts have revealed IMMU-132 is capable of delivering as much as 136-fold more SN-38 to tumor than derived from a maximally tolerated irinotecan therapy (not shown).

[0197] The present Example reports the initial Phase I trial of 25 patients (pts) who had failed multiple prior therapies (some including topoisomerase-I/II inhibiting drugs), and the ongoing Phase II extension now reporting on 69 pts, including in colorectal (CRC), small-cell and non-small cell lung (SCLC, NSCLC, respectively), triple-negative breast (TNBC), pancreatic (PDC), esophageal, and other cancers.

[0198] As discussed in detail below, Trop-2 was not detected in serum, but was strongly expressed ($\geq 2^{+}$) in most archived tumors. In a 3+3 trial design, IMMU-132 was given on days 1 and 8 in repeated 21-day cycles, starting at 8 mg/kg/dose, then 12 and 18 mg/kg before dose-limiting neutropenia. To optimize cumulative treatment with minimal delays, phase II is focusing on 8 and 10 mg/kg (n=30 and 14, respectively). In 49 pts reporting related AE at this time, neutropenia ≥G3 occurred in 28% (4% G4). Most common non-hematological toxicities initially in these pts have been fatigue (55%;≥G3 = 9%), nausea (53%;≥G3=0%), diarrhea (47%;≥G3 = 9%), alopecia (40%), and vomiting (32%;≥G3 = 2%). Homozygous UGT1A1 *28/*28 was found in 6 pts, 2 of whom had more severe hematological and GI toxicities. In the Phase I and the expansion phases, there are now 48 pts (excluding PDC) who are assessable by RECIST/CT for best response. Seven (15%) of the patients had a partial

response (PR), including patients with CRC (N = 1), TNBC (N = 2), SCLC (N = 2), NSCLC (N = 1), and esophageal cancers (N = 1), and another 27 pts (56%) had stable disease (SD), for a total of 38 pts (79%) with disease response; 8 of 13 CT-assessable PDC pts (62%) had SD, with a median time to progression (TTP) of 12.7 wks compared to 8.0 weeks in their last prior therapy. The TTP for the remaining 48 pts is 12.6+ wks *(range 6.0 to 51.4 wks)*. Plasma CEA and CA19-9 correlated with responses. No anti-hRS7 or anti-SN-38 antibodies were detected despite dosing over months. The conjugate cleared from the serum within 3 days, consistent with *in vivo* animal studies where 50% of the SN-38 was released daily, with >95% of the SN-38 in the serum being bound to the IgG in a non-glucoronidated form, and at concentrations as much as 100-fold higher than SN-38 reported in patients given irinotecan. These results show that the hRS7-SN-38-containing ADC is therapeutically active in metastatic solid cancers, with manageable diarrhea and neutropenia.

Pharmacokinetics

[0199] Two ELISA methods were used to measure the clearance of the IgG (capture with anti-hRS7 idiotype antibody) and the intact conjugate (capture with anti-SN-38 IgG/probe with anti-hRS7 idiotype antibody). SN-38 was measured by HPLC. Total IMMU-132 fraction (intact conjugate) cleared more quickly than the IgG (not shown), reflecting known gradual release of SN-38 from the conjugate. HPLC determination of SN-38 (Unbound and TOTAL) showed >95% the SN-38 in the serum was bound to the IgG. Low concentrations of SN-38G suggest SN-38 bound to the IgG is protected from glucoronidation. Comparison of ELISA for conjugate and SN-38 HPLC revealed both overlap, suggesting the ELISA is a surrogate for monitoring SN-38 clearance.

[0200] A summary of the dosing regiment and patient poll is provided in **Table 6.**

Table 6. Clinical Trial Parameters

Dosing regimen	Once weekly for 2 weeks administered every 21 days for up to 8 cycles. In the initial enrollment, the planned dose was delayed and reduced if ≥ G2 treatment-related toxicity; protocol was amended to dose delay and reduction only in the event of ≥ G3 toxicity.
Dose level cohorts	8, 12, 18 mg/kg; later reduced to an intermediate dose level of 10 mg/kg.
Cohort size	Standard Phase I [3+3] design; expansion includes 15 patients in select cancers.

DLT	G4 ANC ≥ 7 d; ≥G3 febrile neutropenia of any duration; G4 Plt ≥ 5 d; G4 Hgb; Grade 4 N/V/D any duration/G3 N/V/D for > 48 h; G3 infusion-related reactions; related ≥G3 non-hematological toxicity.
Maximum Acceptable Dose (MAD)	Maximum dose where ≥2/6 patients tolerate 1st 21-d cycle w/o delay or reduction or ≥ G3 toxicity.
Patients	Metastatic colorectal, pancreas, gastric, esophageal, lung (NSCLC, SCLC), triple-negative breast (TNBC), prostate, ovarian, renal, urinary bladder, head/neck, hepatocellular. Refractory/relapsed after standard treatment regimens for metastatic cancer. Prior irinotecan-containing therapy NOT required for enrollment. No bulky lesion > 5 cm. Must be 4 weeks beyond any major surgery, and 2 weeks beyond radiation or chemotherapy regimen. Gilbert's disease or known CNS metastatic disease are excluded.

Clinical Trial Status

[0201] A total of 69 patients (including 25 patients in Phase I) with diverse metastatic cancers having a median of 3 prior therapies were reported. Eight patients had clinical progression and withdrew before CT assessment. Thirteen CT-assessable pancreatic cancer patients were separately reported. The median TTP (time to progression) in PDC patients was 11.9 wks (range 2 to 21.4 wks) compared to median 8 wks TTP for the preceding last therapy.

