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Guidance and navigation control proteins and method of making and using thereof

Abstract

The application provides guidance and navigation control (GNC) proteins. In one embodiment, the GNC protein Comprises a T-cell binding moiety and a cancer-targeting moiety, wherein the T-cell binding moiety has a binding specificity to a T-cell receptor comprising CD3, CD28, PDL1, PD1, OX40, 4-1BB, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40, VISTA, ICOS, BTLA, Light, NKp30, CD28H, CD27, CD226, CD96, CD112R, A2AR, CD160, CD244, CECAM1, CD200R, TNFRSF25 (DR3), or a combination thereof, and wherein the cancer targeting moiety has a binding specificity to a cancer cell receptor.

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Background/Summary

CROSS REFERENCE TO RELATED APPLICATIONS (1) This application claims the benefit of U.S. Provisional Patent Application No. 62/648,880 filed Mar. 27, 2018, U.S. Provisional Patent Application No. 62/648,888 filed Mar. 27, 2018,

U.S. Provisional Patent Application No. 62/551,032 filed Aug. 28, 2017, U.S. Provisional Patent Application No. 62/524,553 filed Jun. 25, 2017, U.S. Provisional Patent Application No. 62/545,603 filed Aug. 15, 2017, U.S. Provisional Patent Application No. 62/551,035 filed Aug. 28, 2017, U.S. Provisional Patent Application No. 62/551,065 filed Aug. 28, 2017, U.S. Provisional Patent Application No. 62/524,554 filed Jun. 25, 2017, U.S. Provisional Patent Application No. 62/524,557 filed Jun. 25, 2017, and U.S. Provisional Patent Application No. 62/524,558 filed Jun. 25, 2017, the entire disclosures of which are expressly incorporated by reference herein.

TECHNICAL FIELD

(1) The present application generally relates to the technical field of Guidance and Navigation Control (GNC) proteins with multi-specific binding activities against surface molecules on both immune cells and tumor cells, and more particularly relates to making and using GNC proteins.

BACKGROUND

(2) 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].

(3) 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 Mar. 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).

(4) 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 specifically binds to 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. Despite the promising outcome, the cost leading to the combined

therapy must be high due to multiple clinical trials and the difficulty in recruiting representative populations.
(5) Adoptive cell therapy with chimeric antigen receptor T cells (CAR-T) is another promising immunotherapy for treating cancer. The clinical success of CAR-T therapy has revealed durable complete remissions and prolonged survival of patients with CD19-positive treatment-refractory B cell malignancies (Gill & June. 2015. Immunol Rev, 263:68-89). However, the cost and complexity associated with the manufacture of a personalized and genetically modified CAR-T immunotherapy has restricted their production and use to specialized centers for treating relatively small numbers of patients. Cytokine release syndrome (CRS), also known as cytokine storms, is the most notable adverse effect after the infusion of engineered CAR-T cells (Bonifant et al., 201, Mol Ther Oncolytics. 3:16011). In many cases, the onset and severity of CRS seems to be specialized personal events. Current options of mitigating CRS are mainly focused on rapid response and management care because the option of controlling CRS prior to T cell infusion is limited.
(6) While the efficacy of CAR-T therapy specific for a CD19-positive B cell malignancy is now established, the efficacy of CAR-T therapy against solid tumors has not been unequivocally demonstrated to date. Currently, many clinical trials are in progress to explore a variety of solid tumor-associated antigens (TAA) for CAR-T therapy. Inefficient T cell trafficking into the tumors, an immunosuppressive tumor micro-environment, suboptimal antigen recognition specificity, and lack of control over treatment-related adverse events are currently considered as the main obstacles in solid tumor CAR-T therapy (Li et al., 2018, J Hematol Oncol. 11(1): 22-40). The option of managing the therapeutic effect, as well as any adverse effect before and after the CAR-T cell infusion, is limited.

SUMMARY

(7) The present application provides guidance and navigation control (GNC) proteins with multi-specific antigen binding activities to the surface molecules of a T cell and a tumour cell.
(8) In one embodiment, the guidance and navigation control (GNC) protein, comprising a cytotoxic cell binding moiety and a cancer-targeting moiety. Any cytotoxic cells may be a potential binding target by the disclosed GNC proteins. Examples of the cytotoxic cell include, without limitation, T-cell, NK cell, macrophage cell, and dendritic cell.
(9) In one embodiment, the GNC protein includes a T-cell binding moiety. The T-cell binding moiety has a binding specificity to a T-cell receptor. Examples T-cell receptor include without limitation CD3, CD28, PDL1, PD1, OX40, 4-1BB, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40L, VISTA, ICOS, BTLA, Light, CD30, NKp30, CD28H, CD27, CD226, CD96, CD112R, A2AR, CD160, CD244, CECAM1, CD200R, TNFRSF25 (DR3), or a combination thereof.
(10) In one embodiment, the GNC protein includes a NK cell binding moiety. The NK cell binding moiety has a binding specificity to a NK cell receptor. Examples NK cell receptor include, without limitation, receptors for activation of NK cell such as CD16, NKG2D, KIR2DS1, KIR2DS2, KIR2DS4, KIR3DS1, NKG2C, NKG2E, NKG2H; agonist receptors such as NKp30a, NKp30b, NKp46, NKp80, DNAM-1, CD96, CD160, 4-1BB, GITR, CD27, OX-40, CRTAM; and antagonist receptors such as KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1, KIR3DL2, KIR3DL3, NKG2A, NKp30c, TIGIT, SIGLEC7, SIGLEC9, LILR, LAIR-1, KLRG1, PD-1, CTLA-4, CD161.
(11) In one embodiment, the GNC protein includes a macrophage binding moiety. The macrophage binding moiety has a binding specificity to a macrophage receptor. Examples macrophage receptor include, without limitation, agonist receptor on macrophage such as TLR2, TLR4, CD16, CD64, CD40, CD80, CD86, TREM-1, TREM-2, ILT-1, ILT-6a, ILT-7, ILT-8, EMR2, Dectin-1, CD69; antagonist receptors such as CD32b, SIRPa, LAIR-1, VISTA, TIM-3, CD200R, CD300a, CD300f, SIGLEC1, SIGLEC3, SIGLEC5, SIGLEC7, SIGLEC9, ILT-2, ILT-3, ILT-4, ILT-5, LILRB3, LILRB4, DCIR; and other surface receptors such as CSF-1R, LOX-1, CCR2, FRB, CD163, CR3, DC-SIGN, CD206, SR-A, CD36, MARCO.
(12) In one embodiment, the GNC protein includes a dendritic cell binding moiety. The dendritic cell binding moiety has a binding specificity to a dendritic cell receptor. Examples dendritic cell receptor include, without limitation, agonist receptors on dendritic cell such as TLR, CD16, CD64, CD40, CD80, CD86, HVEM, CD70; antagonist receptors such as VISTA, TIM-3, LAG-3, BTLA; and other surface receptors such as CSF-1R, LOX-1, CCR7, DC-SIGN, GM-CSF-R, IL-4R, IL-10R, CD36, CD206, DCIR, RIG-1, CLEC9A, CXCR4.
(13) The cancer targeting moiety has a binding specificity to a cancer cell receptor. Example cancer cell receptor include without limitation BCMA, CD19, CD20, CD33, CD123, CD22, CD30, ROR1, CEA, HER2, EGFR, EGFRvIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2, or a combination thereof.
(14) In one embodiment, GNC proteins comprise at least one T-cell binding moiety and at least one cancer cell binding moiety, wherein the T-cell binding moiety has a binding specificity to a T-cell receptor comprising CD3, CD28, PDL1, PD1, OX40, 4-1BB, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40, VISTA, ICOS, BTLA, Light, CD30, CD27, or a combination thereof, and wherein the cancer cell binding moiety has a binding specificity to a cancer cell receptor.
(15) In one embodiment, the cancer receptor comprises a receptor on a lung cancer cell, a liver cancer cell, a breast cancer cell, a colorectal cancer cell, an anal cancer cell, a pancreatic cancer cell, a gallbladder cancer cell, a bile duct cancer cell, a head and neck cancer cell, a nasopharyngeal cancer cell, a skin cancer cell, a melanoma cell, an ovarian cancer cell, a prostate cancer cell, a urethral cancer cell, a lung cancer cell, a non-small lung cell cancer cell, a small cell lung cancer cell, a brain tumour cell, a glioma cell, a neuroblastoma cell, an esophageal cancer cell, a gastric cancer cell, a liver cancer cell, a kidney cancer cell, a bladder cancer cell, a cervical cancer cell, an endometrial cancer cell, a thyroid cancer cell, an eye cancer cell, a sarcoma cell, a bone cancer cell, a leukemia cell, a myeloma cell, a lymphoma cell, or a combination thereof.
(16) In one embodiment, the GNC protein is capable of activating a T-cell by binding the T-cell binding moiety to a T-cell

