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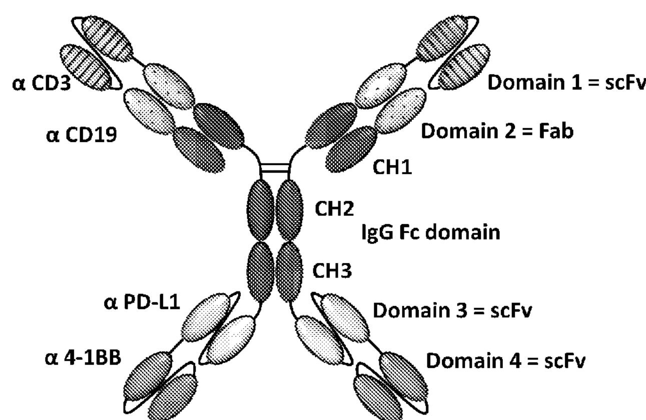
(54) **Title:** METHODS OF MAKING AND USING GUIDANCE AND NAVIGATION CONTROL PROTEINS

FIGURE 1. A GNC protein comprising four antigen-specific binding domains in an antibody structure with targeting specificity to CD19 positive cells.



(57) **Abstract:** The application provides methods for generating a therapeutic composition. The method includes the steps of providing a cell material comprising a cytotoxic cell, incubating the cell material with a first GNC protein to provide an activated cell composition, wherein the activated cell composition comprises a first therapeutic cell, and formulating the activated cell composition to provide a therapeutic composition, wherein the therapeutic composition is substantially free of exogenous viral and non-viral DNA or RNA. The first GNC protein comprises a first cytotoxic binding moiety and a first cancer targeting moiety, wherein the first cytotoxic binding moiety has a specificity to a first cytotoxic cell receptor and is configured to activate the first cytotoxic cell, and wherein the first cancer targeting moiety has a specificity to a first cancer cell receptor. The first therapeutic cell comprises the first GNC protein bound to the cytotoxic cell through the first cytotoxic cell receptor.

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METHODS OF MAKING AND USING GUIDANCE AND NAVIGATION CONTROL PROTEINS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of filing date of U.S. Provisional Patent Application No. 62648888 filed March 27, 2018, and U.S. Provisional Patent Application No. 62648880 filed March 27, 2018, the entire disclosures of which are expressly incorporated by reference herein.

TECHNICAL FIELD

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

Cancer cells develop various strategies to evade the immune system. One of the underlying 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 microenvironments 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 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, Old et al. 2004, Adachi and Tamada 2015).

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, Chesney et al. 2016, Hellmann, Callahan et al. 2018). 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, Makarov et al. 2014). Those patients that have a low tumor mutational load mostly do not show an objective clinical response (Snyder, Makarov et al. 2014, Hellmann, Callahan et al. 2018).

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 an 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 single-chain variable fragment (scFv) specific for CD19 expressed on leukemic cells with the scFv specific for CD3 expressed on T cells (Benjamin and Stein 2016). 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 resistance to Blinatumumab or 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, Kayser et al. 2016). 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). Pembrolizumab specifically binds to PD-1 and blocks the interaction of T cell-expressed PD-1 with tumor cell expressed PD-L1, which 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, Kayser et al. 2016). 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.

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 and June 2015). 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 storm, is considered as the major adverse effect after the infusion of engineered CAR-T cells (Bonifant, Jackson et al. 2016). In many cases, the onset and severity of CRS seems to be personally specific to the patient. 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.

While the efficacy of CAR-T therapy specific for a CD19-positive B cell malignancy is now clearly 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, Li

et al. 2018). The option of managing the therapeutic effect, as well as any adverse effect before and after the CAR-T cell infusion, is limited.

SUMMARY

The application provides, among others, methods for generating therapeutic compositions containing a guidance and navigation (GNC) proteins, methods for treating cancer conditions using a guidance and navigation control (GNC) proteins, and therapeutic compositions containing GNC proteins or therapeutic cells having cytotoxic cells coated (or bound) with GNC proteins.

In one aspect, the application provides therapeutic compositions. In one embodiment, the therapeutic composition comprises a cytotoxic cell, a GNC protein, and a therapeutic cell. The therapeutic cell comprises the GNC protein bound to the cytotoxic cell through the binding interaction with the cytotoxic cell receptor, and the therapeutic cell composition is substantially free exogenous of viral and non-viral DNA and RNA.

In one embodiment, the therapeutic composition may further comprise a second GNC protein, a second therapeutic cell, or a combination thereof, wherein the second therapeutic cell comprises the cytotoxic cells with the second GNC protein bound thereupon or with both the first and the second GNC proteins bound thereupon.

GNC protein includes a cytotoxic binding moiety and a cancer targeting moiety. The cytotoxic binding moiety has a binding specificity to a cytotoxic cell receptor and is configured to activate the cytotoxic cell through the binding with the cytotoxic cell receptor. The cancer targeting moiety has a binding specificity to a cancer cell receptor.

In one embodiment, the GNC protein includes a binding domain for T-cell receptors. 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. 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 is capable of activating a T-cell by binding multiple T-cell binding moieties on the T-cell.

In one embodiment, the GNC protein includes a binding domain for 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.

In one embodiment, the GNC protein includes a binding domain for 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, SIRPα, 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, FRβ, CD163, CR3, DC-SIGN, CD206, SR-A, CD36, MARCO.

In one embodiment, the GNC protein includes a binding domain for 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.

In one embodiment, the GNC protein may include a T-cell binding moiety and a cancer-targeting moiety. In one embodiment, the T-cell binding moiety has a binding specificity to a T-cell receptor comprising CD3, CD28, PDL1, PDL2, PD1, OX40, 4-1BB, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40L, VISTA, ICOS, BTLA, Light, CD30, CD27, or a combination thereof. In one embodiment, the cancer targeting moiety has a binding specificity to a cancer cell receptor. In one embodiment, the cancer cell receptor may include BCMA, CD19, CD20, CD33, CD123, CD22, CD30, ROR1, CEA, HER2, EGFR, EGFRvIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2, as yet to be discovered tumor associated antigens or a combination thereof.

In one embodiment, the GNC protein may have multi-specific antigen binding activities to the surface molecules of a T cell and a tumour cell. In one embodiment, the guidance and navigation control (GNC) protein comprises a binding domain for a T cell activating receptor, a binding domain for a tumor associated antigen, a bind domain for an immune checkpoint receptor, and a binding domain for a T cell co-stimulating receptor.

In one embodiment, the binding domain for the tumor associated antigen is not adjacent to the binding domain for the T cell co-stimulating receptor. In one embodiment, the binding domain for the T cell activating receptor is adjacent to the binding domain for the tumor associated antigen (TAA). The T cell activating receptor may include without limitation CD3. The T cell co-stimulating receptor may include without limitation 4-1BB, CD28, OX40, GITR, CD40L, ICOS, Light, CD27, CD30, or a combination thereof. The immune checkpoint receptor may include without limitation PD-L1, PD-1, TIGIT, TIM-3, LAG-3, CTLA4, BTLA, VISTA, PDL2, or a combination thereof.

The tumor associated antigen (TAA) may include without limitation ROR1, CD19, EGFRVIII, BCMA, CD20, CD33, CD123, CD22, CD30, CEA, HER2, EGFR, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2, or a combination thereof. In one embodiment, the tumor associated antigen may be ROR1. In one embodiment, the tumor associated antigen may be CD19. In one embodiment, the tumor associated antigen may be EGFRVIII.

In one embodiment, the guidance and navigation control (GNC) protein may be an antibody or an antibody monomer or a fragment thereof. In one embodiment, the GNC protein may be a tri-specific antibody. In one embodiment, the GNC protein may be a tetra-specific antibody. In one embodiment, the GNC protein includes Fc domain or a fragment thereof. Any Fc domain from an antibody may be used. Example Fc domains may include Fc domains from IgG, IgA, IgD, IgM, IgE, or a fragment or a combination thereof. Fc domain may be natural or engineered. In one embodiment, the Fc domain may contain an antigen binding site.

