MULTI-SPECIFIC ANTIBODIES AND METHODS OF MAKING AND USING THEREOF

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Patent Application No. 62524558, filed June 25, 2017, which application is expressly incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present disclosure generally relates to the technical field of biologic therapeutics, and more particularly relates to making and using multi-specific antibodies.

BACKGROUND

Cancer cells develop various strategies to evade the immune system. One of the underlaying mechanisms for the immune escape is the reduced recognition of cancer cells by the immune system. Defective presentation of cancer specific antigens or lack of thereof results in immune tolerance and cancer progression. In the presence of effective immune recognition tumors use other mechanisms to avoid elimination by the immune system. Immunocompetent tumors create suppressive microenvironment to downregulate the immune response. Multiple players are involved in shaping the suppressive tumor microenvironment, including tumor cells, regulatory T cells, Myeloid-Derived Suppressor cells, stromal cells, and other cell types. The suppression of immune response can be executed in a cell contact-dependent format as well as in and a contact-independent manner, via secretion of immunosuppressive cytokines or elimination of essential survival factors from the local environment. Cell contact-dependent suppression relies on molecules expressed on the cell surface, e.g. Programmed Death Ligand 1 (PD-L1), T-lymphocyte-associated protein 4 (CTLA-4) and others [Dunn, et al., 2004, Immunity, 21(2): 137-48; Adachi & Tamada, 2015, Cancer Sci., 106(8): 945-50].

As the mechanisms by which tumors evade recognition by the immune system continue to be better understood new treatment modalities that target these mechanisms have recently emerged. On March 25, 2011, the U. S. Food and Drug Administration (FDA) approved ipilimumab injection (Yervoy, Bristol-Myers Squibb) for the treatment of unresectable or metastatic melanoma. Yervoy binds to cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expressed on activated T cells and blocks the interaction of CTLA-4 with CD80/86 on antigen-presenting cells thereby blocking the negative or inhibitory signal delivered into the T cell through CTLA-4 resulting in re-activation of the antigen-specific T cell leading to, in many patients, eradication of the tumor. A few years later in 2014 the FDA approved Keytruda (Pembrolizumab, Merck) and Opdivo (Nivolumab, Bristol-Myers Squibb) for treatment of advanced melanoma. These monoclonal antibodies bind to PD-1 which is expressed on activated and/or exhausted T cells and block the interaction of PD-1 with PD-L1 expressed on tumors thereby eliminating the inhibitory signal through PD-1 into the T cell resulting in re-activation of the antigen-specific T cell leading to again, in many patients, eradication of the tumor. Since then additional clinical trials have been performed comparing the single monoclonal antibody Yervoy to the combination of the monoclonal antibodies Yervoy and Opdivo in the treatment of advanced melanoma which showed improvement in overall survival and progression-free survival in the patients treated with the

combination of antibodies. (Hodi et al., 2016, Lancet Oncol. 17(11):1558-1568, Hellman et al., 2018, Cancer Cell 33(5): 853-861). However, as many clinical trials have shown a great benefit of treating cancer patients with monoclonal antibodies that are specific for one or more immune checkpoint molecules data has emerged that only those patients with a high mutational burden that generates a novel T cell epitope(s) which is recognized by antigen-specific T cells show a clinical response (Snyder et al., 2014, NEJM 371:2189-2199). Those patients that have a low tumor mutational load mostly do not show an objective clinical response (Snyder et al., 2014, NEJM 371:2189-2199, Hellman et al., 2018, Cancer Cell 33(5): 853-861).

In recent years other groups have developed an alternate approach that does not require the presence of neoepitope presentation by antigen-presenting cells to activate T cells. One example is the development of a bi-specific antibody where the binding domain of an antibody which is specific for a tumor associated antigen, e.g., CD19, is linked to and antibody binding domain specific for CD3 on T cells thus creating a bi-specific T cell engager or BiTe molecule. In 2014, the FDA approved a bi-specific antibody called Blinatumumab for the treatment of Precursor B-Cell Acute Lymphoblastic Leukemia. Blinatumumab links the scFv specific for CD19 expressed on leukemic cells with the scFv specific for CD3 expressed on T cells (Bejnjamin and Stein 2016, Ther Adv Hematol 7(3):142-146). However, despite an initial response rate of >50% in patients with relapsed or refractory ALL many patients are resistant to Blinatumumab therapy or relapse after successful treatment with Blinatumumab . Evidence is emerging that the resistant to Blinatumumab or who relapse after Blinatumumab treatment is attributable to the expression of immune checkpoint inhibitory molecules expressed on tumor cells such as PD-L1 that drives an inhibitory signal through PD-1 expressed on activated T cells (Feucht et al., 2016, Oncotarget 7(47):76902-76919). In a case study of a patient who was resistant to therapy with Blinatumumab a second round of Blinatumumab therapy was performed but with the addition of a monoclonal antibody, pembrolizumab (Keytruda, Merck), which specific for PD-1 and blocks the interaction of T cell-expressed PD-1 with tumor cell expressed PD-L1 resulted in a dramatic response and reduction of tumor cells in the bone marrow from 45% to less than 5% in this one patient (Feucht et al., 2016, Oncotarget 7(47):76902-76919). These results show that combining a bi-specific BiTe molecule with one or more monoclonal antibodies can significantly increase clinical activity compared to either agent alone.

SUMMARY

The present disclosure provides, among others, tetra-specific antibody monomers, antibodies containing tetra-specific monomers, antigen-binding fragments thereof, multi-specific antibodies, immuno-conjugates comprising the disclosed antibodies or monomers, methods of making disclosed monomers, antigen-binding fragments, and antibodies, and methods of using the disclosed molecules for treating cancer.

In one aspect, the application provides tetra-specific antibody monomers. In one embodiment, the tetra-specific antibody monomer has a N-terminal and a C-terminal and include in tandem from the N-terminal to the C-terminal, a first scFv domain at the N-terminal, a second scFv domain, a Fab domain, a Fc domain, and a third scFv at the C-terminal. The first scFv domain, the Fab domain, the second scFv domain, and the third scFv domain each has a binding specificity against a different antigen.

In one embodiment, the antigen includes a tumor antigen, an immune signaling antigen, or a combination thereof. In one embodiment, the first scFv domain, the Fab domain, the second scFv domain, and the third scFv domain each has a binding specificity against a tumor antigen or an immune signaling antigen. In one embodiment, the first scFv domain has a binding specificity against a tumor antigen. In one embodiment, the first scFv domain has a binding specificity against an immune signaling antigen. In one embodiment, the second scFv domain has a binding specificity against a tumor antigen. In one embodiment, the second scFv domain has a binding specificity against an immune signaling antigen. In one embodiment, the Fab domain has a binding specificity against a tumor antigen. In one embodiment, the Fab domain has a binding specificity against an immune signaling antigen. In one embodiment, the third scFv domain has a binding specificity against a tumor antigen. In one embodiment, the third scFv domain has a binding specificity against a tumor antigen.

In one embodiment, the tetra-specific monomer includes the first scFv domain, the second scFv domain, the Fab domain, and the third scFv domain, each independently has a binding specificity against an antigen selected from CD19, CD3, CD137, 4-1BB, PD-L1, ROR1, CD28, 41BB, CEA, HER2, EGFRvIII, EGFR, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypimay-3, gpA33, GD2, TROP2, NKG2D, BCMA, CD20, CD33, CD123, CD22, CD30, PD1, OX40, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40, VISTA, ICOS, BTLA, LIGHT, HVEM, CSF1R, CD73, and CD39. In one embodiment, the scFv domain, the second scFv domain, the Fab domain, and the third scFv domain each independently has a binding specificity against tumor specific antigens including, but not limited to, CD19, CD3, CD137, ROR1, CEA, HER2, EGFR, EGFRvIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypimay-3, gpA33, GD2, TROP2, BCMA, CD20, CD33, CD123, CD22, CD30, or immune checkpoint modulators including, without limitation, PD-L1, PD1, OX40, 4-1BB, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40, VISTA, ICOS, BTLA, Light, HVEM, CD73, CD39, etc. In one embodiment, one set of scFv domain may specifically bind to an immune checkpoint modulators or a tumor antigen. The scFv specific to CD3 component may be on either C or N terminal of heave or light chains.

In one embodiment, the first scFv domain, the second scFv domain, the Fab domain, and the third scFv domain each independently has a binding specificity against an antigen selected from CD19, CD3, CD137M , PD-L1, and 4-1BB. In one embodiment, the first scFv domain has a binding specificity against CD19. In one embodiment, the second scFv domain has a binding specificity against CD3. In one embodiment, the Fab domain has a binding specificity against 4-1BB or CD137. In one embodiment, the third scFv domain has a binding specificity against PD-L1.