receptor on the T-cell. In one embodiment, the GNC protein comprises a bi-specific antibody or antibody monomer, a tri-specific antibody or antibody monomer, a tetra-specific antibody or antibody monomer, an antigen-binding fragment thereof, or a combination thereof. In one embodiment, the GNC protein comprises an amino acid sequence having a percentage homology to SEQ ID NO. 49-52, wherein the percentage homology is not less than 70%, 80%, 90%, 95%, 98%, or 99%.

(17) In one embodiment, the GNC protein may have a first moiety and a second moiety. In one embodiment, the first moiety may include a T-cell binding moiety, a NK cell binding moiety, a macrophage binding moiety, or a dendritic cell binding moiety. The second moiety comprises the cancer-targeting moiety.

(18) In one embodiment, the first moiety and the second moiety may have binding specificities toward each other. In these embodiments, the GNC proteins are formed by the binding action between the first moiety and the second moiety. The binding action is a non-covalent bonding. In one embodiment, the GNC protein includes the first moiety bound to the second moiety through a high affinity non-covalent bonding interaction. Examples of high affinity non-covalent bonding interaction include, without limitation, antibody-antigen interaction, biotin-streptavidin interaction, leucine-zipper, and any pair of proteins from a two-hybrid screening assay, non-immunoglobulin protein scaffolds (Hosse et al., 2006, Protein Sci. 15(1): 14-27), or aptamers (Likhin et al., 2013, Acta Naturae. 2013. 5(4): 34-43), or a combination thereof.

(19) In one embodiment, the GNC protein may further include a linker moiety. In one embodiment, the first moiety and the first moiety are joined through a linker moiety to provide the GNC protein. In one embodiment, the linker moiety may covalently link the first and the second moieties together to provide the GNC protein. In one embodiment, the linker moiety may include two complimentary molecules or a stable protein-protein interaction. Examples of complimentary molecules include without limitation the complementary strands of DNA and RNA. Examples of stable protein-protein interaction include, but not limited to, biotin-avidin, leucine-zipper, and any pair of proteins from a two-hybrid screening assay.

(20) In one embodiment, the linker moiety may include the backbone of an immunoglobulin G (IgG), where a GNC proteins may include an immunoglobulin G (IgG) moiety with two heavy chains and two light chains, and at least two scFv moieties being covalently connected to either C or N terminals of the heavy or light chains. The IgG moiety may provide stability to the scFv moiety, and a tri-specific GNC protein may have two moieties for binding the surface molecules on T cells.

(21) In one embodiment, the first moiety comprises an antibody or a fragment thereof, a soluble receptor or a combination thereof. In one embodiment, the second moiety comprises an antibody or a fragment, a soluble receptor or a combination thereof.

(22) The application further provides therapeutic complexes incorporating the GNC protein disclosed herein. In one embodiment, the therapeutic complex includes the GNC protein and a cytotoxic cell. The cytotoxic cell may T cell, NK cell, macrophage, dendritic cell, or a combination thereof. In one embodiment, the T cell may be autologous T cells, allo T cells, or universal donor T cells.

(23) In one embodiment, the therapeutic complex may include the GNC protein and a cancer cell. In one embodiment, the therapeutic complex may include the GNC protein disclosed herein having a T-cell bound to the T-cell binding moiety and a cancer cell bound to the caner-targeting moiety.

(24) The application further provides pharmaceutical compositions. In one embodiment, the pharmaceutical composition includes the therapeutic complex disclosed herein and a pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical composition includes the GNC protein disclosed herein and a pharmaceutically acceptable carrier.

(25) In a further aspect, the application provides methods for making and using the disclosed GNC proteins.

(26) The objectives and advantages of the present application will become apparent from the following detailed description of preferred embodiments thereof in connection with the accompanying drawings.

Description

BRIEF DESCRIPTION OF THE DRAWINGS

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

(2) FIG. **1** shows example GNC proteins, which are characterized by their composition of multiple antigen binding domains (AgBd) and linkers.

(3) FIG. **2** shows an example format of a tetra-specific GNC antibody as an embodiment.

(4) FIG. **3** shows that an example tetra-specific GNC antibody binds to both a T cell and a tumor cell through multiple AgBds.

(5) FIG. **4** shows the example tetra-specific GNC antibodies binding to human ROR1 transfected CHO cells.