In one embodiment, the GNC protein comprises a bi-specific antibody, a tri-specific antibody, a tetra-specific antibody, or a combination thereof yielding up to eight binding motifs on the GNC protein. Examples of antibodies, antibody monomers, antigen-binding fragment thereof are disclosed herein. In one embodiment, 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.

In one embodiment, the guidance and navigation control (GNC) protein may be an antibody. In one embodiment, the tumor associated antigen comprises ROR1, CD19, or EGRFVIII. In on embodiment, the T cell activating receptor comprises CD3 and the binding domain for CD3 may be linked to the binding domain for the tumor associated (TAA) antigen through a linker to form a CD3-TAA pair. In one embodiment, the IgG Fc domain may intermediate the CD3-TAA pair and the binding domain for the immune checkpoint receptor. In one embodiment, the immune checkpoint receptor may be PD-L1.

The linker may be a covalent bond or a peptide linker. In one embodiment, the peptide linker may have from about 2 to about 100 amino acid residues.

In on embodiment, the guidance and navigation control (GNC) protein has a N-terminal and a C-terminal, comprising in tandem from the N-terminal to the C-terminal, the binding domain for CD3, the binding domain for EGFRVIII, IgG Fc domain, the bind domain for PD-L1, and the binding domain for 41-BB. In one embodiment, the guidance and navigation control (GNC) protein has a N-terminal and a C-terminal, comprising in tandem from the N-terminal to the C-terminal, the binding domain for 4-1BB, the binding domain for PD-L1, IgG Fc domain, the bind domain for ROR1, and the binding domain for CD3. In one emobodiment, the guidance and navigation control (GNC) protein has a N-terminal and a C-terminal, comprising in tandem from the N-terminal to the C-terminal, the binding domain for CD3, the binding domain for CD19, IgG Fc domain, the bind domain for PD-L1, and the binding domain for 4-1BB.

In one embodiment, the GNC protein comprises an amino acid having a percentage homology to SEQ ID NO. 50, 52, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, and 110. The percentage homology is not less than 70%. 80%, 90%, 95%, 98% or 99%.

In another aspect, the application provides nucleic acid sequences encoding the GNC protein or its fragments disclosed thereof. In one embodiment, the nucleic acid has a percentage homology to SEQ ID NO. 49, 51, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, and 109. The percentage homology is not less than 70%. 80%, 90%, 95%, 98% or 99%.

In another aspect, the application provides methods for generating a therapeutic composition. In one embodiment, the method may include the steps of providing a cell material comprising a cytotoxic cell, incubating the cell material with a first GNC protein to provide an activated cell composition, and formulating the activated cell composition to provide a therapeutic composition. The activated cell composition contains a first therapeutic cell. The first therapeutic cell comprises the first GNC protein bound to the cytotoxic cell through the binding interaction with the first cytotoxic cell receptor. The therapeutic composition is substantially free of exogenous viral and non-viral DNA or RNA.

In one embodiment, the cell material may include or be derived from PBMC.

The first GNC protein may include a first cytotoxic binding moiety and a first cancer targeting moiety. The first cytotoxic binding moiety has a specificity to a first cytotoxic cell receptor and is configured to activate the first cytotoxic cell through the binding with the first cytotoxic cell receptor. The first cancer targeting moiety has a specificity to a first cancer cell receptor.

In one embodiment, the method may repeat the incubating step by incubating a second GNC protein with the activated cell composition. The second GNC protein comprising a second cytotoxic binding moiety and a second cancer targeting moiety, the second cytotoxic binding moiety has a specificity to a second cytotoxic cell receptor, and the second cancer targeting moiety has a specificity to a second cancer cell receptor. The activated cell composition comprises a second therapeutic cell, and the second

therapeutic cell comprises the second GNC protein bound to the cytotoxic cell or the first therapeutic cell through the binding interaction with the second cytotoxic cell receptor.

In one embodiment, the first and the second cancer-targeting moiety independently has a specificity for CD19, PDL1, or a combination thereof. In one embodiment, the first and the second cytotoxic binding moiety independently has a specificity for CD3, PDL1, 41BB, or a combination thereof.

The method may further include the repeated incubating steps by incubating additional GNC proteins with the activated composition. The additional GNC proteins may be a third GNC protein, a fourth GNC protein, etc. to provide addition therapeutic cells, each having the additional protein bound to the cytotoxic cell.

The first, second, and the additional GNC protein may be the same or may be different. The therapeutic cells may have one GNC protein, multiple same GNC proteins, or multiple different GNC proteins bound thereupon. In one embodiment, the therapeutic cell may have the first GNC protein bound thereupon. In one embodiment, the therapeutic cell may have both the first and the second GNC proteins bound thereupon. In one embodiment, the therapeutic cell may have the first, the second and the additional GNC proteins bound thereupon.

In one embodiment, the therapeutic cell comprises the cytotoxic cell having at least one bound GNC protein. In one embodiment, the therapeutic cell comprises the cytotoxic cell having at least 10, 20, 50, 100, 200, 300, 400 bound GNC proteins.

The therapeutic composition may include the first therapeutic cell, the first GNC protein, the cytotoxic cell, or a combination thereof. In one embodiment, the therapeutic composition may include the second therapeutic cell, the second GNC protein, comprises the first therapeutic cell, the first GNC protein, the cytotoxic cell, or a combination thereof. In one embodiment, the therapeutic composition may include additional GNC proteins and additional therapeutic cells.

In one embodiment, the incubating step may serve to expand the therapeutic cells. In one embodiment, expanding the therapeutic cell may include incubating the therapeutic cells with an additional amount of the GNC protein to provide an expanded cell population. In one embodiment, the expanded cell population comprises at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10} cells per ml. In one embodiment, the expanded cell population comprises the GNC bound cell, the GNC protein, the cytotoxic cell, or a combination thereof. In one embodiment, in order to deplete PD-1+ T cells, a GNC protein may be added to the expansion culture that redirects killing to PD-1+ T cells therefore resulting in reduction in PD-1+ exhausted T cells. In one embodiment, in order to preferentially support PD-1+ T cells, a GNC protein may be added to the expansion culture that relieves checkpoint signaling through PD-1 on T cells therefore resulting in functional improvement of PD-1+ T cells. In one embodiment, in order to isolate 4-1BB mediated co-stimulation through 3rd gen CAR-T, a GNC protein may be added to the expansion culture that redirects killing to 4-1BB+ T cells or resulting in therapeutic composition with controlling level of 4-1BB stimulation in the therapeutic cells, such as CAR-T cells.

In one embodiment, the cancer targeting moiety has the specificity against B cell, and the therapeutic composition is substantially free of B cell. Therefore, the methods disclosed herein couple the activation and purification functions for the therapeutic cells, which allows the methods to produce B cell free therapeutic composition without the need to introduce any foreign materials (such as beads) nor any foreign genetic materials (such as viral and non-viral DNA or RNA vectors).

In one embodiment, the ratio of the GNC protein and the cytotoxic cell is at least 30 to 1 when incubating the cell material with the GNC protein.

In one embodiment, the therapeutic composition may include at least 10^7 cells per ml.

In a further aspect, the application provides methods for using guidance and navigation control (GNC) proteins for cancer treatment. In one embodiment, the method of treating a subject having a cancer, comprises providing a cytotoxic cell, combining a GNC protein with the cytotoxic cell to provide a therapeutic cell, optionally expanding the therapeutic cell to provide an expanded cell population, and administering the therapeutic cell or the expanded cell population to the subject.

In one embodiment, the method include the step of providing a cell material comprising a cytotoxic cell, incubating the cell material with a first GNC protein to provide an activated cell composition, wherein the activated cell composition comprises a first therapeutic cell, formulating the activated cell composition to provide a therapeutic composition, wherein the therapeutic composition is substantially free exogenous of viral and non-viral DNA or RNA, and administering the therapeutic composition to the subject.

In one embodiment, the method may further include the steps of incubating a second GNC protein with the activated cell composition to provide the activated cell composition further comprising a second therapeutic cell. In one embodiment, the method may further include the step of incubating additional GNC proteins with the activated cell composition to provide the activated cell composition further comprising additional therapeutic cells.