[0202] A total of 48 patients with diverse cancers had at least 1 CT-assessment from which Best Response (not shown) and Time to Progression (TTP; not shown) were determined. To summarize the Best Response data, of 8 assessable patients with TNBC (triple-negative breast cancer), there were 2 PR (partial response), 4 SD (stable disease) and 2 PD (progressive disease) for a total response [PR + SD] of 6/8 (75%). For SCLC (small cell lung cancer), of 4 assessable patients there were 2 PR, 0 SD and 2 PD for a total response of 2/4 (50%). For CRC (colorectal cancer), of 18 assessable patients there were 1 PR, 11 SD and 6 PD for a total response of 12/18 (67%). For esophageal cancer, of 4 assessable patients there were 1 PR, 2 SD and 1 PD for a total response of 3/4 (75%). For NSCLC (non-small cell lung cancer), of 5 assessable patients there were 1 PR, 3 SD and 1 PD for a total response of 4/5 (80%). Over all patients treated, of 48 assessable patients there were 7 PR, 27 SD and 14 PD

for a total response of 34/48 (71%). These results demonstrate that the anti-TROP-2 ADC (hRS7-SN-38) showed significant clinical efficacy against a wide range of solid tumors in human patients.

[0203] The reported side effects of therapy (adverse events) are summarized in **Table 7.** The therapeutic efficacy of hRS7-SN-38 was achieved at dosages of ADC showing an acceptably low level of adverse side effects. By comparison, patients receiving a dosage of irinotecan (125 mg/m^2 weekly x 4, Q6W) showed a much higher incidence of adverse effects, with 38% incidence of grade 3/4 diarrhea, 31% neutropenia and 8% neutropenic fever/infection.

Table 7. Related Adverse Events Listing for IMMU-132, Starting does of 8 or 10 mg/kg Criteria: Grade 3-4 Adverse Event for > 5% or any Grade 3 or 4 Adverse Event (N=123 patients)

	Grade 3	Grade 4
Neutropenia	22 (18%)	7 (6%)
Febrile Neutropenia	3 (2%)	2 (2%)
Diarrhea	4 (3%)	0
Anemia	7 (6%)	0
Fatigue	6 (5%)	0
Vomiting	2 (2%)	0
WBC Decrease	2 (2%)	0
Lymphocyte Decrease	2 (2%)	0
Asthenia	1 (1%)	0
Dizziness	1 (1%)	0
Urinary Tract Infection	1 (1%)	0
Alopecia	--	--

[0204] Data on dose reduction is also summarized. Of 76 patients starting at a dose of 8 mg/kg, 12 (16%) were provided with a dose reduction. Of 33 patients at a starting dose of 10 mg/kg, 5 (15%) were provided with a dose reduction. Of 9 patients at a starting dose of 12 mg/kg, 6 (67%) were provided with a dose reduction. Of 3 patients at a starting dose of 18 mg/kg, 3 (100%) were provided with a dose reduction. We conclude that at 8 and 10 mg/kg, there were few dose reductions, reflecting a mild, predictable and manageable toxicity profile at therapeutic levels of ADC. Currently, 425 serum samples from 148 patients have been

analyzed and no evidence of an antibody response to IMMU-132 has been detected, even after repeated administration, with some patients receiving more than 20 doses of ADC.

[0205] Of 46 assessable patients with TNBC treated to date (Phase I and II), an objective response was seen in 12 patients (26%), with disease control in 34 patients (74%), a clinical benefit ratio (CR+PR+(SD≥6 mo)] of 46% and a clinical benefit ratio (CR+PR+(SD≥4 mo)] of 63%.

[0206] Of 19 assessable patients with NSCLC treated to date, an objective response was seen in 6 patients (32%), with disease control in 14 patients (74%), and a clinical benefit ratio (CR+PR+(SD≥4 mo)] of 59%.

[0207] Of 20 assessable patients with SCLC treated to date, an objective response was seen in 6 patients (30%), with disease control in 11 patients (55%), a clinical benefit ratio (CR+PR+(SD≥6 mo)] of 37% and a clinical benefit ratio (CR+PR+(SD≥4 mo)] of 55%.

[0208] Of 16 assessable patients with EAC treated to date, an objective response was seen in 2 patients (13%), with disease control in 9 patients (56%), and a clinical benefit ratio (CR+PR+(SD≥4 mo)] of 44%.

[0209] Exemplary partial responses to the anti-Trop-2 ADC were confirmed by CT data (not shown). As an exemplary PR in CRC, a 62-year-old woman first diagnosed with CRC underwent a primary hemicolectomy. Four months later, she had a hepatic resection for liver metastases and received 7 mos of treatment with FOLFOX and 1 mo 5FU. She presented with multiple lesions primarily in the liver (3+ Trop-2 by immunohistology), entering the hRS7-SN-38 trial at a starting dose of 8 mg/kg about 1 year after initial diagnosis. On her first CT assessment, a PR was achieved, with a 37% reduction in target lesions (not shown). The patient continued treatment, achieving a maximum reduction of 65% decrease after 10 months of treatment (not shown) with decrease in CEA from 781 ng/mL to 26.5 ng/mL), before progressing 3 months later.

[0210] As an exemplary PR in NSCLC, a 65-year-old male was diagnosed with stage IIIB NSCLC (sq. cell). Initial treatment of caboplatin/etoposide (3 mo) in concert with 7000 cGy XRT resulted in a response lasting 10 mo. He was then started on Tarceva maintenance therapy, which he continued until he was considered for IMMU-132 trial, in addition to undergoing a lumbar laminectomy. He received first dose of IMMU-132 after 5 months of Tarceva, presenting at the time with a 5.6 cm lesion in the right lung with abundant pleural effusion. He had just completed his 6^{th} dose two months later when the first CT showed the primary target lesion reduced to 3.2 cm (not shown).