(6) FIG. **5** shows the example tetra-specific GNC antibodies binding to human 41BB transfected CHO cells.

(7) FIG. **6** shows the example tetra-specific GNC antibodies binding to human PD-L1 transfected CHO cells.

(8) FIG. **7** shows the example tetra-specific GNC antibodies with the binding domain 323H7 which is specific for the Ig

domain of ROR1 meditated RTCC of the B-ALL cell line Kasumi2 with PBMC as effectors.
(9) FIG. **8** shows the example tetra-specific GNC antibodies with the binding domain 323H7 which is specific for the Ig domain of ROR1 meditated RTCC of the B-ALL cell line Kasumi2 with CD8+, CD45RO+ memory T cells as effectors.
(10) FIG. **9** shows the example tetra-specific GNC antibodies with the binding domain 323H7 which is specific for the Ig domain of ROR1 meditated RTCC of the B-ALL cell line Kasumi2 with CD8+, CD45RA+ naive T cells as effectors.
(11) FIG. **10** shows the example tetra-specific GNC antibodies with the binding domain 338H4 which is specific for the Frizzled domain of ROR1 meditated RTCC of the B-ALL cell line Kasumi2 with PBMC as effectors.
(12) FIG. **11**. Tetra-specific antibodies with the binding domain 338H4 which is specific for the Frizzled domain of ROR1 mediated RTCC of the B-ALL cell line Kasumi2 with CD8+, CD45RO+ memory T cells as effectors.
(13) FIG. **12** shows the example tetra-specific GNC antibodies with the binding domain 338H4 which is specific for the Frizzled domain of ROR1 meditated RTCC of the B-ALL cell line Kasumi2 with CD8+, CD45RA+ naive T cells as effectors.

DETAILED DESCRIPTION

(14) 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 will 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.
(15) In one embodiment, the guidance navigation control (GNC) proteins are characterized by their composition of multiple antigen-specific binding domains (AgBDs) and by their ability of directing T cells (or other effector cells) to cancer cells (or other target cells) through the binding of multiple surface molecules on a T cell and a tumor cell (FIG. **1**). By this definition, GNC proteins are composed of Moiety 1 for binding at least one surface molecule on a T cell and Moiety 2 for binding at least one surface antigen on a cancer cell (TABLE 1A). In a T cell therapy, the cytotoxic T cells are regulated by T cell proliferation signaling, as well as co-stimulation signaling via either agonist receptors or antagonist receptors on their surface. To regulate these signaling, as well as the interplay between a T cell and a cancer, multiple AgBDs may be necessary for Moiety 1 and Moiety 2, respectively. GNC proteins must have at least one linker to link Moiety 1 and Moiety 2. In a conceptual GNC protein, any linker molecule can be used to link two or more AgBDs together either in vitro or in vivo by using complementary linkers of DNA/RNA or protein-protein interactions, including but not limited to, that of biotin-avidin, leucine-zipper, and any two-hybrid positive protein. However, in the present application all the linkers are either an antibody backbone structure or antibody fragments, so that GNC protein and GNC antibody may have the same meaning, e.g. an example of a tetra-specific GNC antibody structure in FIG. **2**. GNC proteins or antibodies are capable of directing the binding of a T cell to a cancer cell in vivo or ex vivo, mediated by multiple AgBDs (FIG. **3**). The T cells may be derived from the same patient or different individuals, and the cancer cell may exist in vivo, in vitro, or ex vivo. The examples provided in the present application enable GNC proteins as a prime agent in a T cell therapy, i.e. GNC-T therapy, for activating and controlling cytotoxic T cells ex vivo, prior to adoptive transfer.
(16) TABLE-US-00001 TABLE 1A Composition of functional moieties (Moiety 1 and Moiety 2) and antigen binding domain in example GNC proteins with T cell binding domains Moiety 1 Moiety 2 Activation of T cells Agonist receptor Antagonist receptor Tumor Antigen CD3 CD28, 41BB, OX40, PDL1, PD1, TIGIT, TIM- BCMA, CD19, CD20, GITR, CD40L, ICOS, 3, LAG-3, CTLA4, BTLA, CD33, CD123, CD22, Light, CD27, CD30 VISTA, PDL2 CD30, ROR1, CEA, HER2, EGFR, EGFRvIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2
(17) In addition to T cells, other cytotoxic cells may also be utilized by the GNC proteins for cancer killing or preventing purposes. TABLE 1B shows the example compositions of functional moieties (Moiety 1 and Moiety 2) and antigen binding domain in GNC proteins with NK cell binding domains. TABLE 1C shows the example compositions of functional moieties (Moiety 1 and Moiety 2) and antigen binding domain in GNC proteins with macrophage binding domains. TABLE 1D shows the example compositions of functional moieties (Moiety 1 and Moiety 2) and antigen binding domain in GNC proteins with dendritic cell binding domains.
(18) TABLE-US-00002 TABLE 1B Moiety 1 Moiety 2 Activation of NK cell Agonist receptor Antagonist receptor Tumor Antigen CD16, NKG2D, NKp30a, NKp30b, KIR2DL1, KIR2DL2, BCMA, CD19, CD20, KIR2DS1, KIR2DS2, NKp46, NKp80, KIR2DL3, KIR3DL1, CD33, CD123, CD22, KIR2DS4, KIR3DS1, DNAM-1, CD96, KIR3DL2, KIR3DL3, CD30, ROR1, CEA, HER2, NKG2C, NKG2E, CD160, 4-1BB, GITR, NKG2A, NKp30c, TIGIT, EGFR, EGFRvIII, LMP1, NKG2H CD27, OX-40, CRTAM SIGLEC7, SIGLEC9, LILR, LMP2A, Mesothelin, LAIR-1, KLRG1, PD-1, PSMA, EpCAM, CTLA-4, CD161 glypican-3, gpA33, GD2, TROP2
(19) TABLE-US-00003 TABLE 1C Moiety 1 Agonist receptor on Antagonist receptor on Other surface Moiety 2 macrophage macrophage receptors Tumor Antigen TLR2, TLR4, CD16, CD32b, SIRPα, LAIR-1, VISTA, CSF-1R, LOX-1, BCMA, CD19, CD20, CD64, CD40, CD80, TIM-3, CD200R, CD300a, CCR2, FRβ, CD33, CD123, CD22, CD86, TREM-1, TREM-2, CD300f, SIGLEC1, SIGLEC3, CD163, CR3, DC- CD30, ROR1, CEA, ILT-1, ILT-6a, ILT-7, ILT- SIGLEC5, SIGLEC7, SIGLEC9, SIGN, CD206, SR- HER2, EGFR, EGFRvIII, 8, EMR2, Dectin-1, ILT-2, ILT-3, ILT-4, ILT-5, A, CD36, MARCO LMP1, LMP2A, CD69 LILRB3, LILRB4, DCIR Mesothelin, PSMA, EpCAM, glypican-3,