In one embodiment, the method may further comprise isolating the cytotoxic cell from peripheral blood mononuclear cells (PBMC) before providing the cytotoxic cell. In one embodiment, the method may further comprise isolating the peripheral blood mononuclear cells (PBMC) from a blood. In one embodiment, the blood is from the subject. In one embodiment, the blood is not from the subject. In one embodiment, the cytotoxic cells may be from the patient that is under treatment or a different individual, such as a universal donor.

In one embodiment, the cytotoxic cell may be an autologous T cell, an alloreactive T cell, or a universal donor T cell. In one embodiment, when autologous donor T cells are used, in order to prevent infusion of contaminating cancer cells, a GNC protein may be added to the expansion culture that redirects killing to tumor antigens, example tumor antigen may include CD19 for B cell malignancies, Epcam for Breast carcinoma, MCP1 for melanoma.

In one embodiment, the method includes steps of providing a blood from the subject, isolating peripheral blood mononuclear cells (PBMC) from the blood, isolating a cytotoxic cell from the PBMC, combining a GNC protein with the cytotoxic cell to provide a therapeutic cell, optionally expanding the therapeutic cell to provide an expanded cell population, and administering the therapeutic cell or the expanded cell population to the subject.

In one embodiment, the method further comprises administering additional GNC protein to the subject after administering the therapeutic composition to the subject. In one embodiment, the cytotoxic cell may include CD3+ T cell, NK cell, or a combination thereof.

In one embodiment, the isolating of the cytotoxic cell comprises isolating at least one subpopulation of cytotoxic cells to provide the therapeutic T cells. In one embodiment, the subpopulation of cytotoxic cells comprises CD4+ cells, CD8+ cells, CD56+ cells, CD69+ cells, CD107a+ cells, CD45RA+ cells, CD45RO+ cells, CD2+ cells, CD178+ cells, Granzyme+ cells, or a combination thereof.

In one embodiment, the combining of a GNC protein with the cytotoxic cell comprises incubating the GNC protein with the cytotoxic cell for a period of time from about 2 hours to about 14 days, from about 1 day to about 7 days, from about 8 hours to about 24 hours, from about 4 days to about 7 days, or from about 10 days to about 14 days. In one embodiment, the incubating period may be more than 14 days. In one embodiment, the incubating period may be less than 2 hours.

In one embodiment, the ratio between the GNC protein and the cytotoxic cell is at least 600 to 1, 500 to 1, 400 to 1, 300 to 1, 200 to 1, 100 to 1, or 1 to 1. In one embodiment, the ratio between the GNC protein and the cytotoxic cell is from about 1 to 1, 10 to 1, 100 to 1, or to about 1000 to 1 ratio.

In one embodiment, the method may further comprise evaluating therapeutic efficacy after the administering step. In one embodiment, the evaluating therapeutic efficacy includes checking one or more biomarkers of the cancer, monitoring the life span of the therapeutic cells, or a combination thereof. In one embodiment, evaluating therapeutic efficacy comprises checking one or more biomarkers of the cancer, monitoring the life span of the therapeutic cells, or a combination thereof. In one embodiment, the biomarker comprises a tumor antigen, release of cytokines e.g., gamma interferon, IL-2, IL-8, and/or chemokines, and/or CD markers on the surface of various cell types e.g., CD69, PD-1, TIGIT, and/or mutated nucleic acid released into the bloodstream by tumors upon death, circulating tumor cells and their associated nucleic acid, or exosome associated nucleic acid, host inflammatory mediators, or tumor derived analytes, or a combination thereof. In one embodiment, the biomarker comprises a tumor antigen, tumor-associated apoptotic bodies, small molecule metabolites, release of cytokines, lymphocyte surface marker expression, phosphorylated/dephosphorylated signaling molecules, transcription factors, or a combination thereof

The method disclosed herein is free of the step of transfecting the cytotoxic cell with a DNA vector or a viral vector. In one embodiment, the therapeutic cell or the expanded cell population is substantially free of a DNA vector or a viral vector.

The method may be used to treat a human subject suffering from cancer. In one embodiment, the cancer comprises cells expressing ROR1, CEA, HER2, EGFR, EGFRvIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2, BCMA, CD20, CD33, CD123, CD22, CD30, CD19, as yet to be identified tumor associated antigens, or a combination thereof. In one embodiment, the method may be used to treat mammals.

Varieties of cancer may be treated using the methods disclosed herein. Example cancers includes 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 administering an effective amount of a therapeutic agent after the administering the therapeutic cell or the expanded cell population to the subject. In one embodiment, the therapeutic agent comprises a monoclonal antibody, a chemotherapy agent, an enzyme, a protein, a co-stimulator, or a combination thereof. In one embodiment, the co-stimulator is configured to increase the amount of cytotoxic T cells in the subject.

The application further provides a solution comprising an effective concentration of the GNC protein. In one embodiment, the solution is blood plasma in the subject under treatment. In one embodiment, the solution includes the GNC protein bound cells. In one embodiment, the solution includes a GNC cluster including a GNC protein, a T-cell bound to the T-cell binding moiety of the GNC protein, and a cancer cell is bound to the caner-targeting moiety of the GNC protein.

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.

BRIEF DESCRIPTION OF THE DRAWINGS

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:

FIGURE 1 shows a GNC protein comprising four antigen-specific binding domains in an antibody structure with targeting specificity to CD19 positive cells;

FIGURE 2 illustrates that a tetra-specific GNC antibody mediates multi-specific binding between a T cell and a tumor cell;

FIGURE 3 is a flowchart comparing manufacturing processes for GNC-T cell therapy (left) and CAR-T cell therapy (right);

FIGURE 4 is a diagram showing sources of cell material for preparing GNC-activated therapeutic cell composition;

FIGURE 5 is a diagram showing sources of selected T cells for preparing GNC-activated therapeutic composition;

FIGURE 6 is a diagram showing the preparation of GNC-activated therapeutic T cell composition;

FIGURE 7 is a diagram showing the incubating and formulating steps for preparing the first GNC-activated T cells for GNC-T cell therapy;

FIGURE 8 shows that GNC proteins (SI-35E class) induce IL-2 secretion from PBMC;

FIGURE 9 shows that GNC proteins (SI-35E class) induce granzyme B secretion from PBMC;

FIGURE 10 shows that GNC proteins (SI-35E class) induce expression of the activation marker CD69 on CD4+ T cells;

FIGURE 11 shows that GNC proteins (SI-35E class) induce expression of the activation marker CD69 on CD8+ T cells;

FIGURE 12 shows that GNC proteins (SI-35E class) induce expression of the activation marker CD69 on CD56+ NK cells;

FIGURE 13 shows that GNC proteins (SI-35E class) induce expression of the marker of cytotoxic degranulation CD107a on CD4+ T cells;

FIGURE 14 shows that GNC proteins (SI-35E class) induce expression of the marker of cytotoxic degranulation CD107a on CD8+ T cells;

FIGURE 15 shows that GNC proteins (SI-35E class) induce expression of the marker of cytotoxic degranulation CD107a on CD56+ NK cells;

FIGURE 16 shows that GNC proteins (SI-35E class) activate CD3+ T cells to proliferate;

FIGURE 17 shows that GNC proteins (SI-35E class) activate CD3+ T cells to secrete gamma interferon;

FIGURE 18 shows that GNC proteins (SI-35E class) activate naïve CD8+/CD45RA+ T cells to proliferate;

FIGURE 19 shows that GNC proteins (SI-35E class) activate naïve CD8+/CD45RA+ T cells to secrete gamma interferon;

FIGURE 20 shows Images of GNC activated cell growth in 6-well G-Rex plates over time;

FIGURE 21 shows the example process of making the therapeutic composition as disclosed thereof (A), and cell viability of PBMC, GET, and GNC-T cells after thawing (B);

FIGURE 22 shows the result of flow cytometry analyses of PBMC-derived, the first GNC (SI-38E17)-activated therapeutic cell composition (Product A) (22A), the second GNC (SI-38E17)-coated therapeutic cell composition (Product B) (22B), and input PBMC cell material (22C).