In one embodiment, the first scFv domain has a binding specificity against CD19, , the second scFv domain has a binding specificity against CD3, the Fab domain has a binding specificity against 4-1BB, and the third scFv domain has a binding specificity against PD-L1. In one embodiment, the first scFv domain has a binding specificity against CD19, , the second scFv domain has a binding specificity against CD3, the Fab domain has a binding specificity against CD137, and the third scFv domain has a binding specificity against PD-L1.

The scFv domain may include a linker linking the scFv domain to the heavy chain or light chain of the antibody. In one embodiment, the linker may include more than 10 amino acids. In one embodiment,

the linker may include more than 15 amino acids long. In one embodiment, the linker may include less than 20 amino acids.

In one embodiment, the linker may comprises a gly-gly-gly-gly-ser $(G4S)_n$ linker, and n may be an integral between 1 to 20. For example, n may be 2, 4, or 6. In one embodiment, the first scFv domain, the second scFv domain, or the third scFv domain may comprise a gly-gly-gly-gly-ser $(G4S)_n$ linker, wherein n is 2 or 4.

Fc domain may be humanized. In one embodiment, the Fc domain is a human IgG1 Fc.

In one embodiment, the application provides a tetra-specific antibody monomers having an amino acid sequence having a percentage homology to SEQ ID NO. 38 and 39. The percentage homology is not less than 70%, 80%, 90%, 95%, 98% or 99%.

The application further provides antigen-binding fragments. In one embodiment, the application provides scFv domains. In one embodiment, the scFv domain has an amino acid sequence having a percentage homology to SEQ ID No. 2, 4, 6, 8, 10, 12, 26, 28, 30, 32, wherein the percentage homology is not less than 70%, 80%, 90%, 95%, 98% or 99%. In one embodiment, the application provides Fab domains. In one embodiment, the Fab domain includes an amino acid sequence having a percentage homology to SEQ ID NO. 1-12,26-32 , wherein the percentage homology is not less than 70%, 80%, 90%, 95%, 98% or 99%. The antigen-binding fragments disclosed herein may be used to construct the tetra-specific antibody monomers or multi-specific antibodies.

In one aspect, the application provides multi-specific antibodies. In one embodiment, the multi-specific antibody includes tetra-specific antibody monomers. In one embodiment, the multi-specific antibody includes two tetra-specific antibody monomers disclosed herein. As each tetra-specific antibody monomer has four antigen-binding domains, the multi-specific antibody disclosed may include 8 antigen-binding domains. In one embodiment, the antigen binding domains in such multi-specific antibody each independently has a binding specificity against a different antigen therefor providing an octa-specific antibody. In one embodiment, the multi-specific antibody is a penta-specific antibody. In one embodiment, the multi-specific antibody is a penta-specific antibody. In one embodiment, the multi-specific antibody is a penta-specific antibody a hexa-specific antibody. In one embodiment, the multi-specific antibody is a penta-specific antibody a hepta-specific antibody.

In one embodiment, the multi-specific antibody includes a dimer of a tetra-specific antibody monomer therefor providing a tetra-specific antibody. In one embodiment, the application provides an isolated, purified, or non-natural existing multi-specific antibodies. In one embodiment, the application provides a tetra-specific antibody having an amino acid sequence having a percentage homology to SEQ ID No. 37-40. The percentage homology is not less than 70%, 80%, 90%, 95%, 98% or 99%.

The application further provides isolated nucleic acid sequence encoding the tetra-specific antibody monomers, the multi-specific antibodies, or the antigen-binding fragments thereof. In one embodiment, the nucleic acid encodes an amino acid sequence having a percentage homology to the

tetra-specific antibody monomer having a SEQ ID No. 37, 38. The percentage homology is not less than 70%, 80%, 90%, 95%, 98% or 99%.

The application further provides expression vectors and host cells comprising the nucleic acid sequences disclosed herein. In one embodiment, the host cell includes the expression vector. The host cell may be a prokaryotic cell or a eukaryotic cell.

The application further provides immuno-conjugates. In one embodiment, the immuno-conjugate includes a cytotoxic agent or an imaging agent linked to the multi-specific antibody disclosed herein through a linker.

The linker may be cleavable or non-cleavable. In one embodiment, the linker may include a covalent bond such as an ester bond, an ether bond, an amid bond, a disulphide bond, an imide bond, a sulfone bond, a phosphate bond, a phosphorus ester bond, a peptide bond, or a combination thereof. In one embodiment, the linker comprises a hydrophobic poly(ethylene glycol) linker.

The cytotoxic agent may include a chemotherapeutic agent, a growth inhibitory agent, a cytotoxic agent from class of calicheamicin, an antimitotic agent, a toxin, a radioactive isotope, a therapeutic agent, or a combination thereof. In one embodiment, the cytotoxic agent comprises a calicheamicin, ozogamicin, monomethyl auristatin E, emtansine, a derivative or a combination thereof.

The imaging agent may be any compound useful for imaging purpose. In one embodiment, the imaging agent may be radionuclide, a florescent agent, a quantum dots, or a combination thereof.

The application further provides pharmaceutical composition. In one embodiment, the pharmaceutical composition comprises a pharmaceutically acceptable carrier and the tetra-specific antibody monomer disclosed herein. In one embodiment, the pharmaceutical composition comprises a pharmaceutically acceptable carrier and the multi-specific antibody disclosed herein. In one embodiment, the pharmaceutical composition comprises a pharmaceutically acceptable carrier and the antigen-binding fragment disclosed herein. In one embodiment, the pharmaceutical composition comprises a pharmaceutically acceptable carrier and the immuno-conjugate disclosed herein.

In one embodiment, the pharmaceutical composition further includes a therapeutic agent. Example therapeutic agents include without limitation a radioisotope, radionuclide, a toxin, a chemotherapeutic agent, an antibody, an enzyme, or a combination thereof. In one embodiment, the therapeutic agent comprises an anti-estrogen agent, a receptor tyrosine kinase inhibitor, a kinase inhibitor, a cell cycle inhibitor, a DNA, RNA or protein synthesis inhibitor, a RAS inhibitor, or a combination thereof.

In one embodiment, the therapeutic agent comprises a check point inhibitor. In one embodiment, the therapeutic agent comprises an inhibitor of PD1, PDL1, CTLA4, 4-1BB, OX40, GITR, ICOS, LIGHT, TIM3, LAG3, TIGIT, CD40, CD27, HVEM, BTLA, VISTA, B7H4, CSF1R, NKG2D, CD73, a derivative or a combination thereof.

In a further aspect, the application provides methods for making the tetra-specific antibody monomers, the multi-specific antibodies, the antigen-binding fragments thereof, and immuno-conjugates thereof.

In one embodiment, the method includes the steps of culturing the host cell containing the nucleic acid sequences disclosed herein such that the DNA sequence encoding the antibody is expressed and purifying the antibody. In one embodiment, the antibody is a tetra-specific antibody.

In a further aspect, the application provides methods of using the tetra-specific antibody monomers, the multi-specific antibodies, the antigen-binding fragments thereof, and immuno-conjugates thereof for cancer treatment. In one embodiment, the method includes the step of administering tetra-specific antibody monomers, the multi-specific antibodies, the antigen-binding fragments thereof, and immuno-conjugates thereof, or pharmaceutical composition thereof to a subject in need of such treatment. In one embodiment, the method includes the step of administering to the subject an effective amount of the tetra-specific antibody.

In one embodiment, the method includes directly injecting into the tumour site an effective amount of multi-specific monomers, multi-specific antibodies, the immuno-conjugates, the antigen-binding fragment thereof.

Varieties of cancer may be prevented or treated. In one embodiment, the cancer may have cells expressing ROR1, CEA, HER2, EGFR, EGFR VIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypimay-3, gpA33, GD2, TROP2, NKG2D, BCMA, PD-L1, 4-1BB, CD3, CD19, CD20, CD33, CD137, CD123, CD22, or CD30. Example cancers include without limitation breast cancer, colorectal cancer, anal cancer, pancreatic cancer, gallbladder cancer, bile duct cancer, head and neck cancer, nasopharyngeal cancer, skin cancer, melanoma, ovarian cancer, prostate cancer, urethral cancer, lung cancer, non-small lung cell cancer, small cell lung cancer, brain tumor, glioma, neuroblastoma, esophageal cancer, , , gastric cancer, liver cancer, kidney cancer, bladder cancer, cervical cancer, endometrial cancer, thyroid cancer, eye cancer, sarcoma, bone cancer, leukemia, myeloma or lymphoma.