gpA33, GD2, TROP2
(20) TABLE-US-00004 TABLE 1D Moiety 1 Antagonist receptor on Other surface Moiety 2 Agonist receptor on DC DC receptors Tumor Antigen TLR, CD16, CD64, CD40, VISTA, TIM-3, LAG-3, BTLA CSF-1R, LOX-1, BCMA, CD19, CD20, CD80, CD86, HVEM, CD70 CCR7, DC-SIGN, CD33, CD123, CD22, GM-CSF-R, IL-4R, CD30, ROR1, CEA, IL-10R, CD36, HER2, EGFR, EGFRvIII, CD206, DCIR, RIG- LMP1, LMP2A, 1, CLEC9A, CXCR4 Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2
(21) The present application relates to methods of making and using recombinant GNC proteins. Multiple AgBDs can be divided into Moiety 1 and Moiety 2 due to their interface with a cytotoxic cell such as a T cell and a cancer cell, respectively (TABLE 1A). However, the rearrangement of multiple AgBDs may be random and in unequal numbers (TABLE 2). A GNC protein with two AgBDs may simultaneously bind to a surface molecule, such as CD3 on a T cell, and a tumor antigen, such as ROR1 on a tumor cell, for re-directing or guiding the T cell to the tumor cell. The addition of the third AgBD, e.g. specifically bind to 41BB, may help enhance anti-CD3-induced T cell activation because 41BB is a co-stimulation factor and the binding stimulates its agonist activity to activated T cells. The addition of the fourth AgBD to a GNC protein, e.g. specifically bind to PD-L1 on a tumor cell, may block the inhibitory pathway of PD-L1 on tumor cells that is mediated through its binding to PD-1 on the T cells. With these basic principles, GNC proteins may be designed and constructed to acquire multiple AgBDs specifically for binding unequal numbers of T cell antagonists and agonists, not only to re-direct activated T cells to tumor cells but also to control their activity in vivo (TABLE 2). Therefore, the design of GNC proteins may be any multi-specific proteins.
(22) In one embodiment, the GNC protein may be a bi-specific, tri-specific, tetra-specific, penta-specific, hexa-specific, hepta-specific, or octa-specific proteins. In one embodiment, the GNC protein may be a monoclonal antibodies. In one embodiment, the GNC protein may be a bi-specific, tri-specific, tetra-specific, penta-specific, hexa-specific, hepta-specific, or octa-specific antibody monomers. In one embodiment, the GNC protein may be a bi-specific, tri-specific, tetra-specific, penta-specific, hexa-specific, hepta-specific, or octa-specific antibodies. TABLE 3 provides some example GNC proteins and antibodies with the specificity of antibody binding domains.
(23) TABLE-US-00005 TABLE 2 Examples of possible combinations of T cell activation, T cell agonist, T cell antagonist, and tumor antigen binding domains in a single GNC protein. T cell Tumor T cell T cell T cell T cell T cell GNC protein activation antigen antagonist agonist antagonist antagonist antagonist agonist Bi-specific CD3 ROR1 Tri-specific CD3 ROR1 PD1 Tetra-specific CD3 ROR1 PD1 41BB Penta-specific CD3 ROR1 PD1 41BB LAG3 Hexa-specific CD3 ROR1 PD1 41BB LAG3 TLM3 Hepta-specific CD3 ROR1 PD1 41BB LAG3 TLM3 TIGIT Octa-specific CD3 ROR1 PD1 41BB LAG3 TLM3 TIGIT CD28
(24) TABLE-US-00006 TABLE 3 Specificity of antibody binding domains used in GNC proteins. Antibody Name Specificity 460C3 41BB 420H5 41BB 466F6 41BB PL230C6 PD-L1 323H7 ROR1 IgD Domain 338H4 ROR1 Frizzled Domain 330F11 ROR1 Kringle Domain 324C6 ROR1 Frizzled Domain 4420 FITC 284A10 CD3 complex Epsilon chain 480C8 CD3 complex Epsilon chain
(25) In one embodiment, the application provides methods of making and using recombinant GNC proteins. GNC proteins are composed of multi-specific antigen binding moieties characterized by two functional groups: Moiety 1 comprises multiple antigen binding domains (AgBD) whose specificities are implicated in T-cell activation, agonist co-stimulation, and/or inhibitory antagonist activity, and Moiety 2 comprises at least one cancer cell binding specificity. GNC proteins may simultaneously bind to a surface molecule, such as CD3 of a T cell, and a tumor antigen, such as ROR1 of a tumor cell, thereby re-directing or guiding the T cell to the tumor cell. An addition of the third binding domain in a GNC protein may help enhance the CD3-induced T cell activation through its direct binding of 41BB, which is a co-stimulation factor exerting agonist activity. Furthermore, an addition of the fourth binding domain in a GNC protein may bind to PD-L1 on the tumor cell to block the inhibitory pathway of PD-L1 on tumor cells that is mediated through its binding to PD-1 on the T cells. In this way, GNC proteins acquire multiple binding capacities to re-direct activated T cells to tumor cells, and multiple binding may help modulate T cell activation through modulating either agonist or antagonist activity or both. Some binding capacities may be similar to that of either the chimeric antigen receptor on a CAR-T cell or a bi-specific antibody, such as the BiTe antibody. While GNC proteins are unique, their ability of guidance and navigation control of the interaction between activated T cells and tumor cells remains to be demonstrated.
(26) In one embodiment, an example GNC protein with 4 different binding domains is disclosed. This GNC protein is a “tetra-specific antibody” since its linkers and backbone comprises antibody fragments. Of the 4 different antigen binding domains, one specifically binds to CD3 on T cells, the second binding domain is specific against a tumor associated antigen, including but not limited to other tumor antigens, such as ROR1, CEA, HER2, EGFR, EGFRvIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2, BCMA, CD19, CD20, CD33, CD123, CD22, CD30, and the third and fourth binding domains are specific against two distinct immune checkpoint modulators, namely, PD-L1, PD-1, OX40, 4-1BB, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40, VISTA, ICOS, BTLA, Light, HVEM, CD73, CD39, etc. Because of their definition in function and variety in composition, GNC proteins can be classified as a new class of immune-modulators for treating cancer. TABLE 4 shows the list of the example tetra-specific GNC antibodies.
(27) TABLE-US-00007 TABLE 4 List of tetra-specific GNC antibodies. Antibody Domain 1 Humanized Domain 2 Humanized Domain 3 Humanized Domain 4 Humanized ID LH-scFv Variant Fab Variant IgG Fc LH-scFv Variant LH-scFv Variant SI-35E18 460C3 H1L1 PL230C6 H3L3 n2 323H7 H4L1 284A10 H1L1 SI-35E19 420H5 H3L3 PL230C6 H3L3 n2 323H7 H4L1 284A10 H1L1 SI-35E20 466F6 H2L5 PL230C6 H3L3 n2 323H7 H4L1 284A10 H1L1 SI-35E21