[0211] As an exemplary PR in SCLC, a 65-year-old woman was diagnosed with poorly differentiated SCLC. After receiving carboplatin/etoposide (Topo-11 inhibitor) that ended after 2 months with no response, followed with topotecan (Topo-1 inhibitor) that ended after 2 months, also with no response, she received local XRT (3000 cGy) that ended 1 month later. However, by the following month progression had continued. The patient started with IMMU-132 the next month (12 mg/kg; reduced to 6.8 mg/kg; Trop-2 expression 3+), and after two months of IMMU-132, a 38% reduction in target lesions, including a substantial reduction in the main lung lesion occurred (not shown). The patient progressed 3 months later after receiving 12 doses.

[0212] These results are significant in that they demonstrate that the anti-Trop-2 ADC was efficacious, even in patients who had failed or progressed after multiple previous therapies.

[0213] In conclusion, at the dosages used, the primary toxicity was a manageable neutropenia, with few Grade 3 toxicities. IMMU-132 showed evidence of activity (PR and durable SD) in relapsed/refractory patients with triple-negative breast cancer, small cell lung cancer, non-small cell lung cancer, colorectal cancer and esophageal cancer, including patients with a previous history of relapsing on topoisomerase-1 inhibitor therapy. These results show efficacy of the anti-Trop-2 ADC in a wide range of cancers that are resistant to existing therapies.

* * *

[0214] It will be apparent to those skilled in the art that various modifications and variations can be made to the products, compositions, methods and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents.

[0215] Reference to any prior art in the specification is not an acknowledgement or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be combined with any other piece of prior art by a skilled person in the art.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of diagnosing a subject with a Trop-2^+ cancer, consisting of:

a) exposing an anti-Trop-2 antibody or antigen-binding fragment thereof to blood from a subject suspected of having a Trop-2^+ cancer;

b) allowing the anti-Trop-2 antibody or fragment thereof to bind to Trop-2^+ circulating tumor cells (CTCs);

c) detecting CTCs bound to the anti-Trop-2 antibody or fragment thereof; and

d) diagnosing the subject with a Trop-2^+ cancer when CTCs bound to the anti-Trop-2 antibody or fragment thereof are detected,

wherein the presence of Trop-2^+ CTCs with a high copy number of Trop-2 is predictive of response to a therapeutic anti-Trop-2 antibody, and wherein a high copy number is 3 or more per cell.

2. A method of diagnosing a subject with a Trop-2^+ cancer, consisting of:

a) exposing an anti-Trop-2 antibody or antigen-binding fragment thereof to blood from a human subject suspected of having a Trop-2^+ cancer;

b) allowing the anti-Trop-2 antibody or fragment thereof to bind to Trop-2^+ circulating tumor cells (CTCs);

c) detecting CTCs bound to the anti-Trop-2 antibody or fragment thereof;

d) analyzing the copy number of Trop-2 in the CTCs; and

e) diagnosing the subject with a Trop-2^+ cancer when CTCs bound to the anti-Trop-2 antibody or fragment thereof are detected,

wherein the presence of Trop-2^+ CTCs with a high copy number of Trop-2 is predictive of response to a therapeutic anti-Trop-2 antibody, and wherein a high copy number is 3 or more per cell.

3. A method of diagnosing a subject with a Trop-2^+ cancer, comprising:

a) exposing an anti-Trop-2 antibody or antigen-binding fragment thereof to blood from a subject suspected of having a Trop-2^+ cancer, wherein the anti-Trop-2 antibody or fragment thereof is the sole anti-TAA (tumor associated antigen) capture antibody;

b) allowing the anti-Trop-2 antibody or fragment thereof to bind to Trop-2^+ circulating tumor cells (CTCs);

c) detecting CTCs bound to the anti-Trop-2 antibody or fragment thereof; and

d) diagnosing the subject with a Trop-2^+ cancer when CTCs bound to the anti-Trop-2 antibody or fragment thereof are detected,

2016252771 10 Nov 2021

wherein the presence of Trop-2^+ CTCs with a high copy number of Trop-2 is predictive of response to a therapeutic anti-Trop-2 antibody, and wherein a high copy number is 3 or more per cell.

4. A method of treating Trop-2^+ tumors consisting of:

a) exposing an anti-Trop-2 antibody or antigen-binding fragment thereof to blood from a subject suspected of having a Trop-2^+ cancer;

b) allowing the anti-Trop-2 antibody or fragment thereof to bind to Trop-2^+ circulating tumor cells (CTCs);

c) detecting CTCs bound to the anti-Trop-2 antibody or fragment thereof; and

d) treating the subject with an anti-Trop-2 antibody conjugated to at least one therapeutic agent,

wherein the presence of Trop-2^+ CTCs with a high copy number of Trop-2 is predictive of response to a therapeutic anti-Trop-2 antibody, and wherein a high copy number is 3 or more per cell.

5. A method of treating Trop-2^+ tumors consisting of:

a) exposing an anti-Trop-2 antibody or antigen-binding fragment thereof to blood from a human subject suspected of having a Trop-2^+ cancer;

b) allowing the anti-Trop-2 antibody or fragment thereof to bind to Trop-2^+ circulating tumor cells (CTCs);

c) detecting CTCs bound to the anti-Trop-2 antibody or fragment thereof;

d) analyzing the copy number of Trop-2 in the CTCs; and

e) treating the human subject with an anti-Trop-2 antibody conjugated to at least one therapeutic agent,

wherein the presence of Trop-2^+ CTCs with a high copy number of Trop-2 is predictive of response to a therapeutic anti-Trop-2 antibody, wherein a high copy number is 3 or more per cell.