In one embodiment, the method may further include co-administering an effective amount of a therapeutic agent. In one embodiment, the therapeutic agent may include an antibody, a chemotherapy agent, an enzyme, or a combination thereof. In one embodiment, the therapeutic agent may include an anti-estrogen agent, a receptor tyrosine kinase inhibitor, a kinase inhibitor, a cell cycle inhibitor, a DNA, RNA or protein synthesis inhibitor, a RAS inhibitor, or a combination thereof. In one embodiment, the therapeutic agent may include a check point inhibitor. In one embodiment, the therapeutic agent may include an inhibitor of PD1, PD-L1, cd19, cd3, cd137, CTLA4, 4-1BB, OX40, GITR, ICOS, LIGHT, TIM3, LAG3, TIGIT, CD40, CD27, HVEM, BTLA, VISTA, B7H4, CSF1R, NKG2D, CD73, a derivative or a combination thereof.

In one embodiment, the therapeutic agent may include capecitabine, cisplatin, Cyclophosphamide, methotrexate, 5-fluorouracil, Doxorubicin, cyclophosphamide, Mustine, vincristine, procarbazine, prednisolone, bleomycin, vinblastine, dacarbazine, etoposide, Epirubicin, pemetrexed, folinic acid, gemicitabine, oxaliplatin, irinotecan, topotecan, camptothecin, docetaxel, paclitaxel, , fulvestrant, tamoxifen, letrozole, exemestane, anastrozole, aminoglutethimide, testolactone, vorozole, formestane, fadrozole, erlotinib, lafatinib, dasatinib, gefitinib, osimertinib, vandertanib, afatinib, imatinib, pazopinib, lapatinib, sunitinib, nilotinib, sorafenib, nab-palitaxel, Everolimus, temsirolimus, Dabrafenib,

vemurafenib, trametinib, vintafolide, apatinib, crizotinib, periforsine, olaparib, Bortezomib, tofacitinib, trastuzumab, a derivative or a combination thereof.

The subject may be a human. In one embodiment, the subject may be suffering from cancer. The application further provides solutions comprising an effective concentration of the multi-specific antibodies, monomers, or immuno-conjugates disclosed herein. In one embodiment, the solution is blood plasma in a subject.

The objectives and advantages of the disclosure may become apparent from the following detailed description of example embodiments thereof in connection with the accompanying drawings. Still other embodiments may become readily apparent to those skilled in the art from the following detailed description, wherein are described embodiments by way of illustrating the best mode contemplated. As may be realized, other and different embodiments are possible and the embodiments' several details are capable of modifications in various obvious respects, all without departing from their spirit and the scope. Accordingly, the drawings and detailed description are to be regarded as illustrative in nature and not as restrictive.

BRIEF DESCRIPTION OF THE FIGURES

The foregoing and other features of this disclosure may become more fully apparent from the following description and appended claims, taken in conjunction with the accompanying tables and figures. Understanding that these tables and figures depict only several embodiments arranged in accordance with the disclosure and are, therefore, not to be considered limiting of its scope, the disclosure may be described with additional specificity and detail through use of the accompanying drawings, in which:

FIGURE 1 is a diagram of the general format of a guided navigation control (GNC) tetra-specific antibody.

FIGURE 2 depicts experiment results showing re-directed T cell cytotoxicity (RTCC) assay with PBMC (peripheral blood mononuclear cells) as effectors and B-Acute Lymphoblastic Leukemia (B-ALL) cell line Kasumi-2 as targets

FIGURE 3 depicts experiment results showing proliferation of CD8+ T cells induced by tetra-specific GNC antibodies

FIGURE 4 depicts experiment results showing proliferation of CD4+ T cells induced by tetra-specific GNC antibodies

FIGURE 5 depicts experiment results showing secretion of Gamma Interferon from PBMC induced by tetra-specific GNC antibodies

FIGURE 6 depicts experiment results showing secretion of Granzyme B from PBMC induced by tetra-specific GNC antibodies

FIGURE 7 shows example tetra-specific antibodies with CD19 tumor antigen recognition domain.

FIGURE 8 provides a list of example tetra-specific antibodies disclosed herein.

DETAILED DESCRIPTION

In the following detailed description, reference is made to the accompanying drawings, which form a part hereof. In the drawings, similar symbols typically identify similar components, unless context dictates otherwise. The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. Other embodiments may be utilized, and other changes may be made, without departing from the spirit or scope of the subject matter presented herein. It may be readily understood that the aspects of the present disclosure, as generally described herein, and illustrated in the Figures, can be arranged, substituted, combined, separated, and designed in a wide variety of different configurations, all of which are explicitly contemplated herein.

The disclosure provides, among others, isolated antibodies, methods of making such antibodies, tetra-specific or multi-specific molecules, antibody-drug conjugates and/or immuno-conjugates composed from such antibodies or antigen binding fragments, pharmaceutical compositions containing the antibodies, tetra-specific or multi-specific molecules, antibody-drug conjugates and/or immuno-conjugates, method of making thereof, and method of using the disclosed molecules or composition for treatment of cancer.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polyepitopic specificity, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity. In some embodiments, the antibody may be monoclonal, polyclonal, chimeric, single chain, bispecific or bi-effective, simianized, human and humanized antibodies as well as active fragments thereof. Examples of active fragments of molecules that bind to known antigens include Fab, $F(ab')_2$, scFv and Fv fragments, including the products of an Fab immunoglobulin expression library and epitope-binding fragments of any of the antibodies and fragments mentioned above. In some embodiments, antibody may include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e. molecules that contain a binding site that immunospecifically bind an antigen. The immunoglobulin can be of any type (IgG, IgM, IgD, IgE, IgA and IgY) or class (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclasses of immunoglobulin molecule. In one embodiment, the antibody may be whole antibodies and any antigen-binding fragment derived from the whole antibodies. A typical antibody refers to heterotetrameric protein comprising typically of two heavy (H) chains and two light (L) chains. Each heavy chain is comprised of a heavy chain variable domain (abbreviated as VH) and a heavy chain constant domain. Each light chain is comprised of a light chain variable domain (abbreviated as VL) and a light chain constant domain. The VH and VL regions can be further subdivided into domains of hypervariable complementarity determining regions (CDR), and more conserved regions called framework regions (FR). Each variable domain (either VH or VL) is typically composed of three CDRs and four FRs, arranged in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 from amino-terminus to carboxy-terminus. Within the variable regions of the light and heavy chains there are binding regions that interacts with the antigen.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are

identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by the hybridoma method first described by Kohler & Milstein, *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567).

The monoclonal antibodies may include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 [1984]).

Monoclonal antibodies can be produced using various methods including mouse hybridoma or phage display (see Siegel. Transfus. Clin. Biol. 9:15-22 (2002) for a review) or from molecular cloning of antibodies directly from primary B cells (see Tiller. New Biotechnol. 28:453-7 (2011)). In the present disclosure antibodies were created by the immunization of rabbits with both human PD-L1 protein and cells transiently expressing human PD-L1 on the cell surface. Rabbits are known to create antibodies of high affinity, diversity and specificity (Weber et al. Exp. Mol. Med. 49:e305). B cells from immunized animals were cultured in vitro and screened for the production of anti-PD-L1 antibodies. The antibody variable genes were isolated using recombinant DNA techniques and the resulting antibodies were expressed recombinantly and further screened for desired features such as ability to inhibit the binding of PD-L1 to PD-1, the ability to bind to non-human primate PD-L1 and the ability to enhance human T-cell activation. This general method of antibody discovery is similar to that described in Seeber et al. PLOS One. 9:e86184 (2014).

The term "antigen- or epitope-binding portion or fragment" refers to fragments of an antibody that are capable of binding to an antigen (PD-L1 in this case). These fragments may be capable of the antigen-binding function and additional functions of the intact antibody. Examples of binding fragments include, but are not limited to a single-chain Fv fragment (scFv) consisting of the VL and VH domains of a single arm of an antibody connected in a single polypeptide chain by a synthetic linker or a Fab fragment which is a monovalent fragment consisting of the VL, constant light (CL), VH and constant heavy 1 (CH1) domains. Antibody fragments can be even smaller sub-fragments and can consist of domains as small as a single CDR domain, in particular the CDR3 regions from either the VL and/or VH domains (for example see Beiboer et al., J. Mol. Biol. 296:833-49 (2000)). Antibody fragments are produced using conventional

methods known to those skilled in the art. The antibody fragments are can be screened for utility using the same techniques employed with intact antibodies.

The "antigen-or epitope-binding fragments" can be derived from an antibody of the present disclosure by a number of art-known techniques. For example, purified monoclonal antibodies can be cleaved with an enzyme, such as pepsin, and subjected to HPLC gel filtration. The appropriate fraction containing Fab fragments can then be collected and concentrated by membrane filtration and the like. For further description of general techniques for the isolation of active fragments of antibodies, see for example, Khaw, B. A. et al. J. Nucl. Med. 23:1011-1019 (1982); Rousseaux et al. Methods Enzymology, 121:663-69, Academic Press, 1986.