460C3 H1L1 PL230C6 H3L3 n2 338H4 H3L4 284A10 H1L1 SI-35E22 420H5 H3L3 PL230C6 H3L3 n2 338H4 H3L4 284A10 H1L1 SI-35E23 466F6 H2L5 PL230C6 H3L3 n2 338H4 H3L4 284A10 H1L1 SI-35E24 460C3 H1L1 PL230C6 H3L3 n2 330F11 H1L1 284A10 H1L1 SI-35E25 420H5 H3L3 PL230C6 H3L3 n2 330F11 H1L1 284A10 H1L1 SI-35E26 466F6 H2L5 PL230C6 H3L3 n2 330F11 H1L1 284A10 H1L1 SI-27E12 4420 ~ PL230C6 H3L3 n2 324C6 H2L1 480C8 H1L1 SI-27E15 460C3 H1L1 4420 ~ n2 324C6 H2L1 480C8 H1L1 SI-27E13 460C3 H1L1 PL230C6 H3L3 n2 4420 ~ 480C8 H1L1 SI-35E2 460C3 H1L1 PL230C6 H3L3 n2 324C6 H2L1 4420 ~

(28) In one embodiment, GNC-mediated immunotherapy may include types of antibody therapy and cell therapy. Herein, the advantages may include, but not limited to, the inclusion of an IgG Fc domain may confer the characteristic of a longer half-life in serum compared to a bi-specific BiTe molecule; second, the inclusion of two binding domains specific for immune checkpoint modulators may inhibit the suppressive pathways and engage the co-stimulatory pathways at the same time; third, that cross-linking CD3 on T cells with tumor associated antigens re-directs and guides T cells to kill the tumor cells without the need of removing T cells from the patient and genetically modifying them to be specific for the tumor cells before re-introducing them back into the patient, also known as chimeric antigen receptor T cells (CAR-T) therapy; and fourth, that GNC protein-mediated antibody therapy or T cell therapy does not involve genetic modification of T cells, the latter of which may carry the risk of transforming modified T cells to clonal expansion, i.e. T cell leukemia.

(29) With one or more addition of the binding capacity, the advantage of GNC protein-mediated immunotherapy over conventional immunotherapies include, but not limited to, first, that inclusion of an IgG Fc domain may confer the characteristic of a longer half-life in serum compared to a bi-specific BiTe molecule; second, that inclusion of two binding domains specific for immune checkpoint modulators may inhibit the suppressive pathways and engage the co-stimulatory pathways at the same time; third, that cross-linking CD3 on T cells with tumor associated antigens re-directs and guides T cells to kill the tumor cells without the need of removing T cells from the patient and genetically modifying them to be specific for the tumor cells before re-introducing them back into the patient, also known as chimeric antigen receptor T cells (CAR-T) therapy; and fourth, that GNC protein-mediated antibody therapy or T cell therapy does not involve genetic modification of T cells, the latter of which may carry the risk of transforming modified T cells to clonal expansion, i.e. T cell leukemia.

(30) 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

(31) While the following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially the same or similar results.

Example 1: FACS Analysis of Tetra-Specific Specific Antibody Binding to Human ROR1 Transfected CHO Cells

(32) The tetra-specific GNC antibodies listed in TABLEs 3 and 4 were tested for binding to Chinese hamster ovary cells (CHO) cells stably expressing a full-length human ROR1. Antibodies were prepared at 2× final concentration and titrated 1:5 across 3 wells of a 96 well plate in 50 ul of PBS/2% FBS and then 5,000 ROR1-CHO cells in 50 ul PBS/2% FBS were added. This mixture was incubated for 30 minutes on ice, washed once with 200 ul PBS/2% FBS, and then the secondary antibody PE Goat anti-Human IgG Fc at 1:1000 dilution of stock was added, and this mixture was incubated for 30 minutes on ice. Cells were washed 2×200 ul PBS/2% FBS, resuspended in 50 ul PBS/2% FBS and analyzed on a BD LSRFORTESSA and the binding profile is shown in FIG. **4**. The tetra-specific antibodies SI-35E18, 19, and 20, with the 323H7 binding domain specific for the Ig domain of ROR1, showed higher binding than the tetra-specific GNC antibodies SI-3521, 22, and 23, with the 338H4 binding domain specific for the frizzled domain of ROR1, and the tetra-specific GNC antibodies SI-3524, 25, and 26, with the 330F11 binding domain specific for the kringle domain of ROR1, did not bind.

Example 2: FACS Analysis of Tetra-Specific GNC Antibody Binding to Human 41BB Transfected CHO Cells

(33) The tetra-specific GNC antibodies listed in TABLEs 3 and 4 were tested for binding to Chinese hamster ovary cells (CHO) cells stably expressing a full-length human ROR1. Antibodies were prepared at 2× final concentration and titrated 1:5 across 3 wells of a 96 well plate in 50 ul of PBS/2% FBS and then 5,000 ROR1-CHO cells in 50 ul PBS/2% FBS were added. This mixture was incubated for 30 minutes on ice, washed once with 200 ul PBS/2% FBS, and then the secondary antibody PE Goat anti-Human IgG Fc at 1:1000 dilution of stock was added, and this mixture was incubated for 30 minutes on ice. Cells were washed 2×200 ul PBS/2% FBS, resuspended in 50 ul PBS/2% FBS and analyzed on a BD LSRFORTESSA and the binding profile is shown in FIG. **5**. All of the tetra-specific GNC antibodies except for the control SI-27E12 contain a 41BB binding domain, 460C3, 420H5, or 466F6 and bound to 41BB expressing CHO cells with varying intensity.