6. A method of treating Trop-2^+ tumors consisting of:

a) exposing an anti-Trop-2 antibody or antigen-binding fragment thereof to blood from a human subject suspected of having a Trop-2^+ cancer;

b) allowing the anti-Trop-2 antibody or fragment thereof to bind to Trop-2^+ circulating tumor cells (CTCs);

c) detecting CTCs bound to the anti-Trop-2 antibody or fragment thereof;

d) treating the human subject with an anti-Trop-2 antibody conjugated to at least one therapeutic agent; and

2016252771 10 Nov 2021

e) monitoring the presence of Trop-2^+ CTCs in the circulation to determine the response of the tumor to therapeutic anti-Trop-2 antibody,

wherein the presence of Trop-2^+ CTCs with a high copy number of Trop-2 is predictive of response to a therapeutic anti-Trop-2 antibody, wherein a high copy number is 3 or more per cell.

7. A method of treating Trop-2^+ tumors comprising:

a) exposing an anti-Trop-2 antibody or antigen-binding fragment thereof to blood from a subject suspected of having a Trop-2^+ cancer, wherein the anti-Trop-2 antibody or fragment thereof is the sole anti-TAA (tumor associated antigen) capture antibody;

b) allowing the anti-Trop-2 antibody or fragment thereof to bind to Trop-2^+ circulating tumor cells (CTCs);

c) detecting CTCs bound to the anti-Trop-2 antibody or fragment thereof; and

d) treating the subject with an anti-Trop-2 antibody conjugated to at least one therapeutic agent,

wherein the presence of Trop-2^+ CTCs with a high copy number of Trop-2 is predictive of response to a therapeutic anti-Trop-2 antibody, wherein a high copy number is 3 or more per cell.

8. The method of claim 7, further comprising analyzing the copy number of Trop-2 in the CTCs.

9. The method of claim 7, further comprising monitoring the presence to Trop-2^+ CTCs in the circulation to determine the response of the tumor to therapeutic anti-Trop-2 antibody.

10. The method of any one of claims 4-9, wherein the therapeutic agent is selected from the group consisting of an antibody, an antibody fragment, a drug, a toxin, a hormone, an immunomodulator, a pro-apoptotic agent, an anti-angiogenic agents, a boron compound, a photoactive agent and a radionuclide.

11. The method of claim 10, wherein the drug is selected from the group consisting of an anthracycline, a camptothecin, a tubulin inhibitor, a maytansinoid, a calicheamycin, an auristatin, a nitrogen mustard, an ethylenimine derivative, an alkyl sulfonate, a nitrosourea, a triazene, a folic acid analog, a taxane, a COX-2 inhibitor, a pyrimidine analog, a purine analog, an antibiotic, an enzyme inhibitor, an epipodophyllotoxin, a platinum coordination complex, a vinca alkaloid, a substituted urea, a methyl hydrazine derivative, an adrenocortical suppressant, a hormone antagonist, an antimetabolite, an alkylating agent, an antimitotic, an anti-angiogenic agent, a tyrosine kinase inhibitor, an mTOR inhibitor, a heat shock protein (HSP90) inhibitor, a proteosome inhibitor, an HDAC inhibitor, and a pro-apoptotic agent.

12. The method of claim 10, wherein the drug is selected from the group consisting of 5-fluorouracil, afatinib, aplidin, azaribine, anastrozole, anthracyclines, axitinib, AVL-101,

AVL-291, bendamustine, bleomycin, bortezomib, bosutinib, biyostatin-1, busulfan, calicheamycin, camptothecin, carboplatin, 10-hydroxy camptothecin, carmustine, celecoxib, chlorambucil, cisplatinum, COX-2 inhibitors, irinotecan (CPT-11), SN-38, carboplatin, cladribine, camptothecans, crizotinib, cyclophosphamide, cytarabine, dacarbazine, dasatinib, dinaciclib, docetaxel, dactinomycin, daunorubicin, DM1, DM3, DM4, doxorubicin, 2-pyrrolinodoxorubicine (2-PDox), a pro-drug form of 2-PDox (pro- 2-PDox), cyano-morpholino doxorubicin, doxorubicin glucuronide, endostatin, epirubicin glucuronide, erlotinib, estramustine, epidophyllotoxin, erlotinib, entinostat, estrogen receptor binding agents, etoposide (VP 16), etoposide glucuronide, etoposide phosphate, exemestane, fingolimod, floxuridine (FUdR), 3',5'-0-dioleoyl-FudR (FUdR-dO), fludarabine, flutamide, farnesyl-protein transferase inhibitors, flavopiridol, fostamatinib, ganetespib, GDC-0834, GS-1101, gefitinib, gemcitabine, hydroxyurea, ibrutinib, idarubicin, idelalisib, ifosfamide, imatinib, lapatinib, lenolidamide, leucovorin, LFM- A13, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mitoxantrone, mithramycin, mitomycin, mitotane, monomethylauristatin F (MMAF), monomethylauristatin D (MMAD), monomethylauristatin E (MMAE), navelbine, neratinib, nilotinib, nitrosurea, olaparib, plicomycin, procarbazine, paclitaxel, PCI-32765, pentostatin, PSI-341, raloxifene, semustine, SN-38, sorafenib, streptozocin, SU11248, sunitinib, tamoxifen, temazolomide, transplatinum, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vatalanib, vinorelbine, vinblastine, vincristine, vinca alkaloids and ZD1839.