Papain digestion of antibodies produces two identical antigen binding fragments, called "Fab" fragments, each with a single antigen binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragment may contain the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other, chemical couplings of antibody fragments are also known.

"Fv" is the minimum antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, delta, epsilon, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

A “humanized antibody” refers to a type of engineered antibody having its CDRs derived from a non-human donor immunoglobulin, the remaining immunoglobulin-derived parts of the molecule being derived from one (or more) human immunoglobulin(s). In addition, framework support residues may be altered to preserve binding affinity. Methods to obtain “humanized antibodies” are well known to those skilled in the art. (see, e.g., Queen et al., Proc. Natl Acad Sci USA, 86:10029-10032 (1989), Hodgson et al., Bio/Technology, 9:421 (1991)). In one embodiment, the “humanized antibody” may be obtained by genetic engineering approach that enables production of affinity-matured humanlike polyclonal antiboies in large animals such as, for example, rabbits (see, e.g. U.S. Pat. No. 7,129,084).

The terms “polypeptide”, “peptide”, and “protein”, as used herein, are interchangeable and are defined to mean a biomolecule composed of amino acids linked by a peptide bond.

The terms “a”, “an” and “the” as used herein are defined to mean “one or more” and include the plural unless the context is inappropriate.

By “isolated” is meant a biological molecule free from at least some of the components with which it naturally occurs. "Isolated," when used to describe the various polypeptides disclosed herein, means a polypeptide that has been identified and separated and/or recovered from a cell or cell culture from which it was expressed. Ordinarily, an isolated polypeptide may be prepared by at least one purification step. An "isolated antibody," refers to an antibody which is substantially free of other antibodies having different antigenic specificities.

"Recombinant" means the antibodies are generated using recombinant nucleic acid techniques in exogeneous host cells.

The term “antigen” refers to an entity or fragment thereof which can induce an immune response in an organism, particularly an animal, more particularly a mammal including a human. The term includes immunogens and regions thereof responsible for antigenicity or antigenic determinants.

Also as used herein, the term “immunogenic” refers to substances which elicit or enhance the production of antibodies, T-cells or other reactive immune cells directed against an immunogenic agent and contribute to an immune response in humans or animals. An immune response occurs when an individual produces sufficient antibodies, T-cells and other reactive immune cells against administered immunogenic compositions of the present disclosure to moderate or alleviate the disorder to be treated.

"Specific binding" or "specifically binds to" or is "specific for" a particular antigen or an epitope means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target.

Specific binding for a particular antigen or an epitope can be exhibited, for example, by an antibody having a KD for an antigen or epitope of at least about 10^{-4} M, at least about 10^{-5} M, at least about 10^{-6}

M, at least about 10^{-7} M, at least about 10^{-8} M, at least about 10^{-9}, alternatively at least about 10^{-10} M, at least about 10^{-11} M, at least about 10^{-12} M, or greater, where KD refers to a dissociation rate of a particular antibody-antigen interaction. In some embodiments, an antibody that specifically binds an antigen may have a KD that is 20-, 50-, 100-, 500-, 1000-, 5,000-, 10,000- or more times greater for a control molecule relative to the antigen or epitope.

Also, specific binding for a particular antigen or an epitope can be exhibited, for example, by an antibody having a KA or Ka for an antigen or epitope of at least 20-, 50-, 100-, 500-, 1000-, 5,000-, 10,000- or more times greater for the epitope relative to a control, where KA or Ka refers to an association rate of a particular antibody-antigen interaction.

"Homology" between two sequences is determined by sequence identity. If two sequences which are to be compared with each other differ in length, sequence identity preferably relates to the percentage of the nucleotide residues of the shorter sequence which are identical with the nucleotide residues of the longer sequence. Sequence identity can be determined conventionally with the use of computer programs. The deviations appearing in the comparison between a given sequence and the above-described sequences of the disclosure may be caused for instance by addition, deletion, substitution, insertion or recombination.

In one aspect, the application provides tetra-specific antibody monomers, antigen-binding fragments, and multi-specific antibodies. In one embodiment, the application provides tetra-specific antibodies.

In one embodiment, the disclosure provides tetra-specific antibodies with a binding specificity against four different antigen targets. In one embodiment, the antigen targets are tumor specific antigens, T cell receptor CD3 component, or immune checkpoint molecules. The tetra-specific antibodies may directly engage body's endogenous T cells to kill tumor cells independent of tumor antigen presentation by MHC to the antigen specific T cell receptors. In addition, the immune checkpoint modulating component of the tetra-specific antibodies may overcome the immunosuppressive tumor microenvironment to fully activate the exhausted T cells within the tumor microenvironment.

In one embodiment, the tetra-specific antibodies may have unique properties of directly engaging T cells at the same time modulating immune checkpoint or inhibiting Treg or other inhibitory immune cells or targeting tumor with component against tumor antigens. It will show benefit to the patients where BiTE or CAR-T treatment isn't appropriate. Particularly, the tetra-specific antibodies could demonstrate clinical benefit in solid tumor where BiTE-like technology or CAR-T treatment, which yet to show clinical benefit due to the limitations imposed by the inhibitory tumor microenvironment.

In one embodiment, the disclosure provides an engineered antibody with 4 different binding domains or a "tetra-specific antibody". One binding domain is specific for CD3 on T cells, a second binding domain is specific for a tumor associated antigen including but not limited to ROR1, CEA, HER2, EGFR, EGFRvIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2, BCMA, CD19, CD20, CD33, CD123, CD22, CD30, and a third and fourth binding domains are specific for two distinct immune checkpoint modulators such as PD-L1, PD-1, OX40, 4-1BB, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40, VISTA, ICOS, BTLA, Light, HVEM, CD73, CD39, etc.

In one embodiment, the tetra-specific molecules (FIGURE 1) target human CD19 SEQ ID NO. 25-32 a tumor associated antigen. In some embodiments, these targeted tetra-specific proteins carry an anti-human PD-L1 (SEQIDs 9-12), an anti-human 4-1BB (SEQIDs 13-24), an anti-human CD3 binding domain (SEQIDs 1-8). The tetra-specific molecule binding domains are arrayed such that the binding domain placement proceeds from the N-terminus to D1, scFv VLVH, followed by D2, scFv VLVH, D3 which is in the Fab position in this class of tetra-specific proteins, followed by the human IgG1 Fc and an scFv, VHVL, in D4.

In one embodiment, tetra-specific protein SI-38E34 (SEQIDs 37-40) is composed of an anti-human CD19 21D4 scFv, anti-human CD3 284A10 scFv, anti-human CD137 (Fab) and anti-human PD-L1 clone PL221G5 scFv occupying positions D1, D2, D3 and D4, respectively. D1, D2 and D3 are genetically linked through a 10 amino acid (G4S) x 2 linker, as is the C-terminus of the human IgG1 Fc and D4, resulting in a contiguous ~150 kDa heavy chain monomer peptide containing the binding specificities outlined above. All scFv molecules described herein contain a 20 amino acid flexible gly-gly-gly-gly-ser (G4S) X4 linker that operably links the VH and VL, regardless of the V-region orientation (LH or HL). The remaining position in the tetra-specific protein, Domain 3 (D3), consists of an IgG1 heavy chain, VH-CH1-Hinge-CH2-CH3, and its corresponding light chain, VL-CL, which can be either a kappa or lambda chain. D1 and D2 are genetically linked through a 10 amino acid (G4S) x 2 linker, as are D2, D3 and D4 resulting in a contiguous ~150 kDa heavy chain monomer peptide. When co-transfected with the appropriate light chain, the final symmetric tetra-specific peptide can be purified through the IgG1 Fc (Protein A/Protein G) and assayed to assess functional activity. Heavy and light chain gene "cassettes" were previously constructed such that V-regions could be easily cloned using either restriction enzyme sites (HindIII/NheI for the heavy chain and HindIII/BsiWI for the light chain) or "restriction-free cloning" such as Gibson Assembly (SGI-DNA, La Jolla, CA), Infusion (Takara Bio USA) or NEBuilder (NEB, Ipswich, MA), the latter of which was used here.

In one embodiment, tetra-specific proteins are produced through a process that involves design of the intact molecule, synthesis and cloning of the nucleotide sequences for each domain, expression in mammalian cells and purification of the final product. Nucleotide sequences were assembled using the Geneious 10.2.3 software package (Biomatters, Auckland, NZ) and broken up into their component domains for gene synthesis (Genewiz, South Plainsfield, NJ).