Example 3: FACS Analysis of Tetra-Specific GNC Antibody Binding to Human PDL1 Transfected CHO Cells

(34) The tetra-specific GNC antibodies listed in TABLEs 3 and 4 were tested for binding to Chinese hamster ovary cells (CHO) cells stably expressing full length human ROR1. Antibodies were prepared at 2× final concentration and titrated 1:5 across 3 wells of a 96 well plate in 50 ul of PBS/2% FBS and then 5,000 ROR1-CHO cells in 50 ul PBS/2% FBS were added. This mixture was incubated for 30 minutes on ice, washed once with 200 ul PBS/2% FBS, and then the secondary antibody PE Goat anti-Human IgG Fc at 1:1000 dilution of stock was added, and this mixture was incubated

for 30 minutes on ice. Cells were washed 2×200 ul PBS/2% FBS, resuspended in 50 ul PBS/2% FBS and analyzed on a BD LSRFORTESSA and the binding profile is shown in FIG. **6**. All of the tetra-specific GNC antibodies except for the control SI-27E15 contain the same PDL1 binding domain, PL230C6, and showed very similar binding intensity to PDL1 expressing CHO cells.

Example 4: Re-Directed T Cell Cytotoxicity (RTCC) Assay with Peripheral Blood Mononuclear Cells as Effectors and B-Acute Lymphoblastic Leukemia (B-ALL) Cell Line Kasumi-2 as Targets

(35) The tetra-specific GNC antibodies listed in TABLEs 3 and 4 were tested for RTCC activity against the B-ALL cell line Kasumi 2 using human peripheral blood mononuclear cells (PBMC) as effectors. The Kasumi 2 target cells, 5×10.sup.6, were labeled with CFSE (Invitrogen, #C34554) at 0.5 uM in 10 ml of culture media for 20 minutes at 37° C. The cells were washed 3 times with 50 ml of culture media before resuspending in 10 ml then counted again. Antibodies were prepared at 2× final concentration and titrated 1:3 across 10 wells of a 96 well plate in 200 ul of RPMI+10% FBS. Human PBMC were purified by standard ficoll density gradient from a "leukopak" which is an enriched leukapheresis product collected from normal human peripheral blood. In the final destination 96 well plate the target cells, PBMC, and serially titrated antibodies were combined by adding 100 μl of target cells (5,000), 50 ul of PBMC (25,000), and 100 ul of each antibody dilution to each well of the assay. The assay plate was incubated at 37° C. for approximately 72 hours and then the contents of each assay well were harvested and analyzed for the number of CFSE-labeled target cells remaining. As shown on FIG. **7**, the tetra-specific GNC antibodies all contain the same PDL1 binding domain PL230C6, the same ROR1 binding domain 323H7, and the same CD3 binding domain 284A10, but have one of the 41BB binding domains 460C3, 420H5, and 466F6 and showed greater RTCC activity compared to the controls except for the control SI-27E12 which does not have a 41BB binding domain but appeared to be similarly potent at the tetra-specific GNC antibodies SI-35E18, 19, and 20.

Example 5: Re-Directed T Cell Cytotoxicity (RTCC) Assay with CD8+, CD45RO+ Memory T Cells as Effectors and B-Acute Lymphoblastic Leukemia (B-ALL) Cell Line Kasumi-2 as Targets

(36) The tetra-specific GNC antibodies listed in TABLE 3 and 4 were tested for RTCC activity against the B-ALL cell line Kasumi 2 using human CD8+, CD45RO+ memory T cells as effectors. The Kasumi 2 target cells, 5×10.sup.6, were labeled with CFSE (Invitrogen, #C34554) at 0.5 uM in 10 ml of culture media for 20 minutes at 37° C. The cells were washed 3 times with 50 ml of culture media before resuspending in 10 ml then counted again. Antibodies were prepared at 2× final concentration and titrated 1:3 across 10 wells of a 96 well plate in 200 ul of RPMI+10% FBS. Human CD8+, CD45RO+ memory T cells were enriched from PBMC from a normal donor using the EasySep™ Human Memory CD8+ T Cell Enrichment Kit (Stemcell Technologies, #19159) as per the manufacturers protocol. The final cell population was determined to be 98% CD8+, CD45RO+ T cells by FACS analysis. In the final destination 96 well plate the target cells, T cells, and serially titrated antibodies were combined by adding 100 μl of target cells (5,000), 50 ul of CD8+, CD45RO+ memory T cells (25,000), and 100 ul of each antibody dilution to each well of the assay. The assay plate was incubated at 37 C for approximately 72 hours and then the contents of each assay well were harvested and analyzed for the number of CFSE-labeled target cells remaining. As shown on FIG. **8**, the tetra-specific antibodies all contain the same PDL1 binding domain PL230C6, the same ROR1 binding domain 323H7, and the same CD3 binding domain 284A10, but have one of the 41BB binding domains 460C3, 420H5, and 466F6 and showed greater RTCC activity compared to the controls that do not contain one of the 41BB, PDL1, ROR1, or CD3 binding domains.

Example 6: Re-Directed T Cell Cytotoxicity (RTCC) Assay with CD8+, CD45RA+ Naive T Cells as Effectors and B-Acute Lymphoblastic Leukemia (B-ALL) Cell Line Kasumi-2 as Targets

(37) The tetra-specific-specific antibodies listed in TABLEs 3 and 4 were tested for RTCC activity against the B-ALL cell line Kasumi 2 using human CD8+, CD45RA+ memory T cells as effectors. The Kasumi 2 target cells, 5×10e6, were labeled with CFSE (Invitrogen, #C34554) at 0.5 uM in 10 ml of culture media for 20 minutes at 37 C. The cells were washed 3 times with 50 ml of culture media before resuspending in 10 ml then counted again. Antibodies were prepared at 2× final concentration and titrated 1:3 across 10 wells of a 96 well plate in 200 ul of RPMI+10% FBS. Human CD8+, CD45RA+ memory T cells were enriched from peripheral blood mononuclear cells from a normal donor using the EasySep™ Human Naïve CD8+ T Cell Isolation Kit (Stemcell Technologies, #19258) as per the manufacturers protocol. The final cell population was determined to be 98% CD8+, CD45RA+ T cells by FACS analysis (data not shown). In the final destination 96 well plate the target cells, T cells, and serially titrated antibodies were combined by adding 100 μl of target cells (5,000), 50 ul of CD8+, CD45RO+ T cells (25,000), and 100 ul of each antibody dilution to each well of the assay. The assay plate was incubated at 37 C for approximately 72 hours and then the contents of each assay well were harvested and analyzed for the number of CFSE-labeled target cells remaining. As shown on FIG. **9**, the tetra-specific antibodies all contain the same PDL1 binding domain PL230C6, the same ROR1 binding domain 323H7, and the same CD3 binding domain 284A10, but have one of the 41BB binding domains 460C3, 420H5, and 466F6 and showed greater RTCC activity compared to the controls that do not contain one of the 41BB, PDL1, ROR1, or CD3 binding domains.