FIGURE 23 shows GNC-T therapeutic cell composition of GET cells and formulated GNC-T cells from G-Rex 100M bioreactor after thawing;

FIGURE 24 shows the result of RTCC of CHO-ROR1 cells by using GNC (SI-35E class)-coated PBMC cells;

FIGURE 25 shows kinetics of PBMC-derived, SI-38E17 GNC-activated therapeutic cells on killing precursor B cell leukemia Kasumi over time;

FIGURE 26 shows efficacy of killing Nalm-6, MEC-1, Daudi, and Jurkat cells by using PMBC-derived, SI-38E17 GNC-activated therapeutic cells; and

FIGURE 27 shows the killing of Nalm-6, MEC-1, Daudi, and Jurkat leukemic cells by using PBMC-derived, SI-38E17 GNC-activated therapeutic cells in a spike-in model.

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 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.

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 such as bystander suppressor cells) through the binding of multiple surface molecules on a T cell and a tumor cell. In one embodiment, 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 as shown in TABLE 1. FIGURE 1 shows the structure of an example tetra-specific GNC antibody comprising AgBDs for binding to both a T cell expressing CD3, PD-L1, and/or 4-1BB and a target B cell expressing CD19, as illustrated in FIGURE 2.

In a T cell therapy, the cytotoxic T cells are regulated by T cell receptor complex proteins, as well as co-stimulation signaling proteins via either agonist receptors or antagonist receptors on their surface. To regulate this signaling, as well as the interaction between a T cell and a cancer cell, multiple AgBDs may

compose Moiety 1 and Moiety 2, respectively. Examples of molecules that can be targeted by agonistic or antagonistic binding domains in Moiety 1 and 2 are shown in TABLE 1. In one embodiment, the GNC proteins may have at least one linker to link Moiety 1 and Moiety 2. In one example 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. In some embodiments, the linkers may be an antibody backbone structure or antibody fragments, so that GNC protein and GNC antibody may have the same meaning, e.g. the structure of the example tetra-specific GNC antibody in FIGURE 1.

GNC proteins or antibodies are capable of directing a T cell to a cancer cell, *in vivo* or *ex vivo*, through the binding function of multiple AgBDs (FIGURE 2). 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 cell therapy, for activating and controlling cytotoxic T cells *ex vivo*, prior to adoptive transfer.

The present application relates to methods of making GNC-activated therapeutic cell composition. Multiple AgBDs can be divided into Moiety 1 and Moiety 2 due to their interface with a T cell and a cancer cell, respectively (TABLE 1). 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 the T cell to the tumor cell.

The addition of a third AgBD, for example, one that specifically binds 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 a fourth AgBD to a GNC protein, for example, one that specifically binds to PD-L1 on a tumor cell, may block the inhibitory pathway of PD-L1 on tumor cells or that is mediated through its binding to PD-1 on the T cells.

in some embodiments, with these basic principles, GNC proteins are 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, in some embodiments, GNC proteins may be bi-specific, tri-specific, tetra-specific, penta-specific, hexa-specific, hepta-specific, or octa-specific proteins.

In one embodiment, the application relates to a GNC-T cell therapy where GNC proteins are used to expand the T cells *ex vivo* prior to adoptive transfer (FIGURE 3). The *ex vivo* priming of autonomous T cells provides the cytotoxic T cells guidance and navigation control. For example, peripheral blood mononuclear cells (PBMC) or specific types of cell populations within PBMC e.g., CD8+, CD45RO+ memory T cells may be isolated and primed *ex vivo* by GNC proteins. These expanded cytotoxic T cells can be formulated and infused back to the patient through adoptive transfer. While attacking the cancer *in vivo*, additional GNC proteins may be infused into the patient for managing the efficacy and lifespan of cytotoxicity. Thus, GNC-T cell therapy is different from GNC protein-based immunotherapy, where GNC proteins are directly administered into patients. However, GNC-T cell therapy does not rule out the direct administration of GNC proteins for managing the efficacy of infused cytotoxic T cells *in vivo* in a controlled manner. Additional GNC protein can both promote cytolytic activity and encourage T cell proliferation dependent of the configuration of AgBDs.

In one aspect, the application relates to the production of therapeutic GNC-T cells. In comparison with and to distinguish from the production of therapeutic CAR-T cells, their general processes are shown

in FIGURE 3, for comparison purpose. In CAR-T therapy, cell material, for example patient leukocytes, are collected by apheresis, and a subset of CD3+ T cells is selected and activated to facilitate gene transfer to the cellular material, which is then expanded in number by the introduction of foreign material scaffold for support to the T cell populations, for example, by using anti-CD3/anti-CD28 antibody coated beads. Advantageously, GNC-T cell material does not require the introduction of scaffold impurities for T cell expansion from patient leukocytes.

The CAR-T therapy cellular material must undergo the gene transfer that involves the preparation and transfection of CAR-T vector DNA, which results in genetically modifying the genome of the T cells. Furthermore, these genetically modified T cells may undergo another round of T cell expansion before being transferred back into the patient. The random integration of CAR-T vector DNA carries a risk of transformation of the T cells leading to primary leukemogenesis or introduction of the CAR-T vector to leukemia cells increasing the risk of relapse by mechanism of internal sequestration of the CAR target antigen (Zhang, Liu et al. 2017).

In contrast, GNC-T cell therapy has the advantages of not involving the transfection of any vector DNA, therefore there is no risk of genetic modification prior to adoptive transfer, which provides one of the significant advantages and technical improvements over the existing CAR-T therapy. Besides the advantage of GNC-T cell therapy being free of exogenous generic material contamination and cancer risk, the efficacy of GNC-T cell therapy may be improved when PBMC or different T cell subsets are being primed and activated *ex vivo* as shown in FIGURE 5 & 6. Similar approaches have been explored in the use of CAR-T therapy, where selected specific ratios of some subsets of T cells may be transferred back to the patient (Turtle, Hanafi et al. 2016, Turtle, Hanafi et al. 2016).

In some embodiments, it may be beneficial to remove leukemia or other cancer cells from the cellular material prior to cell expansion (FIGURE 7). The PBMC of a patient with circulating leukemic cells, in particular from B cell malignancy, may profoundly alter the cellular composition and thus affect the suitability of the final therapeutic cellular products. For example, a high level of circulating leukemic blast cells (greater that 10% of WBC) may require a depletion of leukemic cells prior to GNC mediated cell expansion. The percentage of leukemic cells in the PBMC derived from a patient may be reduced by using cell fractionation methods. These methods may include steps involving density gradient separation, or immunofluorescent cell separation or fluorescent activated cells sorting, immunomagnetic cell separation, or microfluidic flow chambers methods. These methods may be preceded by or follow centrifugation, cell washing, incubation, or temperature modulation. These methods may utilize non-cellular substrates (magnetic beads, Plastic, polymers), modification of non-cellular substrates (protein, antibodies, charge state), antibody treatment, multiple antibody treatments, multi-specific antigen binding proteins and cell surface antigen-based cell coupling. These methods may use enzymatic digestion or, ionic chelation, or mechanical agitation or cell vessel rotation. The method for reduction of leukemic blasts may utilize antibody drug conjugates, or leukemia sensitizing agents. The method may consist of a combination of these approaches.

In one embodiment, to enable the production of therapeutic T cells primed (or coated or bound) with GNC proteins, a tetra-specific antibody is produced and used as the GNC protein. In one embodiment, the tetra-specific antibody/GNC protein comprises 4 different binding domains linked by antibody fragments as its backbone. 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-L2, PD-1, OX40, 4-1BB, GITR, TIGIT, TIM-3, LAG-3, CTLA4, CD40L, VISTA, ICOS, BTLA, Light, etc.

Without being bound by theory, the advantages of GNC protein-mediated GNC-T cell therapy over conventional CAR-T therapies include, but are 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.