In one embodiment, SI-35E18 (SEQID 65 and 67) was split into its component domains where the anti-4-1BB scFv, VLVH, occupies D1, anti-human PD-L1 clone PL230C6 occupies D2 (Fab position), anti-human ROR1 Ig domain-specific clone 323H7 VHVL scFv occupies D3, and anti-human CD3 scFv, VHVL, occupies the C-terminal D4. Using NEBuilder web-based tools, 5' and 3' nucleotides were appended to each of the domains depending on their position in the larger protein so that each domain overlaps its flanking domains by 20-30 nucleotides which direct site-specific recombination, thus genetically fusing each domain in a single gene assembly step. Due to the high number of homologous regions in the tetra-specific nucleotide sequence, the N-terminal domains 1 and 2 are assembled separately from the C-terminal D3 and D4. The N- and C-terminal fragments were then assembled together in a second NEBuilder reaction. A small aliquot was transformed into *E.coli* DH10b (Invitrogen, Carlsbad, CA) and plated on TB + carbenicillin 100ug/ml plates (Teknova, Hollister, CA) and incubated at 37C overnight.

Resultant colonies were selected and 2ml overnight cultures inoculated in TB + carbenicillin. DNA was prepared (Thermo-Fisher, Carlsbad, CA) from overnight cultures and subsequently sequenced (Genewiz, South Plainsfield, NJ) using sequencing primers (Sigma,St. Louis, MO) flanking each domain. All DNA sequences were assembled and analyzed in Geneious.

In another aspect, the application provides pharmaceutical compositions including the multi-specific antibody monomers, the multi-specific antibodies, the antigen-binding fragments, and the immuno-conjugates thereof, and methods of using the disclosed antibodies or pharmaceutical compositions for treatment of cancer.

The advantages of treating cancer using the disclosed multi-specific antibody monomers, the multi-specific antibodies, the antigen-binding fragments, the immuno-conjugates and composition thereof over currently existing therapies include, without limitation: 1) Inclusion of an IgG Fc domain will confer the characteristic of a longer half-life in serum compared to a bi-specific BiTe molecule; 2) Inclusion of two binding domains that are specific for immune checkpoint modulators, that can inhibit the suppressive pathways and engage the co-stimulatory pathways at the same time; and 3) Cross-link CD3 on T cells with tumor associated antigens thus “re-directing” T cells to kill the tumor without the need to remove T cells from the patient and genetically modify them to be specific for the tumor cell before re-introducing them back into the patient as done for chimeric antigen receptor T cells (CAR-T).

Formulation of the pharmaceutical composition may be accomplished according to standard methodology know to those of ordinary skill in the art.

In one embodiment, the antibodies and monomers according to the disclosure may be prepared in a physiologically acceptable formulation and may comprise a pharmaceutically acceptable carrier, diluent and/or excipient using known techniques. For example, the antibody according to the disclosure and as described herein including any functionally equivalent antibody or functional parts thereof, in particular, the monoclonal antibody including any functionally equivalent antibody or functional parts thereof is combined with a pharmaceutically acceptable carrier, diluent and/or excipient to form a therapeutic composition. Formulation of the pharmaceutical composition according to the disclosure may be accomplished according to standard methodology know to those of ordinary skill in the art.

With respect to the formulation of suitable compositions for administration to a subject such as a human patient in need of treatment, the antibodies disclosed herein may be mixed or combined with pharmaceutically acceptable carriers known in the art dependent upon the chosen route of administration. There are no particular limitations to the modes of application of the antibodies disclosed herein, and the choice of suitable administration routes and suitable compositions are known in the art without undue experimentation.

Suitable pharmaceutical carriers, diluents and/or excipients are well known in the art and include, for example, phosphate buffered saline solutions, water, emulsions such as oil/water emulsions.

“Pharmaceutically acceptable” refers to those compounds, materials, compositions, and dosage forms which are, within the scope of sound medical judgment, suitable for use contact with the tissues of

human beings or animals without excessive toxicity, irritation, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

In one embodiment, the pharmaceutical composition may include proteinaceous carriers such as, for example, serum albumin or immunoglobulin, particularly of human origin. Further biologically active agents may be present in the pharmaceutical composition of the disclosure dependent on the intended use. In one embodiment, the proteinaceous pharmaceutically active matter may be present in amounts between 1 ng and 10 mg per dose. Generally, the regime of administration should be in the range of between 0.1 μg and 10 mg of the antibody according to the disclosure, particularly in a range 1.0 μg to 1.0 mg, and more particularly in a range of between 1.0 μg and 100 μg, with all individual numbers falling within these ranges also being part of the disclosure. If the administration occurs through continuous infusion a more proper dosage may be in the range of between 0.01 μg and 10 mg units per kilogram of body weight per hour with all individual numbers falling within these ranges also being part of the disclosure.

The compositions of the present disclosure may be administered to a subject in the form of a solid, liquid or aerosol at a suitable, pharmaceutically effective dose. Examples of solid compositions include pills, creams, and implantable dosage units. Pills may be administered orally. Therapeutic creams may be administered topically. Implantable dosage units may be administered locally, for example, at a tumor site, or may be implanted for systematic release of the therapeutic composition, for example, subcutaneously. Examples of liquid compositions include formulations adapted for injection intramuscularly, subcutaneously, intravenously, intra-arterially, and formulations for topical and intraocular administration. Examples of aerosol formulations include inhaler formulations for administration to the lungs.

It is well known to those of ordinary skill in the art that the dosage of the composition may depend on various factors such as, for example, the condition of being treated, the particular composition used, and other clinical factors such as weight, size, sex and general health condition of the patient, body surface area, the particular compound or composition to be administered, other drugs being administered concurrently, and the route of administration.

The term “therapeutically effective amount” or “effective amount” refers to the amount of antibody which, when administered to a human or animal, elicits a response which is sufficient to result in a therapeutic effect in said human or animal, e.g., to ameliorate disease in a subject. The effective amount is readily determined by one of ordinary skill in the art following routine procedures. Where the disease is a cancer, the effective amount of the drug may inhibit (for example, slow to some extent, inhibit or stop) one or more of the following example characteristics including, without limitation, cancer cell growth, cancer cell proliferation, cancer cell motility, cancer cell infiltration into peripheral organs, tumor metastasis, and tumor growth. Wherein the disease is a mayer, the effective amount of the drug may alternatively do one or more of the following when administered to a subject: slow or stop tumor growth, reduce tumor size (for example, volume or mass), relieve to some extent one or more of the symptoms associated with the cancer, extend progression free survival, result in an objective response (including, for example, a partial response or a complete response), and increase overall survival time.

To the extent the drug may prevent growth and/or kill existing cancer cells, it is cytostatic and/or cytotoxic.

A person skilled in the art have the ability to determine the effective amount or concentration of the antibodies disclosed therein to effective treat a condition such as a cancer. Other parameters such as the proportions of the various components in the pharmaceutical composition, administration does and frequency may be obtained by a person skilled in the art without undue experimentation. For example, a suitable solution for injection may contain, without limitation, from about 1 to about 20, from about 1 to about 10 mg antibodies per ml. The example dose may be, without limitation, from about 0.1 to about 20, from about 1 to about 5mg/Kg body weight. The example administration frequency could be, without limitation, once per day or three times per week.

The compositions may be administered by standard routes of administration. In general, the composition may be administered by topical, oral, rectal, nasal, interdermal, intraperitoneal, or parenteral (for example, intravenous, subcutaneous, or intramuscular) routes. In addition, the composition may be incorporated into sustained release matrices such as biodegradable polymers, the polymers being implanted in the vicinity of where delivery is desired, for example, at the site of a tumor. The method includes administration of a single dose, administration of repeated doses at predetermined time intervals, and sustained administration for a predetermined period of time.

Although many forms of administration are possible, an example administration form would be a solution for injection, in particular for intravenous or intra-arterial injection. Usually, a suitable pharmaceutical composition for injection may include pharmaceutically suitable carriers or excipients such as, without limitation, a buffer, a surfactant, or a stabilizer agent. Example buffers may include, without limitation, acetate, phosphate or citrate buffer. Example surfactants may include, without limitation, polysorbate. Example stabilizer may include, without limitation, human albumin.

In one embodiment, the administration may be parenterally, e.g. intravenously. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions and emulsions. Non-aqueous solvents include without being limited to it, propylene glycol, polyethylene glycol, vegeTABLE oil such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous solvents may be chosen from the group consisting of water, alcohol/aqueous solutions, emulsions or suspensions including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose) and others. Preservatives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, inert gases, etc.

The antibody monomers, antibodies, antigen-binding fragments and immuno-conjugates thereof may be used in combination with a therapeutic agent or a composition comprising a therapeutic agent for treatment purpose.