Example 7: Re-Directed T Cell Cytotoxicity (RTCC) Assay with Peripheral Blood Mononuclear Cells as Effectors and B-Acute Lymphoblastic Leukemia (B-ALL) Cell Line Kasumi-2 as Targets

(38) The tetra-specific-specific antibodies listed in TABLEs 3 and 4 were tested for RTCC activity against the B-ALL cell line Kasumi 2 using human peripheral blood mononuclear cells (PBMC) as effectors. The Kasumi 2 target cells, 5×10.sup.6, were labeled with CFSE (Invitrogen, #C34554) at 0.5 M in 10 ml of culture media for 20 minutes at 37° C. The cells were washed 3 times with 50 ml of culture media before resuspending in 10 ml then counted again. Antibodies

were prepared at 2× final concentration and titrated 1:3 across 10 wells of a 96 well plate in 200 ul of RPMI+10% FBS. Human PBMC were purified by standard ficoll density gradient from a "leukopak" which is an enriched leukapheresis product collected from normal human peripheral blood. In the final destination 96 well plate the target cells, PBMC, and serially titrated antibodies were combined by adding 100 μl of target cells (5,000), 50 ul of PBMC (25,000), and 100 ul of each antibody dilution to each well of the assay. The assay plate was incubated at 37° C. for approximately 72 hours and then the contents of each assay well were harvested and analyzed for the number of CFSE-labeled target cells remaining. As shown on FIG. **10**, the tetra-specific GNC antibodies all contain the same PDL1 binding domain PL230C6, the same ROR1 binding domain 338H4, and the same CD3 binding domain 284A10, but have one of the 41BB binding domains 460C3, 420H5, and 466F6 and showed greater RTCC activity compared to the controls except for the control SI-35E36 which does not have a 41BB binding domain but appeared to be similarly potent at the tetra-specific GNC antibodies SI-35E18, 19, and 20.

Example 8: Re-Directed T Cell Cytotoxicity (RTCC) Assay with CD8+, CD45RO+ Memory T Cells as Effectors and B-Acute Lymphoblastic Leukemia (B-ALL) Cell Line Kasumi-2 as Targets

(39) The tetra-specific GNC antibodies listed in TABLEs 3 and 4 were tested for RTCC activity against the B-ALL cell line Kasumi 2 using human CD8+, CD45RO+ memory T cells as effectors. The Kasumi 2 target cells, 5×10.sup.6, were labeled with CFSE (Invitrogen, #C34554) at 0.5 uM in 10 ml of culture media for 20 minutes at 37° C. The cells were washed 3 times with 50 ml of culture media before resuspending in 10 ml then counted again. Antibodies were prepared at 2× final concentration and titrated 1:3 across 10 wells of a 96 well plate in 200 ul of RPMI+10% FBS. Human CD8+, CD45RO+ memory T cells were enriched from PBMC from a normal donor using the EasySep™ Human Memory CD8+ T Cell Enrichment Kit (Stemcell Technologies, #19159) as per the manufacturers protocol. The final cell population was determined to be 98% CD8+, CD45RO+ T cells by FACS analysis (data not shown). In the final destination 96 well plate the target cells, T cells, and serially titrated antibodies were combined by adding 100 ul of target cells (5,000), 50 ul of CD8+, CD45RO+ memory T cells (25,000), and 100 ul of each antibody dilution to each well of the assay. The assay plate was incubated at 37° C. for approximately 72 hours and then the contents of each assay well were harvested and analyzed for the number of CFSE-labeled target cells remaining. As shown on FIG. **11**, the tetra-specific GNC antibodies all contain the same PDL1 binding domain PL230C6, the same ROR1 binding domain 338H4, and the same CD3 binding domain 284A10, but have one of the 41BB binding domains 460C3, 420H5, and 466F6 and showed greater RTCC activity compared to the controls that do not contain one of the 41BB, PDL1, ROR1, or CD3 binding domains.

Example 9: Re-Directed T Cell Cytotoxicity (RTCC) Assay with CD8+, CD45RA+ Naive T Cells as Effectors and B-Acute Lymphoblastic Leukemia (B-ALL) Cell Line Kasumi-2 as Targets

(40) The tetra-specific GNC antibodies listed in TABLEs 3 and 4 were tested for RTCC activity against the B-ALL cell line Kasumi 2 using human CD8+, CD45RA+ memory T cells as effectors. The Kasumi 2 target cells, 5×10.sup.6, were labeled with CFSE (Invitrogen, #C34554) at 0.5 uM in 10 ml of culture media for 20 minutes at 37° C. The cells were washed 3 times with 50 ml of culture media before resuspending in 10 ml then counted again. Antibodies were prepared at 2× final concentration and titrated 1:3 across 10 wells of a 96 well plate in 200 ul of RPMI+10% FBS. Human CD8+, CD45RA+ memory T cells were enriched from PBMC from a normal donor using the EasySep™ Human Naïve CD8+ T Cell Isolation Kit (Stemcell Technologies, #19258) as per the manufacturers protocol. The final cell population was determined to be 98% CD8+, CD45RA+ T cells by FACS analysis. In the final destination 96 well plate the target cells, T cells, and serially titrated antibodies were combined by adding 100 μl of target cells (5,000), 50 ul of CD8+, CD45RO+ T cells (25,000), and 100 ul of each antibody dilution to each well of the assay. The assay plate was incubated at 37° C. for approximately 72 hours and then the contents of each assay well were harvested and analyzed for the number of CFSE-labeled target cells remaining. As shown on FIG. **12**, the tetra-specific GNC antibodies all contain the same PDL1 binding domain PL230C6, the same ROR1 binding domain 338H4, and the same CD3 binding domain 284A10, but have one of the 41BB binding domains 460C3, 420H5, and 466F6 but did not show greater RTCC activity compared to the controls that do not contain one of the 41BB, PDL1, ROR1, or CD3 binding domains. This is in contrast to the tetra-specific GNC antibodies described in Example 6 and shown in FIG. **6** that do show RTCC activity with CD8+, CD45RA+naïve T cells.

(41) 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.

(42) 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).

(43) 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]).

(44) 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).

(45) 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.

(46) 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.

(47) 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.

(48) 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.

(49) “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.
(50) The “light chains” of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda (A), based on the amino acid sequences of their constant domains.
(51) 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 a, delta, epsilon, y, and u, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.
(52) 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 antibodies in large animals such as, for example, rabbits (see, e.g. U.S. Pat. No. 7,129,084).
(53) 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.
(54) 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.
(55) 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 will 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.
(56) “Recombinant” means the antibodies are generated using recombinant nucleic acid techniques in exogeneous host cells.
(57) 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.
(58) 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.
(59) “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.
(60) 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 will 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.
(61) 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.
(62) “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.
(63) 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.