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

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. GNC proteins and tetra-specific GNC antibodies

In the present application, the examples of GNC proteins are classes of tetra-specific GNC antibodies, of which 4 AgBDs are covalently linked using an IgG antibody as its backbone (FIGURE 1). From the N-terminal of this protein, the first scFv is linked to the Fab domain of the constant domains C_H1, 2, and 3 of IgG antibody which is then linked to another scFv at the C-terminal. Because each of the scFv domains display independent binding specificity, linking of these AgBDs does not need to be done using the constant domains of an IgG antibody. Structured as a tetra-specific GNC antibody, a GNC protein can directly bind to tumor-associated antigen (TAA) and engage the host endogenous T cells to kill tumor cells independent of tumor antigen presentation by MHC to the antigen specific T cell receptors (FIGURE 2). As shown in FIGURE 1, CD19 is a TAA targeting CD19 positive B cells and tumor cells. In addition, PD-L1 is an example of the immune checkpoint modulating component for tetra-specific GNC antibodies that may overcome the immunosuppressive tumor microenvironment and fully activate the exhausted T cells within the tumor microenvironment.

Of tetra-specific GNC antibodies, the SI-35E class comprises targets an anti-human CD3 binding domain (SEQIDs 1-4), an anti-human PD-L1 (SEQIDs 5-12), an anti-human 4-1BB (SEQIDs 13-24), and targets a human ROR1 (SEQIDs 25-32), i.e. a TAA. In this context, the classes of SI-38E and SI-39E target CD19 (SEQIDs 47-50) and EGFR (SEQID 51-54), respectively.

To construct tetra-specific GNC antibodies, AgBDs were converted to scFv and VLVH for placement at the N-terminal Domain 1 (D1) or scFv and VHVL for placement at the C-terminal Domains 3

(D3) and 4 (D4) of the GNC protein. 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 GNC antibody, Domain 2 (D2), 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 linkers, 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 GNC 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.

The tetra-specific GNC antibodies can be 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. Herein, 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 this example, SI-35E18 (SEQID 65 and 67) was split into its component domains where the anti-41BB scFv, VL-VH, 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 37°C overnight. Resultant colonies were selected and 2 mL 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 tetra-specific GNC protein, SI-38E17 targeting human CD19 (SEQIDs 47-50), multiple AgBDs carry an anti-human 4-1BB (scFv 466F6, SEQIDs 17-20) as well as an anti-human PD-L1 (scFv PL221G5 SEQIDs 9-13), and an anti-human CD3 binding domain (SEQIDs 1-4). The methods and procedures for producing this tetra-specific antibody were the same.

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). The tetra-specific GNC antibodies can be used to directly engage the body's endogenous T cells to kill tumor cells independent of tumor antigen presentation by MHC to the antigen specific T cell receptors. This is in contrast to therapies based solely on immune checkpoint blockade, which have been limited by antigen recognition. In context, the immune checkpoint modulating component may be constructed as a part of tetra-specific GNC antibodies, which may provide benefits similar to that in a standard checkpoint blockade therapy.

In addition to T cells, other cytotoxic cells may also be targeted by 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.

GNC proteins are constructed to acquire multiple AgBDs specifically for binding unequal numbers of T cell antagonists and agonists. In this way, GNC proteins may re-direct activated T cells to tumor cells with certain levels of control of their activity *in vivo* (TABLE 2). Therefore, GNC proteins may be bi-specific, tri-specific, tetra-specific, penta-specific, hexa-specific, hepta-specific, or even octa-specific proteins. In the present invention, three classes of tetra-specific GNC antibodies, i.e. SI-39E, SI-35E, and SI-38E, were created to enable GNC-T cell therapy, of which antibody domains and its specificity is listed in TABLE 3. The structures of tetra-specific GNC antibodies targeting EGFRvIII (SI-39E), ROR1 (SI-35E), and CD19 (SI-38E) are listed in TABLE 4.

Example 2: GNC-activated, PBMS-derived cell composition.

The SI-35 class listed in Table 4 were tested for their ability to activate and induce proliferation of different cell types, such as CD4+ and /or CD8+ T cells and/or CD56+ natural killer cells (NK) within PBMC. The tetra-specific GNC antibodies were prepared at 2X final concentration and titrated in 1:10 serial dilutions across 6 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 PBMC and serially titrated GNC proteins were combined by adding 100 µL of PBMC (100,000), and 100 µL 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 by FACS for the number of CD4+ T cells, CD8+ T cells, and CD56+ NK cells. Cells were harvested from each well and transferred to a new 96 well V-bottom plate then centrifuged at 400 x g for 3 minutes. Supernatant was transferred to a 96 well plate for analysis of IL-2 and Granzyme B. Cells were re-suspended in 200 µL of 2%FBS/PBS of FACS antibodies and incubated on ice for 30 minutes. The plate was centrifuged at 400 x g for 3 minutes and the supernatant was aspirated. This wash step was repeated once more and then the cells were re-suspended in 100 µL 2%FBS/PBS and analyzed on a BD LSR FORTESSA.

As shown in FIGURE 8, all SI-35E tetra-specific GNC antibodies, with the exception of those that had the scFv binding domain replaced with FITC at positions 2 (SI-35E37) and 4 (SI-35E39), induced production of IL-2 from PBMC. These two proteins lacked the binding domains for PD-L1 or CD3 respectively. The secretion of Granzyme B into the culture supernatant followed a similar pattern as that for IL-2 production as shown in FIGURE 9. Both SI-35E37 and SI-35E3 were also much less potent at inducing cell-surface expression of the activation marker CD69 on CD4+ (FIGURE 10), CD8+ (FIGURE 11), and CD56+ (FIGURE 12) cells in the PBMC culture. Surface expression of the cytotoxic degranulation marker CD107a (LAMP-1) was induced by all GNC proteins tested except those lacking binding at positions 2 and 4 on CD4+ (FIGURE 13), CD8+ (FIGURE 14), but less consistently on CD56+ (FIGURE 15) in the culture. At lower concentrations, 3 of the GNC proteins (SI-35E42, SI-35E43, and SI-35E46) induced expression of

CD69 on CD4+ T cells, CD8+ T cells, and CD56+ NK cells, which correlated well with the level of IL-2 and granzyme B secretion (FIGUREs 8 and 9) induced by these GNC.

Proliferation and production of gamma interferon was measured from cultures of $CD3^+$ or naïve $CD8^+$ T cells (70,000 cells/well) stimulated for 5 days with a panel of SI-35 class antibodies. Human $CD3^+$ or $CD8^+CD45RA^+$ naive T cells were enriched from peripheral blood mononuclear cells from a normal donor using the EasySep™ Human $CD3^+$ or Naïve $CD8^+$ T Cell Isolation Kits (StemCell Technologies) as per the manufacturer protocols. The final cell populations were determined to be >98% $CD3^+$ or $CD8^+CD45RA^+$ T cells by flow cytometry. Proliferation in the culture was measured after stain with Alamar blue (ThermoFisher Cat. No. DAL1100) for 1 hour at 37°C, and then read on a Spectramax plus 384 well reader (Molecular Devices). Proliferation of GNC-expanded $CD3^+$ T cells was expressed as a fold increase in cell number over background of $CD3^+$ T cells in cell culture without GNC (FIGURE 16). Proliferation was induced by all constructs tested except the one lacking CD3 binding domain. Culture supernatants were also collected from these cultures and analyzed for the presence of gamma interferon by ELISA. Secretion of gamma interferon (FIGURE 17) was high unless CD3 or ROR1 binding domains were changed to FITC in the GNC constructs. Proliferation of naïve $CD8^+CD45RA^+$ T cells (FIGURE 18) was more sensitive to the presence or absence of 4-1BB binding domain compared to total $CD3^+$ T cells as shown by addition of soluble anti-4-1BB monoclonal antibody to the culture in which 4-1BB binding on the GNC was absent. A similar pattern was found for secretion of gamma interferon from the naïve $CD8^+$ T cells (FIGURE 19).

Example 3. Scale up and formulation of a first GNC-activated therapeutic cell composition.