In some embodiments, the multi-specific antibody molecule is used in combination with one or more additional therapeutic agents at an effective amount. The additional therapeutic agent includes an

antibody, a chemotherapy agent, an enzyme, or a combination thereof. In some embodiment, the additional therapeutic agent may be an anti-estrogen agent, a receptor tyrosine kinase inhibitor, a kinase inhibitor, a cell cycle inhibitor, a DNA, RNA or protein synthesis inhibitor, a RAS inhibitor, or a combination thereof. In some embodiments, the additional therapeutic agent may be a check point inhibitor. In some embodiments, therapeutic agent comprises inhibitors of PD1, PDL1, CTLA4, 4-1BB, OX40, GITR, ICOS, LIGHT, TIM3, LAG3, TIGIT, CD40, CD27, HVEM, BTLA, VISTA, B7H4, CSF1R, NKG2D, CD73, a derivative or a combination thereof.

In one embodiment, the therapeutic agent may include capecitabine, cisplatin, trastuzumab, fulvestrant, tamoxifen, letrozole, exemestane, anastrozole, aminoglutethimide, testolactone, vorozole, formestane, fadrozole, letrozole, erlotinib, lafatinib, dasatinib, gefitinib, imatinib, pazopinib, lapatinib, sunitinib, nilotinib, sorafenib, nab-palitaxel, a derivative or a combination thereof. In one embodiment, the therapeutic agent may include capecitabine, cisplatin, Cyclophosphamide, methotrexate, 5-fluorouracil, Doxorubicin, cyclophosphamide, Mustine, vincristine, procarbazine, prednisolone, bleomycin, vinblastine, dacarbazine, etoposide, Epirubicin, pemetrexed, folinic acid, gemicitabine, oxaliplatin, irinotemay, topotemay, camptothecin, docetaxel, paclitaxel, , fulvestrant, tamoxifen, letrozole, exemestane, anastrozole, aminoglutethimide, testolactone, vorozole, formestane, fadrozole, letrozole, erlotinib, lafatinib, dasatinib, gefitinib, osimertinib, vandertanib, afatinib, imatinib, pazopinib, lapatinib, sunitinib, nilotinib, sorafenib, nab-palitaxel, Everolimus, temsirolimus, Dabrafenib, vemurafenib, trametinib, vintafolide, apatinib, crizotinib, periforsine, olaparib, Bortezomib, tofacitinib, a derivative or a combination thereof.

Cancers, including breast cancer, colorectal cancer, pancreatic cancer, head and neck cancer, melanoma, ovarian cancer, prostate cancer, non-small lung cell cancer, glioma, esophageal cancer, nasopharyngeal cancer, anal cancer, rectal cancer, gastric cancer, bladder cancer, cervical cancer, or brain cancer, may express cancer-associated genes. Inhibition of cancer-associated activity with specific monoclonal antibodies or antigen-binding fragment may have therapeutic effect on cancers. Furthermore, administering a therapeutically effective amount of composition comprising monoclonal antibodies or antigen-binding fragment specific for cancer-associated protein may cure, prevent, ameliorate, and delay the development or metastasis of cancers, through the effect of the cytotoxic agent.

The present disclosure may be understood more readily by reference to the following detailed description of specific embodiments and examples included herein. Although the present disclosure has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the disclosure.

EXAMPLES

Example 1: Re-directed T cell cytotoxicity (RTCC) assay with PBMC (peripheral blood mononuclear cells) as effectors and B-Acute Lymphoblastic Leukemia (B-ALL) cell lines Kasumi-2 and NALM-6 as targets.

The Tetra-specific antibodies listed in tables 1 and 2 were tested for RTCC activity against the B-ALL cell lines Kasumi-2 and Nalm-6 using human PBMC as effectors. Both the Kasumi-2 and Nalm-6 target cells

were previously transfected with green fluorescent protein (GFP) and FACS-sorted to create a population of cells where greater than 99% expressed GFP. The GFP+ Kasumi-2 and GFP+ Nalm-6 cells were counted and set to a density of 100,000 cells/ml in assay media. Human PBMC were counted and set to a density of 100,000 cells/ml. Antibodies were prepared at 2X final concentration and titrated 1:10 across 6 wells of a 96 well plate in assay media. In the destination 96 well plate the target cells, PBMC, and serially titrated antibodies were combined by adding 50ul of target cells (5,000), 50 ul of PBMC cells (5,000), and 100 ul of each antibody dilution to each well of the assay. The assay plate was incubated at 37C for 8 days and then 100ul of supernatant was transferred into a new 96 well and frozen at -80C for later analysis. The cells were re-suspended by pipetting and transferred to a 384 well plate. Cells were stained with an antibody cocktail that included anti-CD4 and anti-CD8 directly conjugated commercial antibodies. The cells remaining in the wells were washed and re-suspended in assay buffer containing anti-CD4 (biolegend Cat#317436) and anti-CD8 (biolegend Cat#557746) directly conjugated commercial antibodies plus 7AAD live/dead stain and counting beads and then analyzed on a BD LSRII Fortessa. The number of CD4+, CD8+ and GFP+ target cells per well were determined. As shown in FIGURE 2, the tetra-specific antibodies SI-38X34, 35, and 36 induce T cell killing of most of the target NALM-6 cells at a concentration of 0.05 pM which is roughly 10-fold more potent than the bi-specific antibodies SI-38X19 and HD37 x I2C. Since the bi-specific SI-38X19 has the 21D4 (CD19) and 284A10 (CD3) binding domains as in the tetra-specific antibodies SI-38E34, 35, and 36 but the tetra-specific antibodies have the additional domains of 420H5, 466F6, 460C3 (41BB) and PL221 (PDL1) this suggests that the additional 41BB and/or PDL1 binding domains in the tetra-specific antibodies have an enhancing effect on T cell killing of the target cells. In addition, as shown in FIGURE 3 the bi-specific antibody 21D4 x 284A10 strongly induces proliferation of CD8+ T cells as does the bi-specific HD37 x I2C at 10-fold higher concentration than the 21D4 x 284A10 bid-specific. However, the tetra-specific antibodies SI-38E34, 35, and 36 induce much lower levels of CD8+ T cell proliferation at similar concentrations. These data, combined with the RTCC data in FIGURE 2 suggest that the tetra-specific antibodies induce differentiation of CD8+ T cells into terminal cytotoxic T cells better than the bi-specific antibodies. As shown in FIGURE 4, the bi-specific antibodies tested also induce greater proliferation of CD4+ T cells, similar to the effect on proliferation of CD8+ T cells, and the tetra-specific antibodies induce much lower levels of CD4+ T cell proliferation.

Example 2: ELISA analysis of gamma interferon and granzyme B in the culture supernatants from day 8 RTCC with CD19-specific GNC antibodies.

The well supernatants that were stored at -80 were thawed and analyzed for the level of interferon gamma and granzyme B using the g-IFN and GrB kits from R&D systems (no.DY285B and no.DY2906-05) as per the manufacturers recommended protocol. QuantaRed™ Enhanced Chemifluorescent HRP Substrate (ThermoFisher Scientific no.15159) was added to each well of the ELISA plates and used as per the manufactures instructions. As shown in figure 6, the bi-specific 21D4 x 284A10 induced a high level of gamma interferon secretion from PBMC at 50 pM of antibody, almost identical to the tetra-specific antibody SI-34E34, whereas the other tetra-specific antibodies SI-34E35 and 36 as well as the bi-specific HD37 x I2C did induce gamma interferon secretion from PBMC but a much lower levels. As shown in figure 6, the bi-specific 21D4 x 284A10 induced a high level of granzyme B secretion from PBMC at 50

pM of antibody, almost identical to the tetra-specific antibody SI-34E34, whereas the other tetra-specific antibodies SI-34E35 and 36 as well as the bi-specific HD37 x I2C did induce granzyme B secretion from PBMC but at slightly lower levels. While the tumor cell killing mediated by the tetra-specific antibodies SI-38E34, 35, and 36 was quite similar as shown in FIGURE 3, the amount of granzyme B secreted from PBMC was highest with the tetra-specific antibody SI-38E34 at about 2-fold higher levels compare to the other 2 tetra-specific antibodies SI-38E35 and 36.

While the present disclosure has been described with reference to particular embodiments or examples, it may be understood that the embodiments are illustrative and that the disclosure scope is not so limited. Alternative embodiments of the present disclosure may become apparent to those having ordinary skill in the art to which the present disclosure pertains. Such alternate embodiments are considered to be encompassed within the scope of the present disclosure. Accordingly, the scope of the present disclosure is defined by the appended claims and is supported by the foregoing description. All references cited or referred to in this disclosure are hereby incorporated by reference in their entireties.