13. The method of claim 10, wherein the drug is selected from the group consisting of SN- 38, pro-2-pyrrolinodoxorubicin (pro-2-PDox), paclitaxel, calichemicin, DM1, DM3, DM4, MMAE, MMAD and MMAF.

14. The method of any one of claims 1-13, wherein the cancer is resistant to treatment with at least one anti-cancer therapy.

15. The method of any one of claims 1-14, wherein the cancer is pancreatic cancer.

16. The method of claim 10, wherein the radionuclide is selected from the group consisting of ^{11}C, ^{13}N, ^{15}O, ^{32}P, ^{33}P, ^{47}Sc, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{62}Cu, ^{67}Cu, ^{67}Ga, ^{67}Ga, ^{75}Br, ^{75}Se, ^{75}Se, ^{76}Br, ^{77}As, ^{77}Br, ^{80}mBr, ^{89}Sr, ^{90}Y, ^{95}Ru, ^{97}Ru, ^{99}Mo, ^{99}mTc, ^{103}mRh, ^{103}Ru, ^{105}Rh, ^{105}Ru, ^{107}Hg, ^{109}Pd, ^{109}Pt, ^{111}Ag, ^{111}In, ^{113}mIn, ^{119}Sb, ^{121}mTe, ^{122}mTe, ^{125}I, ^{125}mTe, ^{126}I, ^{131}I, ^{133}I, ^{142}Pr, ^{143}Pr, ^{149}Pm, ^{152}Dy, ^{153}Sm, ^{161}Ho, ^{161}Tb, ^{165}Tm, ^{166}Dy, ^{166}Ho, ^{167}Tm, ^{168}Tm, ^{169}Er, ^{169}Yb, ^{177}Lu, ^{186}Re, ^{188}Re, ^{189}mOs, ^{189}Re, ^{192}Ir, ^{194}Ir, ^{197}Pt, ^{198}Au, ^{199}Au, ^{199}Au, 201Tl, 203Hg, ^{211}At, ^{211}Bi, ^{211}Pb, ^{212}Bi, ^{212}Pb, ^{213}Bi, ^{215}Po, ^{217}At, ^{219}Rn, ^{221}Fr, ^{223}Ra, ^{224}Ac, ^{225}Ac, ^{255}Fm and ^{227}Th.

2016252771 10 Nov 2021

17. The method of claim 10, wherein the toxin is selected from the group consisting of ricin, abrin, alpha toxin, saporin, ribonuclease (RNase), DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtheria toxin, Pseudomonas exotoxin, and Pseudomonas endotoxin.

18. The method of claim 10, wherein the immunomodulator is selected from the group consisting of a cytokine, a stem cell growth factor, a lymphotoxin, a hematopoietic factor, a colony stimulating factor (CSF), an interferon (IFN), an interleukin, erythropoietin and thrombopoietin.

19. The method of claim 18, wherein the cytokine is selected from the group consisting of human growth hormone, N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), hepatic growth factor, prostaglandin, fibroblast growth factor, prolactin, placental lactogen, OB protein, tumor necrosis factor-a, tumor necrosis factor-β, mullerian- inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, integrin, thrombopoietin (TPO), NGF-β, platelet-growth factor, TGF-a, TGF-β, insulin-like growth factor-I, insulin-like growth factor-II, erythropoietin (EPO), osteoinductive factors, interferon-a, interferon-β, interferon-γ, macrophage-CSF (M-CSF), IL-1, IL-la, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-21, IL-25, LIF, FLT-3, angiostatin, thrombospondin, endostatin, tumor necrosis factor and lymphotoxin.

20. Use of an anti-Trop-2 antibody or antigen-binding fragment thereof conjugated to at least one therapeutic agent, in the manufacture of a medicament for treating Trop-2^+ tumors in a subject, wherein prior to treatment with the medicament,

a) an anti-Trop-2 antibody or antigen-binding fragment thereof is exposed to blood from the subject suspected of having a Trop-2^+ cancer; and

b) the anti-Trop-2 antibody or fragment thereof is allowed to bind to Trop-2^+ circulating tumor cells (CTCs);

c) CTCs bound to the anti-Trop-2 antibody or fragment thereof are detected; and

d) the copy number of Trop-2 in the CTCs is determined to be 3 or more per cell.

21. The use of claim 20, further comprising analyzing the copy number of Trop-2 in the CTCs.

22. The use according to claim 20 or 21 wherein the subject is a human subject.

2016252771 10 Nov 2021

23. The use according to any one of claims 20 to 22 wherein the therapeutic agent is selected from the group consisting of an antibody, an antibody fragment, a drug, a toxin, a hormone, an immunomodulator, a pro-apoptotic agent, an anti-angiogenic agents, a boron compound, a photoactive agent and a radionuclide.