The manufacture of GNC-activated and -coated T cells at clinically significant dosage of 10E9 was achieved after 7 days culture. Human PBMC were isolated from LRS cone leukocytes by standard Ficoll density gradient from leukopaks which are enriched leukapheresis product collected from normal human peripheral blood. After collection the cells were frozen at -80°C and then later thawed before putting in culture. Using the G-Rex plate and bioreactor culture systems, the growth of SI-38E17 GNC-stimulated PBMC cultures was monitored for up to 14 days. The culture medium consisted of RPMI 1640, 10% fetal calf serum, 1% non-essential amino acids, 1% GlutaMax, 0.6% glutamine-alanine supplement, 15 ng/mL human IL-2, and 1nM GNC protein. The 6-well G-Rex cultures tolerated seeding densities of 25-100 million PBMC/well for six days, which greatly exceeded recommended amounts, but was tolerated by the cells in the system with a single 50% medium change on day 7. Clustering of cells was indicative of their activation in the culture (FIGURE 20). At least 250 million cells from one leukapheresis donor were seeded into two G-Rex 100M bioreactors and cultured in 1 liter of culture medium for seven days. The larger volume of medium allowed the culture to continue without needing to exchange the culture medium. Cell yield in each of the 100M bioreactors was between 1.2-1.4 billion cells with greater than 88% viability.

Example 4. A second GNC-activated therapeutic cell composition.

The cells from the bioreactor were harvested as the first GNC-activated therapeutic cell composition, which were optionally concentrated using LOVO Automated Cell Processing System (Fresenius Kabi). One sample (Product B) was exposed to 1 nM SI-38E17, which is identical to the first GNC in this case for preparing a second GNC-activated therapeutic cell composition, potential for being used to target treat patients harboring CD19 positive malignancies (FIGURE 21A).

After the second concentration step (100 mL volume) during the processing in the LOVO system, the second GNC-activated therapeutic cells were washed twice before eluting to a final volume of 54 mL in a sterile processing bag. The other sample (Product A) was only exposed to the first GNC protein during

the culture phase and not re-exposed during processing in the LOVO system (FIGURE 21A). Cells were removed from bags, mixed 1:1 with CryoStor CS10 reagent, and frozen to -80°C. The processed cells were thawed and compared to the thawed unstimulated PBMC from the same donor before culture.

Cell viability from the GNC-expanded T cell (GET) culture was >75% and was not affected by exposure to additional GNC reagent (GNC-T, Product B) during processing (FIGURE 21B). The mean diameter of the cells increased during culture, indicative of cell activation. Flow cytometry was performed on the input PBMC cell material and the two formulations after thawing using a multi-color panel of antibodies to stain for: live/dead (e780), CD45, TCRα/β, CD56, CD4, CD8, CD14, TCRγ/δ, and CD20. Gating for quantification of the different cell subsets is shown on the GNC-activated T cells (Product A) and the additional GNC-coated GNC-T cells (Product B) (FIGURE 22A and 22B). The percentages of each subpopulation of cells were similar between Product A and Product B, but very different from those of input PBMC (FIGURE 22C). FIGURE 23 summaries the total number and percentage of each subpopulation of cells. Compared to the input PBMC cell material, while the total number of leukocytes increased from 250 to 1000 millions or four-fold, the total number of each subpopulation of T cells was vastly increased by 55-fold for α/β T cells, 45-fold for CD4+ T cells, and 78-fold for CD8+ T cells. In this context, the increase of γ/δ T cells was modest at 5-fold, and TCRα/β-/lo, γ/δ+, CD8+ T cells seemed to the most abundant. Finally, the characteristic feature of both Product A and Product B cell compositions is the fact that there were no detectable B cells.

This example illustrates a number of advantages of GNC-T cells in comparison to CAR-T cell preparations. First, the cell composition of the starting material was fresh PBMC from the donor and did not need to be pre-selected for particular subsets of cells or require addition of feeder cells or synthetic beads. The GNC protein was 100% non-nucleotide biological material, and did not require the transfer of RNA or DNA into the cells, or transfection with a viral vector. The GNC-induced expansion yielded a therapeutic dose in 9 days, compared to the average of 40 days for CAR-T cell expansion. The resulting cells were devoid of B cells and highly enriched for activated CD4+ and CD8+ T cells that had potent killing potential against their specific targets. The GNC therapeutic composition was viable and bioactive upon thaw from -80°C. Together these advantages are expected to significantly lower waiting times, costs and issues related to infrastructure and training related to CAR-T cell therapy. Improvements in the purity, safety and quantity of the end product will be of significant benefit to the patient.

Example 5. PBMC pre-activated with GNC proteins are redirected to potently kill tumor cells.

Six of the GNC SI-35 class proteins listed in Table 4 were tested for the ability to activate PBMC for redirected T cell cytotoxicity (RTCC) activity against a human ROR1-transduced CHO cell line (FIGURE 24). GNC proteins were prepared at 2X final concentration and titrated 1:3 across 10 wells of a 96 well plate in 200 ul of RPMI + 10%FBS. In the final destination 96 well plate, the PBMC and serially titrated antibodies were combined by adding 100 µL of PBMC (200,000), and 100 µL of each antibody dilution to each well of the assay. The assay plate was incubated at 37°C for approximately 72 hours before the addition of CFSE-labeled CHO-ROR1 cells. CHO-ROR1 target cells, 5 x 10e6, were labeled with CFSE (Invitrogen, #C34554) at 0.5 µM in 10 mL of culture media for 20 minutes at 37°C. The CHO-ROR1 cells were washed 3 times with 50 mL of culture media before resuspending in 10 mL, counted again and then 5,000 CFSE-labeled CHO-ROR1 cells were added to each well of GNC-activated PBMC. Cells were incubated for another 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 FIGURE 24, all of the GNC proteins tested directed RTCC activity with SI-35E42, SI-35E43, and SI-35E46 being the most potent in reducing the number of CHO-ROR1 cells in the well.

To further demonstrate the killing effects of GNC-labeled PBMC against human tumor cells, a GNC-dose and effector:target ratio escalation experiment was performed using an IncuCyte S3 Live Cell Analysis System (Sartorius/Essen Biosciences) to monitor the cells over time. PBMC from a healthy donor were labeled with GNC protein SI-38E17 at 10-fold serial doses ranging from 0.01 to 100 nM for 30 minutes at 37°C and then washed prior to culture. The GNC SI-38E17 targets the CD19 antigen expressed on B cell surfaces, and therefore, the Kasumi-2 precursor B cell leukemia line was chosen as a target cell. The Kasumi-2 cell used was transduced to express green fluorescence protein (GFP) and therefore the presence of tumor cells was tracked by measuring the average green fluorescence in 4 images/well collected 9 times over a six-day period. The effector:target (E:T) ratios were escalated by adding GNC-labeled PBMC in a serial 2-fold dilution of 5,000 (1:1) to 160,000 (32:1) cells to duplicate wells. As shown in FIGURE 25, Kasumi-2 cells increased in number in the wells that had from 1:1 to 8:1 E:T ratios of unlabeled PBMC. Exposure to as little as 0.1 nM GNC led to decreased growth of Kasumi-2 in the 1:1 culture with suppression increasing at each 2-fold increase in the E:T ratio. Coating of PBMC with 1nM or greater concentrations of GNC led to nearly complete elimination of Kasumi-2 cells after 42 hours of culture at all E:T ratios.

As a follow up experiment, three other transformed B cell lines: NALM-6, MEC-1, and Daudi and the acute T cell leukemia line, Jurkat, were used as target cells. These target cells were previously transduced by lentivirus to constitutively express the NucRed 647 molecule. In this assay, PBMC were exposed to 10-fold doses of GNC protein SI-38E17 for 30 minutes at 37°C and then washed as before. PBMC were plated at $1.2x10^6$ cells/well and 50,000 target tumor cells were added. Cells were placed in IncuCyte S3 set to collect red fluorescence images (4 images/well) collected at 10 time points over a 5.5-day period (FIGURE 26). Growth curves were established for all four tumor cell lines in the absence of PBMC (null). Labeling of PBMC with 1 nM or more of GNC protein SI-38E17 led to arrested growth of all three B cell lines but not Jurkat T cell leukemia. The B cell lines varied in their susceptibility to PBMC cells pre-exposed to 0.1 nM of GNC protein.