(64) Guidance and Navigation Control Proteins and Method of Making and Using Thereof

(65) TABLE-US-00008 SEQUENCE LIST SEQ ID Description 1 anti-CD3 284A10 VHv1 nt 2 anti-CD3 284A10 VHv1 aa 3 anti-CD3 284A10 VLv1 nt 4 anti-CD3 284A10 VLv1 aa 5 anti-CD3 480C8 VHv1 nt 6 anti-CD3 480C8 VHv1 aa 7 anti-CD3 480C8 VLv1 nt 8 anti-CD3 480C8 VLv1 aa 9 anti-PD-L1 PL230C6 VHv3 nt 10 anti-PD-L1 PL230C6 VHv3 aa 11 anti-PD-L1 PL230C6 VLv2 nt 12 anti-PD-L1 PL230C6 VLv2 aa 13 anti-4-1BB 420H5 VHv3 nt 14 anti-4-1BB 420H5 VHv3 aa 15 anti-4-1BB 420H5 VLv3 nt 16 anti-4-1BB 420H5 VHLv3 aa 17 anti-4-1BB 466F6 VHv2 nt 18 anti-4-1BB 466F6 VHv2 aa 19 anti-4-1BB 466F6 VLv5 nt 20 anti-4-1BB 466F6 VLv5 aa 21 anti-4-1BB 460C3 VHv1 nt 22 anti-4-1BB 460C3 VHv1 aa 23 anti-4-1BB 460C3 VLv1 nt 24 anti-4-1BB 460C3 VLv1 aa 25 anti-ROR1 324C6 VHv2 nt 26 anti-ROR1 324C6 VHv2 aa 27 anti-ROR1 324C6 VLv1 nt 28 anti-ROR1 324C6 VLv1 aa 29 anti-ROR1 323H7 VHv4 nt 30 anti-ROR1 323H7 VHv4 aa 31 anti-ROR1 323H7 VLv1 nt 32 anti-ROR1 323H7 VLv1 aa 33 anti-ROR1 338H4 VHv3 nt 34 anti-ROR1 338H4 VHv3 aa 35 anti-ROR1 338H4 VLv4 nt 36 anti-ROR1 338H4 VLv4 aa 37 anti-ROR1 330F11 VHv1 nt 38 anti-ROR1 330F11 VHv1 aa 39 anti-ROR1 330F11 VLv1 nt 40 anti-ROR1 330F11 VLv1 aa 41 anti-FITC 4-4-20 VH nt 42 anti-FITC 4-4-20 VH aa 43 anti-FITC 4-4-20 VL nt 44 anti-FITC 4-4-20 VL aa 45 human IgG1 null2 (G1m-fa with ADCC/CDC null mutations) nt 46 human IgG1 null2 (G1m-fa with ADCC/CDC null mutations) aa 47 human Ig Kappa nt 48 human Ig Kappa aa 49 SI-35E18 (460C3-L1H1-scFv × PL230C6-Fab × 323H7-H4L1-scFv × 284A10-H1L1-scFv) heavy chain nt 50 SI-35E18 (460C3-L1H1-scFv × PL230C6-Fab × 323H7-H4L1-scFv × 284A10-H1L1-scFv) heavy chain aa 51 SI-35E18 (460C3-L1H1-scFv × PL230C6-Fab × 323H7-H4L1-scFv × 284A10-H1L1-scFv) light chain nt 52 SI-35E18 (460C3-L1H1-scFv × PL230C6-Fab × 323H7-H4L1-scFv × 284A10-H1L1-scFv) light chain aa >SEQ ID 01 anti-CD3 284A10 VHv1 nt

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Claims

1. A guidance and navigation control (GNC) protein, comprising a cytotoxic cell binding moiety and a cancer-targeting moiety, wherein the cytotoxic cell binding moiety has a binding specificity to a T-cell receptor, a NK cell receptor, a macrophage receptor, a dendritic cell receptor, or a combination thereof, and wherein the cancer-targeting moiety has a binding specificity to a cancer cell receptor, wherein the GNC protein comprises the amino acid sequences of SEQ ID NO: 50 and SEQ ID NO: 52.
2. The GNC protein of claim 1, wherein the T-cell receptor comprises CD3.
3. The GNC protein of claim 1, wherein the NK cell receptor comprises 4-1BB.
4. The GNC protein of claim 1, wherein the macrophage receptor comprises PD-L1.
5. The GNC protein of claim 1, wherein the dendritic cell receptor comprises PDL1 or 4-1BB.
6. The GNC protein of claim 1, wherein the cancer cell receptor is a receptor on a lung cancer cell, a liver cancer cell, a breast cancer cell, a colorectal cancer cell, an anal cancer cell, a pancreatic cancer cell, a gallbladder cancer cell, a bile duct cancer cell, a head and neck cancer cell, a nasopharyngeal cancer cell, a skin cancer cell, a melanoma cell, an ovarian cancer cell, a prostate cancer cell, a urethral cancer cell, a lung cancer cell, a non-small cell lung cancer cell, a small cell lung cancer cell, a brain tumour cell, a glioma cell, a neuroblastoma cell, an esophageal cancer cell, a gastric cancer cell, a liver cancer cell, a kidney cancer cell, a bladder cancer cell, a cervical cancer cell, an endometrial cancer cell, a thyroid cancer cell, an eye cancer cell, a sarcoma cell, a bone cancer cell, a leukemia cell, a myeloma cell, a lymphoma cell, or a combination thereof.
7. The GNC protein of claim 1, wherein the cancer cell receptor comprises ROR1.
8. The GNC protein of claim 1, wherein the GNC protein is capable of activating a T-cell by binding the cytotoxic cell binding moiety to the T-cell receptor.
9. The GNC protein of claim 1, comprising a tetra-specific antibody or antibody monomer.
10. A therapeutic complex, comprising the GNC protein of claim 1 and a cytotoxic cell, wherein the cytotoxic cell comprises a T cell, a NK cell, a macrophage, a dendritic cell, or a combination thereof.
11. A therapeutic complex, comprising the GNC protein of claim 1 and a cancer cell.
12. A therapeutic complex, comprising the GNC protein of claim 1, a T-cell bound to the T-cell binding moiety and a cancer cell bound to the cancer-targeting moiety.
13. A pharmaceutical composition, comprising the therapeutic complex of claim 10 and a pharmaceutically acceptable carrier.