FIG. 1

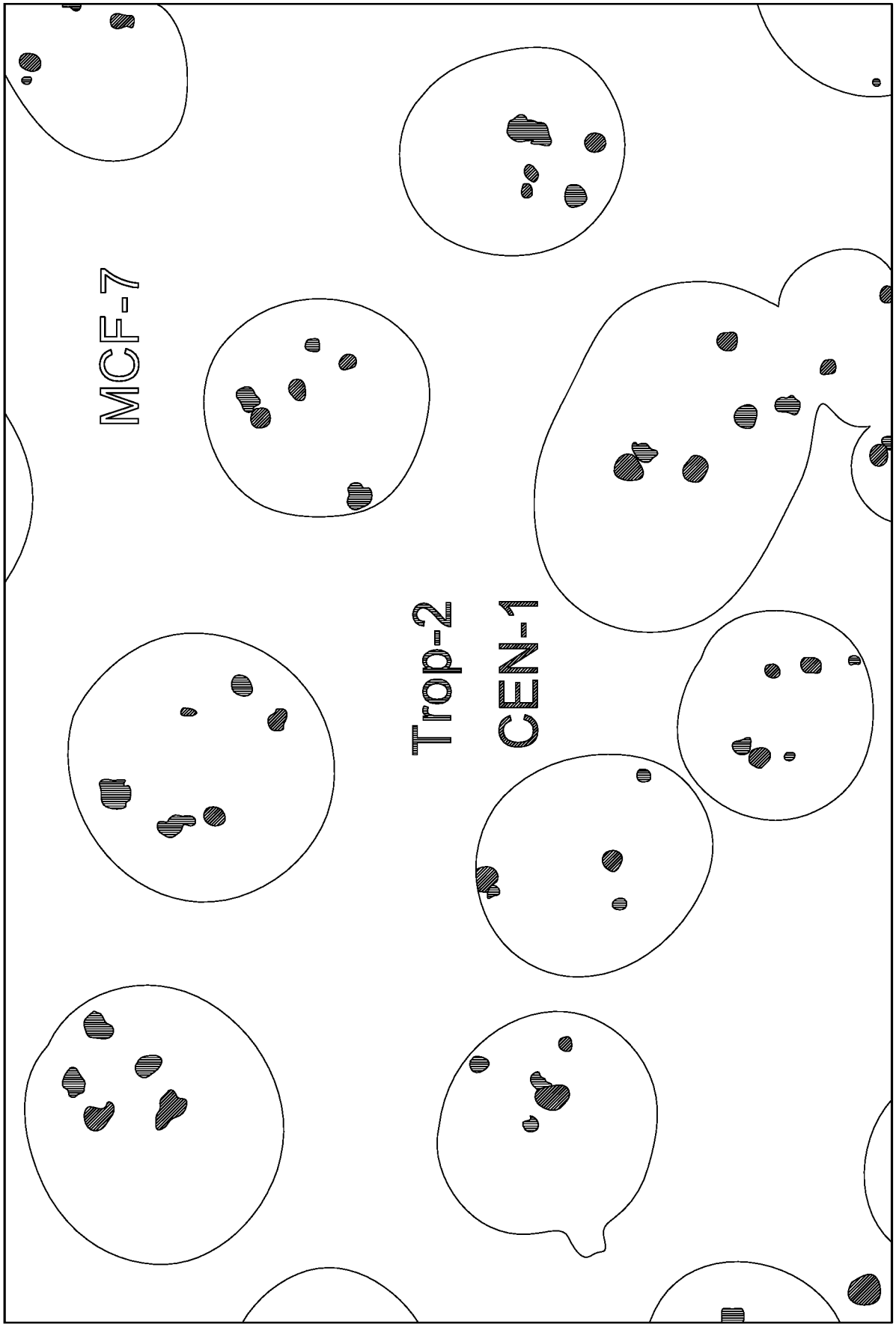


FIG. 2

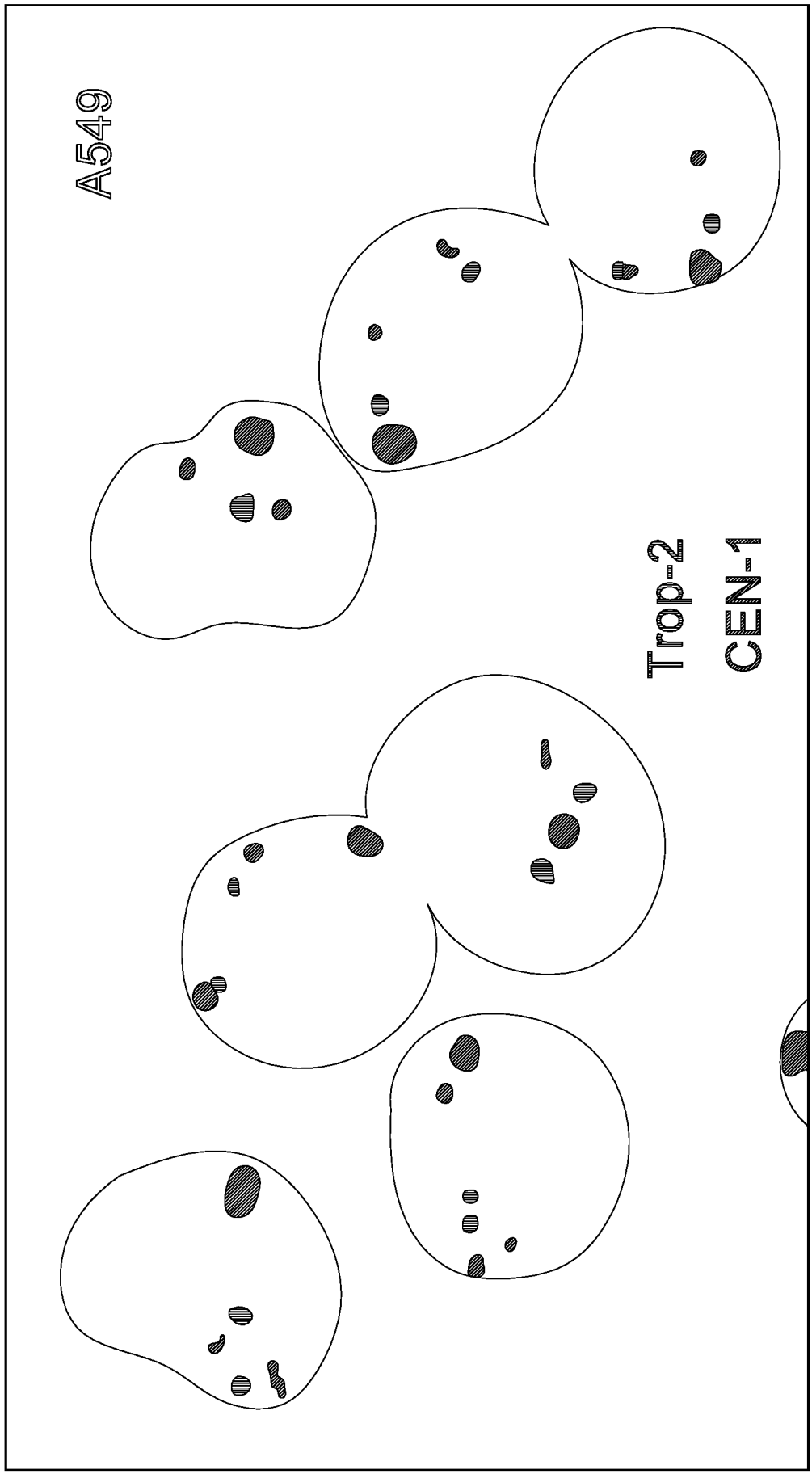