As a different method of quantifying the outcome of cultures of GNC-T cells with tumor cells, we established a limit of quantification (LOQ) curve for detection by flow cytometry. Daudi-Red cells were serially diluted 10-fold in a range from 200,000 to 20 cells and then mixed 1:1 with 1 million PBMC to create samples of 10%, 1.0%, 0.1%, 0.01% and 0.001% tumor cells, which were then analyzed by flow cytometry (FIGURE 27). Next, cells were harvested from a 15 day 6-well G-Rex culture of 1 nM GNC-expanded T cells that had been spiked with 10%, 1% or 0.1% of NALM-6, MEC-1, Daudi, or Jurkat (all NucRed-transduced) tumor cells at time 0 and analyzed using the same flow cytometry settings as above. Tumor cells were reduced to less than 0.001% in all conditions with the exception of the culture in which the MEC-1 tumor line was spiked in at 10% were 44 cells were detected. In this condition the MEC-1 cells were reduced to <0.01% in the culture.

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.

TABLES

TABLE 1A. Composition of 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, GITR, CD40L, ICOS, Light, CD27, CD30	PDL1, PD1, TIGIT, TIM-3, LAG-3, CTLA4, BTLA, VISTA, PDL2	BCMA, CD19, CD20, CD33, CD123, CD22, CD30, ROR1, CEA, HER2, EGFR, EGFRvIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2

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.

GNC protein	T cell activation	Tumor antigen	T cell antagonist	T cell agonist	T cell antagonist	T cell antagonist	T cell antagonist	T cell 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	TIM3		
Hepta-specific	CD3	ROR1	PD1	41BB	LAG3	TIM3	TIGIT	
Octa-specific	CD3	ROR1	PD1	41BB	LAG3	TIM3	TIGIT	CD28

TABLE 3. Specificity of antibody binding domains used in GNC proteins.

AgBD Specificity	Antibody Name
CD3ε	284A10 480C8
4-1BB	460C3 420H5 466F6
FITC	4420
PD-L1	PL230C6
CD19	21D4
ROR1 IgD Domain Kringle Domain Frizzled Domain	 323H7 330F11 338H4 324C6
EGFRvIII	806

Table 4. Classes of tetra-specific GNC antibodies targeting EGFRvIII (SI-39E), ROR1 (SI-35E), and CD19 (SI-38E).

GNC ID	AgBD 1 (LH-scFv)	Humanized Variant	AgBD 2 (Fab)	Humanized Variant	IgG1 Fc	AgBD 3 (HL-scFv)	Humanized Variant	AgBD 4 (HL-scFv)	Humanized Variant
SI-39E18	284A10	L1H1	806	-	n2	PL221G5	H1L1	420H5	H3L3
SI-39E29	806	-	284A10	H1L1	n2	PL221G5	H1L1	420H5	H3L3
SI-35E20	466F6	L5H2	PL230C6	H3L2	n2	323H7	H4L1	284A10	H1L1
SI-35E58	284A10	L1H1	PL230C6	H3L2	n2	323H7	H4L1	466F6	H2L5
SI-35E88	284A10	L1H1	323H7	H4L1	n2	PL230C6	H3L2	466F6	H2L5
SI-35E99	284A10	L1H1	323H7	H4L1	n2	PL221G5	H1L1	466F6	H2L5
SI-35E18	460C3	L1H1	PL230C6	H3L2	n2	323H7	H4L1	284A10	H1L1
SI-35E19	420H5	L3H3	PL230C6	H3L2	n2	323H7	H4L1	284A10	H1L1
SI-35E36	4420	-	PL230C6	H3L2	n2	338H4	H3L4	284A10	H1L1
SI-35E37	460C3	L1H1	4420	-	n2	338H4	H3L4	284A10	H1L1
SI-35E38	460C3	L1H1	PL230C6	H3L2	n2	4420	-	284A10	H1L1
SI-35E39	460C3	L1H1	PL230C6	H3L2	n2	338H4	H3L4	4420	-
SI-38E17	284A10	H1L1	21D4	-	n2	PL221G5	H1L1	466F6	H2L5
SI-38E33	21D4	-	284A10	H1L1	n2	PL221G5	H1L1	466F6	H2L5

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SEQUENCE LISTING

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-PD-L1 PL230C6 VHv3 nt
6	anti-PD-L1 PL230C6 VHv3 aa
7	anti-PD-L1 PL230C6 VLv2 nt
8	anti-PD-L1 PL230C6 VLv2 aa
9	anti-PD-L1 PL221G5 VHv1 nt
10	anti-PD-L1 PL221G5 VHv1 aa
11	anti-PD-L1 PL221G5 VLv1 nt
12	anti-PD-L1 PL221G5 VLv1 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 323H7 VHv4 nt
26	anti-ROR1 323H7 VHv4 aa
27	anti-ROR1 323H7 VLv1 nt
28	anti-ROR1 323H7 VLv1 aa
29	anti-ROR1 338H4 VHv3 nt
30	anti-ROR1 338H4 VHv3 aa
31	anti-ROR1 338H4 VLv4 nt
32	anti-ROR1 338H4 VLv4 aa
33	anti-FITC 4-4-20 VH nt
34	anti-FITC 4-4-20 VH aa
35	anti-FITC 4-4-20 VL nt
36	anti-FITC 4-4-20 VL aa
37	human IgG1 null2 (G1m-fa with ADCC/CDC null mutations) nt

38	human IgG1 null2 (G1m-fa with ADCC/CDC null mutations) aa
39	human Ig Kappa nt
40	human Ig Kappa aa
41	SI-35E18 (460C3-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) heavy chain nt
42	SI-35E18 (460C3-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) heavy chain aa
43	SI-35E18 (460C3-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) light chain nt
44	SI-35E18 (460C3-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) light chain aa
45	anti-CD3 284A10 VHv1b nt
46	anti-CD3 284A10 VHv1b aa
47	anti-huCD19 21D4 VH nt
48	anti-huCD19 21D4 VH aa
49	anti-huCD19 21D4 VL nt
50	anti-huCD19 21D4 VL aa
51	anti-huEGFRvIII 806 VH nt
52	anti-huEGFRvIII 806 VH aa
53	anti-huEGFRvIII 806 VL nt
54	anti-huEGFRvIII 806 VL aa
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56	GGGGSGGGGSG linker aa
57	GGGSGGGGS linker 01 nt
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64	SI-39E18 (284A10-L1H1-scFv x 806-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) heavy chain aa
65	SI-39E18 (284A10-L1H1-scFv x 806-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) light chain nt
66	SI-39E18 (284A10-L1H1-scFv x 806-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) light chain aa
67	SI-39E29 (806-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) heavy chain nt
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74	SI-35E20 (466F6-L5H2-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) light chain aa
75	SI-35E58 (284A10-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 466F6-H2L5-scFv) heavy chain nt
76	SI-35E58 (284A10-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 466F6-H2L5-scFv) heavy chain aa
77	SI-35E58 (284A10-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 466F6-H2L5-scFv) light chain nt
78	SI-35E58 (284A10-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 466F6-H2L5-scFv) light chain aa

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83	SI-35E99 (284A10-L1H1-scFv x 323H7-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) heavy chain nt
84	SI-35E99 (284A10-L1H1-scFv x 323H7-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) heavy chain aa
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91	SI-38E33 (21D4-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) heavy chain nt
92	SI-38E33 (21D4-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) heavy chain aa
93	SI-38E33 (21D4-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) light chain nt
94	SI-38E33 (21D4-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) light chain aa

GNC-T Sequence listing of tetra-specific GNC antibodies

CDR’s underlined in amino acid sequences

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CTGCAGCCTGAAGATTTTGCAACTTACTATTGTCAACAGGGTTATGGTAAAAATAATGTTGATAATGCTTTCGGCGG
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>SEQ ID 08 anti-PD-L1 PL230C6 VLv2 aa
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AGGGACCAAGGTGGAGATCAAA

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

>SEQ ID 13 anti-4-1BB 420H5 VHv3 nt
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ATGTTGGTAGTAGTGGTGACACTTACTACGCGAGCTCCGCGAAAGGCCGGTTCACCATCTCCAGAGACAATTCCAAG
AACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAGAGATAGTAGTAG
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>SEQ ID 14 anti-4-1BB 420H5 VHv3 aa
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>SEQ ID 15 anti-4-1BB 420H5 VLv3 nt
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>SEQ ID 16 anti-4-1BB 420H5 VLv3 aa