MULTI-SPECIFIC ANTIBODIES AND METHODS OF MAKING AND USING THEREOF

SEQUENCE LISTING

tetra-specific sequence listing

CDR's underlined in amino acid sequences

>SEQ ID:01 anti-CD3 284A10 VHv1 nt

GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTCCAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTC
TGGATTCACCATCAGTACCAATGCAATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGATCGGAG
TCATTACTGGTCGTGATATCACATACTACGCGAGCTGGGCGAAAGGCAGATTCACCATCTCCAGAGACAATTCCAA
GAACACGCTGTATCTTCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGCGCGACGGTGG
ATCATCTGCTATTACTAGTAACAACATTTGGGGCCAAGGAACTCTGGTCACCGTTTCTTCA

>SEQ ID:02 anti-CD3 284A10 VHv1 aa

EVQLVESGGGLVQPGGSLRLSCAASGFTISTNAMSWVRQAPGKGLEWIGVITGRDITYYASWAKGRFTISRDNSKNTLY
LQMNSLRAEDTAVYYCARDGGSSAITSNNIWGQGTLVTVSS

>SEQ ID:03 anti-CD3 284A10 VLv1 nt

GACGTCGTGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCAATTGCCAAGCCA
GTGAGAGCATTAGCAGTTGGTTAGCCTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGAAG
CATCCAAACTGGCATCTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGAGTTCACTCTCACCATCA
GCAGCCTGCAGCCTGATGATTTTGCAACTTATTACTGCCAAGGCTATTTTTATTTTATTAGTCGTACTTATGTAAATT
CTTTCGGCGGAGGGACCAAGGTGGAGATCAAA

>SEQ ID:04 anti-CD3 284A10 VLv1 aa

DVVMTQSPSTLSASVGDRVTINCQASESISSWLAWYQQKPGKAPKLLIYEASKLASGVPSRFSGSGSGTEFTLTISSLQPD
DFATYYCQGYFYFISRTYVNSFGGGTKVEIK

>SEQ ID:05 anti-CD3 I2C VH nt

CAGGTGCAATTGGTGGAAAGCGGAGGGGGACTGGTGCAGCCCGGGGGAAGTCTGAAGCTGTCCTGTGCCGCCA
GCGGCTTTACCTTCAACAAGTACGCCATGAATTGGGTCCGACAGGCCCCAGGGAAAGGCCTGGAATGGGTGGCA
CGGATTCGGTCCAAGTACAACAACTACGCCACCTACTACGCTGACTCCGTGAAGGACAGATTCACCATCAGCCGGG
ACGACTCTAAGAACACCGCCTATCTGCAGATGAACAACCTGAAAACCGAGGATACAGCTGTGTACTATTGTGTGCG
GCACGGCAACTTCGGCAACTCCTACATCTCCTACTGGGCCTATTGGGGACAGGGAACACTGGTCACCGTGTCTAGC

>SEQ ID:06 anti-CD3 I2C VH aa

QVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDS
KNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGTLVTVSS

>SEQ ID:07 anti-CD3 I2C VL nt

CAGACCGTGGTCACCCAGGAACCTTCCCTGACCGTCTCCCCAGGCGGCACCGTGACCCTGACCTGTGGCTCCTCTA
CCGGCGCTGTGACCTCCGGCAACTACCCTAACTGGGTGCAGCAGAAACCCGGACAGGCTCCTAGAGGCCTGATCG
GCGGCACCAAGTTTCTGGCCCCTGGCACCCCTGCCAGATTCTCCGGCTCCCTGCTGGGAGGCAAGGCCGCTCTGAC
CCTGTCTGGCGTGCAGCCTGAGGACGAGGCCGAGTACTACTGTGTGCTGTGGTACTCCAACAGATGGGTGTTCGG
AGGCGGCACAAAGCTGACCGTGCTGTCCTCG

>SEQ ID:08 anti-CD3 I2C VL aa

QTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQQKPGQAPRGLIGGTKFLAPGTPARFSGSLLGGKAALTLS
GVQPEDEAEYYCVLWYSNRWVFGGGTKLTVLSS

>SEQ ID:09 anti-PD-L1 PL221G5 VHv1 nt

GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCT
GGATTCTCCTTCAGTAGCGGGTACGACATGTGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGATCGC
ATGCATTGCTGCTGGTAGTGCTGGTATCACTTACGACGCGAACTGGGCGAAAGGCCGGTTCACCATCTCCAGAGA
CAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAG
ATCGGCGTTTTCGTTCGACTACGCCATGGACCTCTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGC

>SEQ ID:10 anti-PD-L1 PL221G5 VHv1 aa

EVQLLESGGGLVQPGGSLRLSCAASGFSFSSGYDMCWVRQAPGKGLEWIACIAAGSAGITYDANWAKGRFTISRDNSK
NTLYLQMNSLRAEDTAVYYCARSAFSFDYAMDLWGQGTLVTVSS

>SEQ ID:11 anti-PD-L1 PL221G5 VLv1 nt

GACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCCAGGCCA
GTCAGAGCATTAGTTCCCACTTAAACTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATAAGGC
ATCCACTCTGGCATCTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGAATTTACTCTCACCATCAGC
AGCCTGCAGCCTGATGATTTTGCAACTTATTACTGCCAACAGGGTTATAGTTGGGGTAATGTTGATAATGTTTTCG
GCGGAGGGACCAAGGTGGAGATCAAA

>SEQ ID:12 anti-PD-L1 PL221G5 VLv1 aa

DIQMTQSPSTLSASVGDRVTITCQASQSISSHLNWYQQKPGKAPKLLIYKASTLASGVPSRFSGSGSGTEFTLTISSLQPD
DFATYYCQQGYSWGNVDNVFGGGTKVEIK

>SEQ ID:13 anti-4-1BB 420H5 VHv3 nt

CAGTCGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGG
ATTCTCCTTCAGTAGCAACTACTGGATATGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGATCGCATG
CATTTATGTTGGTAGTAGTGGTGACACTTACTACGCGAGCTCCGCGAAAGGCCGGTTCACCATCTCCAGAGACAAT

TCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAGAGAT
AGTAGTAGTTATTATATGTTTAACTTGTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGC

>SEQ ID:14 anti-4-1BB 420H5 VHv3 aa

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LYLQMNSLRAEDTAVYYCARDSSSYYMFNLWGQGTLVTVSS

>SEQ ID:15 anti-4-1BB 420H5 VLv3 nt

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ATCCGATCTGGCATCTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACCATCAGC
AGCCTGCAGCCTGATGATTTTGCAACTTATTACTGCCAAGGCGGTTACTATACTAGTAGTGCTGATACGAGGGGTG
CTTTCGGCGGAGGGACCAAGGTGGAGATCAAA

>SEQ ID:16 anti-4-1BB 420H5 VLv3 aa

ALVMTQSPSTLSASVGDRVTINCQASEDIDTYLAWYQQKPGKAPKLLIFYASDLASGVPSRFSGSGSGTEFTLTISSLQPD
DFATYYCQGGYYTSSADTRGAFGGGTKVEIK

>SEQ ID:17 anti-4-1BB 466F6 VHv2 nt

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AGTAGTGGTGGTAATGTATACTACGCGAGCTCCGCGAGAGGCAGATTCACCATCTCCAGACCCTCGTCCAAGAAC
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AGTGATCCTATGTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGC

>SEQ ID:18 anti-4-1BB 466F6 VHv2 aa

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>SEQ ID:19 anti-4-1BB 466F6 VLv5 nt

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AGCCAATCTGGCATCTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGC
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>SEQ ID:20 anti-4-1BB 466F6 VLv5 aa

DVVMTQSPSSVSASVGDRVTITCQASQNIRTYLSWYQQKPGKAPKLLIYAAANLASGVPSRFSGSGSGTDFTLTISDLEP
GDAATYYCQSTYLGTDYVGGAFGGGTKVEIK

>SEQ ID:21 anti-4-1BB 460C3 VHv1 nt

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AATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAGA
GAAGGTAGCCTGTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGC

>SEQ ID:22 anti-4-1BB 460C3 VHv1 aa

EVQLLESGGGLVQPGGSLRLSCAASGIDFSRRYYMCWVRQAPGKGLEWIACIYTGSRDTPHYASSAKGRFTISRDNSKN
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>SEQ ID:23 anti-4-1BB 460C3 VLv1 nt

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TGCATCCACTCTGGCATCTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACCATC
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>SEQ ID:24 anti-4-1BB 460C3 VLv1 aa

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>SEQ ID:25 anti-CD19 21D4 VH nt

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ATCAGGACTGCCTACCTGCAGTGGAGTAGCCTGAAGGCCTCGGACACCGCTATGTATTACTGTGCGAGACATGTT
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>SEQ ID:26 anti-CD19 21D4 VH aa