FIG. 3

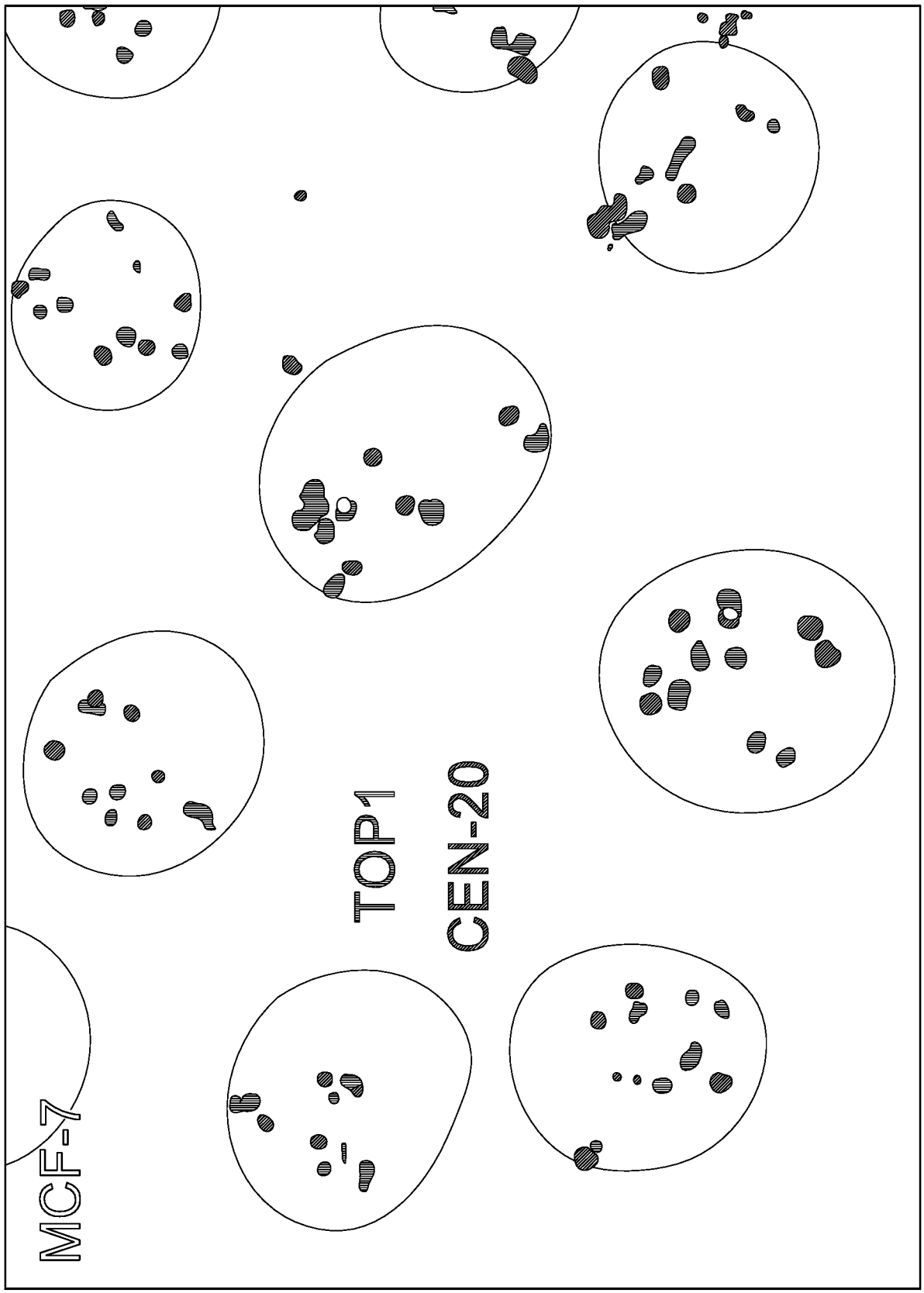
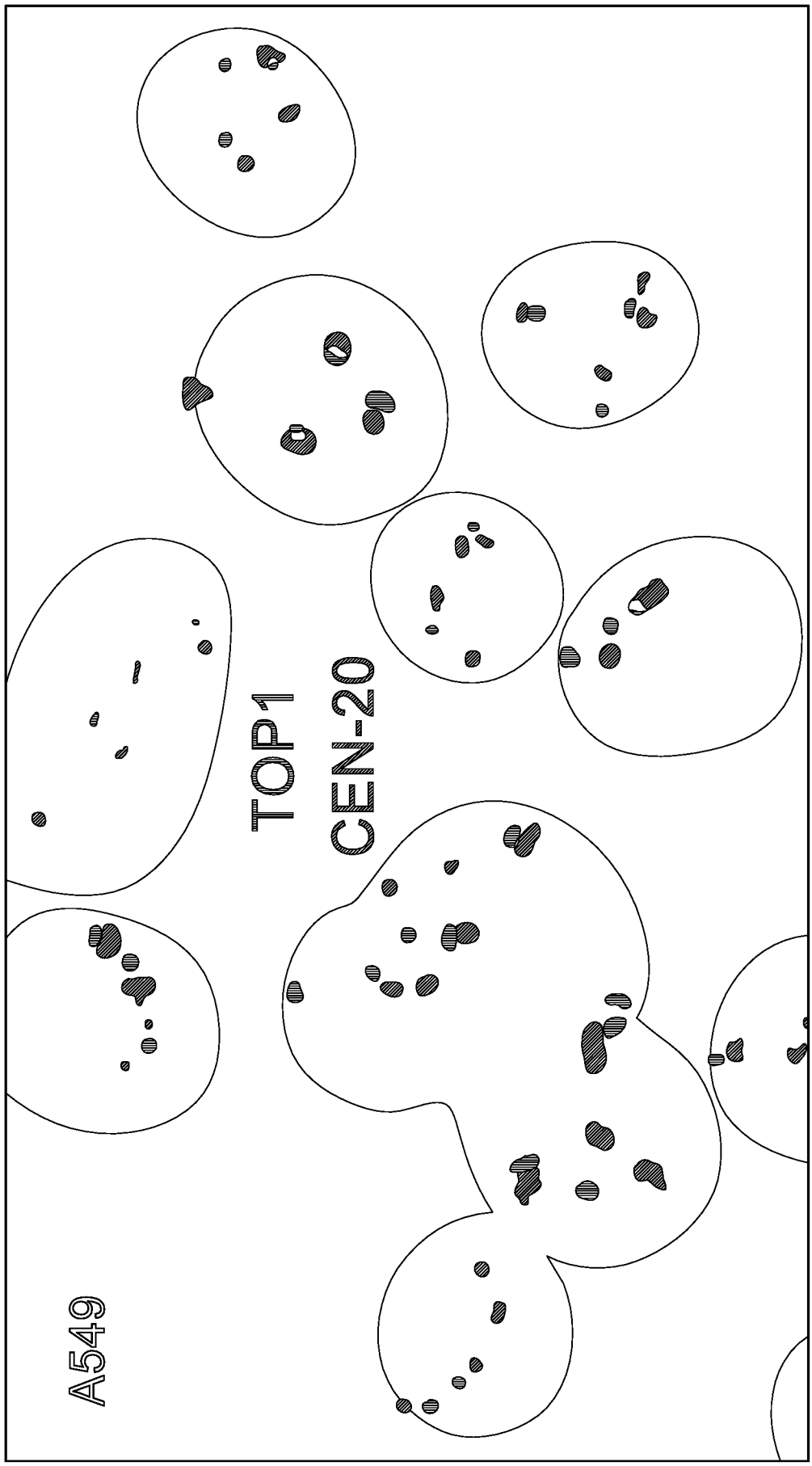