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CCTCCAGTTTGGAAAGTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAG
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>SEQ ID:28 anti-CD19 21D4 VL aa

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>SEQ ID:29 anti-CD19 HD37 VH nt

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GATTTGGCCTGGAGATGGTGATACTAACTACAATGGAAAGTTCAAGGGGAAAGCCACTCTGACTGCAGACGAATC
CTCCAGCACAGCCTACATGCAACTCAGCAGCCTAGCATCTGAGGACTCTGCGGTCTATTTCTGTGCAAGACGGGAG
ACTACGACGGTAGGCCGTTATTACTATGCTATGGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCC

>SEQ ID:30 anti-CD19 HD37 VH aa

QVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLTADESS
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>SEQ ID:31 anti-CD19 HD37 VL nt

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CATCTATGATGCTTCCAATCTAGTTTCTGGGATCCCACCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTTCACC
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>SEQ ID:32 anti-CD19 HD37 VL nt

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>SEQ ID:33 human IgG1 null (G1m-fa with ADCC/CDC null mutations) nt

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GTCTCCGGGT

>SEQ ID:34 human IgG1 null (G1m-fa with ADCC/CDC null mutations) aa

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HYTQKSLSLSPG

>SEQ ID:35 human Ig Kappa nt

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CTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCA
AAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGA
GCTTCAACAGGGGAGAGTGT

>SEQ ID:36 human Ig Kappa aa

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE
KHKVYACEVTHQGLSSPVTKSFNRGEC

>SEQ ID:37 SI-38E34 (21D4-LH-scFv x 284A10-L1H1-scFv x 420H5-Fab x PL221G5-H1L1-scFv) heavy chain nt

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CCTCCAGTTTGGAAAGTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAG
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>SEQ ID:38 SI-38E34 (21D4-LH-scFv x 284A10-L1H1-scFv x 420H5-Fab x PL221G5-H1L1-scFv) heavy chain aa

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NLWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
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>SEQ ID:39 SI-38E34 (21D4-LH-scFv x 284A10-L1H1-scFv x 420H5-Fab x PL221G5-H1L1-scFv) light chain nt

GCCCTTGTGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCAATTGCCAGGCCA
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>SEQ ID:40 SI-38E34 (21D4-LH-scFv x 284A10-L1H1-scFv x 420H5-Fab x PL221G5-H1L1-scFv) light chain aa

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SGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

MULTI-SPECIFIC ANTIBODIES AND METHODS OF MAKING AND USING THEREOF

CLAIMS

What is claimed is:

1. A tetra-specific antibody monomer having a N-terminal and a C-terminal, comprising in tandem from the N-terminal to the C-terminal,

a first scFv domain at the N-terminal,

a second scFv domain

a Fab domain,

a Fc domain, and

a third scFv at the C-terminal,

wherein the first scFv domain, the second scFv domain, the Fab domain, and the third scFv domain each has a binding specificity against a different antigen, and wherein the antigen is a tumor antigen, an immune signaling antigen, or a combination thereof.

2. The tetra-specific antibody monomer of Claim 1, wherein the first scFv domain, the second scFv domain, the Fab domain, and the third scFv domain each independently has a binding specificity against an antigen selected from ROR1, PD-L1, CD3, CD28, 41BB, CEA, HER2, EGFRvIII, EGFR, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypimay-3, gpA33, GD2, TROP2, NKG2D, BCMA, CD19, CD20, CD33, CD123, CD22, CD30, PD1, OX40, 4-1BB, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40, VISTA, ICOS, BTLA, LIGHT, HVEM, CSF1R, CD73, and CD39, and wherein the Fc domain comprises a human IgG Fc domain.

3. The tetra-specific antibody monomer of Claim 1, wherein the first scFv domain, the second scFv domain, the Fab domain, and the third scFv domain each independently has a binding specificity against an antigen selected from CD19, CD3, CD137, 4-1BB, and PD-L1.

4. The tetra-specific antibody monomer of Claim 1, wherein the first scFv domain has a binding specificity against CD19.

5. The tetra-specific antibody monomer of Claim1, wherein the second scFv domain has a binding specificity against CD3.

6. The tetra-specific antibody monomer of Claim 1, wherein the Fab domain has a binding specificity against 4-1BB or CD137.

7. The tetra-specific antibody monomer of Claim1, wherein the third scFv domain has a binding specificity against PD-L1.

8. The tetra-specific antibody monomer of Claim 1, wherein the first scFv domain has a binding specificity against CD19, wherein the second scFv domain has a binding specificity against CD3, wherein the Fab domain has a binding specificity against 4-1BB or CD137, and wherein the third scFv domain has a binding specificity against PD-L1.

9. The tetra-specific antibody monomer of Claim 1, wherein the first scFv domain, the second scFv domain, or the third scFv domain comprises a gly-gly-gly-gly-ser $(G4S)_n$ linker, wherein n is 2, 3 or 4.

10. The tetra-specific antibody monomer of Claim 1, comprising an amino acid sequence having a percentage homology to SEQ ID No. 37-40, wherein the percentage homology is not less than 98%.

11. A scFv domain, comprising an amino acid sequence having a percentage homology to SEQ ID No. 2, 4, 6, 8, 10, 12, 26, 28, 30, 32, wherein the percentage homology is not less than 98%.

12. A Fab domain for the tetra-specific antibody monomer of Claim 1, comprising an amino acid sequence having a percentage homology to SEQ ID NO. 1-12,26-32 , wherein the percentage homology is not less than 90%.

13. A tetra-specific antibody, comprising a tetra-specific antibody monomer of Claim 1.

14. The tetra -specific antibody of Claim 13, comprising an amino acid sequence having a percentage homology to SEQ ID NO. 38 and 40, wherein the percentage homology is not less than 98%.

15. An isolated nucleic acid sequence, encoding an amino acid sequence having a percentage homology to SEQ ID NO. 37 and 39, wherein the percentage homology is not less than 98%.

16. An expression vector, comprising the isolated nucleic acid sequence of Claim 15.

17. A host cell comprising the isolated nucleic acid sequence of Claim 15, wherein the host cell is a prokaryotic cell or a eukaryotic cell.

18. A method for producing a tetra-specific antibody or monomer, comprising culturing a host cell comprising an isolated nucleic acid sequence such that the DNA sequence encoding the tetra-specific antibody or monomer is expressed, and purifying said tetra-specific antibody, wherein the isolated nucleic acid sequence encodes an amino acid having a percentage homology to SEQ ID NO. 37-40, wherein the percentage homology is not less than 98%.

19. A method for treating or preventing a cancer, said method comprising administering a pharmaceutical composition comprising a purified tetra-specific antibody of Claim 13.

20. An immuno-conjugate comprising a cytotoxic agent or an imaging agent linked to the tetra-specific antibody of Claim 13 through a linker, wherein the linker comprises an ester bond, an ether bond, an amid bond, a disulphide bond, an imide bond, a sulfone bond, a phosphate bond, a phosphorus ester bond, a peptide bond, a hydrophobic poly(ethylene glycol) linker, or a combination thereof.

21. The immuno-conjugate of Claim 20, wherein the cytotoxic agent comprises a chemotherapeutic agent, a growth inhibitory agent, a cytotoxic agent from class of calicheamicin, an antimitotic agent, a toxin, a radioactive isotope, a toxin, a therapeutic agent, or a combination thereof.

22. A pharmaceutical composition, comprising a pharmaceutically acceptable carrier and one of the tetra-specific antibody of Claim 13, the immuno-conjugate of Claim 20, or both.

23. The pharmaceutical composition of Claim 22, further comprising a therapeutic agent selected from a radioisotope, radionuclide, a toxin, an antibody, an enzyme, a chemotherapeutic agent or a combination thereof.

24. A method of treating a human subject with a cancer, comprising administering to the subject an effective amount of the tetra-specific antibody according to Claim 13.

25. The method of Claim 24, further comprising co-administering an effective amount of a therapeutic agent, wherein the therapeutic agent comprises an antibody, a chemotherapy agent, an enzyme, an anti-estrogen agent, a receptor tyrosine kinase inhibitor, a kinase inhibitor, a cell cycle inhibitor, a check point inhibitor, a DNA, RNA or protein synthesis inhibitor, a RAS inhibitor, an inhibitor of PD1, PD-L1, CTLA4, 4-1BB, OX40, GITR, ICOS, LIGHT, TIM3, LAG3, TIGIT, CD40, CD27, HVEM, BTLA, VISTA, B7H4, CSF1R, NKG2D, CD73, CD3, CD19 or a combination thereof.

26. A solution comprising an effective concentration of the tetra-specific antibody of Claim 13, wherein the solution is blood plasma in a human subject.