FIG. 4



SEQUENCE LISTING

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<120> COMPOSITIONS AND METHODS USING IL-8 TO INCREASE MILK PRODUCTION AND IMPROVE REPRODUCTIVE HEALTH AND INHIBIT HYPERKETONEMIA IN MAMMALS

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Glu Ile Ile Val Lys Leu Thr Asn Gly Asn Glu Val Cys Leu Asn Pro
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Glu Ile Ile Val Lys Leu Thr Asn Gly Lys Glu Val Cys Leu Asn Pro
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Arg Cys Gln Cys Ile Lys Thr His Ser Thr Pro Phe His Pro Lys Phe
35 40 45

Ile Lys Glu Leu Arg Val Ile Glu Ser Gly Pro His Cys Glu Asn Ser
50 55 60

Glu Ile Ile Val Lys Leu Thr Asn Gly Lys Glu Val Cys Leu Asn Pro
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Ile Lys Glu Leu Arg Val Ile Glu Ser Gly Pro His Cys Ala Asn Thr
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Glu Ile Ile Val Lys Leu Ser Asp Gly Arg Glu Leu Cys Leu Asp Pro
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Lys Glu Asn Trp Val Gln Arg Val Val Glu Lys Phe Leu Lys Arg Ala
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Glu Asn Ser Leu
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Glu Ile Ile Val Lys Leu Phe Asn Gly Asn Glu Val Cys Leu Asp Pro
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Arg Cys Gln Cys Ile Lys Thr His Ser Thr Pro Phe Asn Pro Lys Leu
        35                  40                  45

Ile Lys Glu Leu Thr Val Ile Asp Ser Gly Pro His Cys Glu Asn Ser
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Glu Ile Ile Val Lys Leu Val Asn Gly Lys Glu Val Cys Leu Asp Pro
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Arg Cys Gln Cys Ile Lys Thr His Ser Thr Pro Phe His Pro Lys Phe
        35                  40                  45


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    50                  55                  60


Glu Ile Ile Val Lys Leu Xaa Asn Gly Lys Xaa Val Cys Leu Xaa Pro
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Glu Lys Gln Asp Pro
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