ALVMTQSPSTLSASVGDRVTINCQASEDIDTYLAWYQQKPGKAPKLLIFYASDLASGVPSRFSGSGSGTEFTLTISS
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>SEQ ID 17 anti-4-1BB 466F6 VHv2 nt
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>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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>SEQ ID 20 anti-4-1BB 466F6 VLv5 aa
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>SEQ ID 21 anti-4-1BB 460C3 VHv1 nt
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>SEQ ID 22 anti-4-1BB 460C3 VHv1 aa
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>SEQ ID 23 anti-4-1BB 460C3 VLv1 nt
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>SEQ ID 24 anti-4-1BB 460C3 VLv1 aa
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>SEQ ID 25 anti-ROR1 323H7 VHv4 nt
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ACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCACCTATTTCTGTGCGAGATTGGATGTTGGTGG
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>SEQ ID 26 anti-ROR1 323H7 VHv4 aa
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>SEQ ID 27 anti-ROR1 323H7 VLv1 nt
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>SEQ ID 28 anti-ROR1 323H7 VLv1 aa
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>SEQ ID 29 anti-ROR1 338H4 VHv3 nt
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>SEQ ID 30 anti-ROR1 338H4 VHv3 aa
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>SEQ ID 31 anti-ROR1 338H4 VLv4 nt
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>SEQ ID 32 anti-ROR1 338H4 VLv4 aa
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>SEQ ID 33 anti-FITC 4420 VH nt
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>SEQ ID 34 anti-FITC 4420 VH aa
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>SEQ ID 35 anti-FITC 4420 VL nt
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CGGTGGAGGCACCAAGCTGGAAATCAAA

>SEQ ID 36 anti-FITC 4420 VL aa
DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLRWYLQKPGQSPKVLIYKVSNRFSGVPDRFSGSGSGTDFT
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>SEQ ID 37 human IgG1 null (G1m-fa with ADCC/CDC null mutations) nt

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>SEQ ID 38 human IgG1 null (G1m-fa with ADCC/CDC null mutations) aa
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>SEQ ID 39 human Ig Kappa nt
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>SEQ ID 40 human Ig Kappa aa
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>SEQ ID 41 SI-35E18 (460C3-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x
284A10-H1L1-scFv) heavy chain nt

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>SEQ ID 42 SI-35E18 (460C3-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x
284A10-H1L1-scFv) heavy chain aa

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>SEQ ID 43 SI-35E18 (460C3-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x
284A10-H1L1-scFv) light chain nt

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>SEQ ID 44 SI-35E18 (460C3-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) light chain aa

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>SEQ ID 45 anti-CD3 284A10 VHv1b nt
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>SEQ ID 46 anti-CD3 284A10 VHv1b aa
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>SEQ ID 47 anti-huCD19 21D4 VH nt
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>SEQ ID 48 anti-huCD19 21D4 VH aa
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>SEQ ID 49 anti-huCD19 21D4 VL nt
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>SEQ ID 50 anti-huCD19 21D4 VL aa
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>SEQ ID 51 anti-huEGFRvIII 806 VH nt
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>SEQ ID 52 anti-huEGFRvIII 806 VH aa
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>SEQ ID 53 anti-huEGFRvIII 806 VL nt
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>SEQ ID 54 anti-huEGFRvIII 806 VL aa
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>SEQ ID 55 GGGGSGGGGSG linker nt
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>SEQ ID 56 GGGGSGGGGSG linker aa
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>SEQ ID 57 GGGGSGGGGS linker 01 nt
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>SEQ ID 58 GGGGSGGGGS linker 01 aa
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>SEQ ID 59 GGGGSGGGGS linker 02 nt
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>SEQ ID 60 GGGGSGGGGS linker 02 aa
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>SEQ ID 61 GGGGSGGGGSGGGGSGGGGS linker nt
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>SEQ ID 62 GGGGSGGGGSGGGGSGGGGS linker aa
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>SEQ ID 63 SI-39E18 (284A10-L1H1-scFv x 806-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) heavy chain nt
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>SEQ ID 64 SI-39E18 (284A10-L1H1-scFv x 806-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) heavy chain aa
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>SEQ ID 65 SI-39E18 (284A10-L1H1-scFv x 806-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) light chain nt
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>SEQ ID 66 SI-39E18 (284A10-L1H1-scFv x 806-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) light chain aa
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>SEQ ID 67 SI-39E29 (806-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) heavy chain nt
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>SEQ ID 68 SI-39E29 (806-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) heavy chain aa
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>SEQ ID 69 SI-39E29 (806-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) light chain nt
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>SEQ ID 70 SI-39E29 (806-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 420H5-H3L3-scFv) light chain aa
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>SEQ ID 71 SI-35E20 (466F6-L5H2-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) heavy chain nt
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>SEQ ID 72 SI-35E20 (466F6-L5H2-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) heavy chain aa
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>SEQ ID 73 SI-35E20 (466F6-L5H2-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) light chain nt
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>SEQ ID 74 SI-35E20 (466F6-L5H2-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 284A10-H1L1-scFv) light chain aa
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>SEQ ID 75 SI-35E58 (284A10-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 466F6-H2L5-scFv) heavy chain nt
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>SEQ ID 76 SI-35E58 (284A10-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 466F6-H2L5-scFv) heavy chain aa

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>SEQ ID 77 SI-35E58 (284A10-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 466F6-H2L5-scFv) light chain nt
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>SEQ ID 78 SI-35E58 (284A10-L1H1-scFv x PL230C6-Fab x 323H7-H4L1-scFv x 466F6-H2L5-scFv) light chain aa
AYDMTQSPSSVSASVGDRVTIKCQASEDIYSFLAWYQQKPGKAPKLLIHSASSLASGVPSRFSG
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>SEQ ID 79 SI-35E88 (284A10-L1H1-scFv x 323H7-Fab x PL230C6-H3L2-scFv x 466F6-H2L5-scFv) heavy chain nt
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>SEQ ID 80 SI-35E88 (284A10-L1H1-scFv x 323H7-Fab x PL230C6-H3L2-scFv x 466F6-H2L5-scFv) heavy chain aa
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>SEQ ID 81 SI-35E88 (284A10-L1H1-scFv x 323H7-Fab x PL230C6-H3L2-scFv x 466F6-H2L5-scFv) light chain nt
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>SEQ ID 82 SI-35E88 (284A10-L1H1-scFv x 323H7-Fab x PL230C6-H3L2-scFv x 466F6-H2L5-scFv) light chain aa
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>SEQ ID 83 SI-35E99 (284A10-L1H1-scFv x 323H7-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) heavy chain nt

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>SEQ ID 84 SI-35E99 (284A10-L1H1-scFv x 323H7-Fab x PL221G5-
H1L1-scFv x 466F6-H2L5-scFv) heavy chain aa
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>SEQ ID 85 SI-35E99 (284A10-L1H1-scFv x 323H7-Fab x PL221G5-
H1L1-scFv x 466F6-H2L5-scFv) light chain nt
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>SEQ ID 86 SI-35E99 (284A10-L1H1-scFv x 323H7-Fab x PL221G5-
H1L1-scFv x 466F6-H2L5-scFv) light chain aa
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>SEQ ID 87 SI-38E17 (284A10-L1H1-scFv x 21D4-Fab x PL221G5-H1L1-
scFv x 466F6-H2L5-scFv) heavy chain nt
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>SEQ ID 88 SI-38E17 (284A10-L1H1-scFv x 21D4-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) heavy chain aa
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>SEQ ID 89 SI-38E17 (284A10-L1H1-scFv x 21D4-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) light chain nt
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>SEQ ID 90 SI-38E17 (284A10-L1H1-scFv x 21D4-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) light chain aa
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>SEQ ID 91 SI-38E33 (21D4-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) heavy chain nt
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SEQ ID 94 SI-38E33 (21D4-LH-scFv x 284A10-Fab x PL221G5-H1L1-scFv x 466F6-H2L5-scFv) light chain aa
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CLAIMS

What we claim is:

1. A method for generating a therapeutic composition, comprising

providing a cell material comprising a cytotoxic cell,

incubating the cell material with a first GNC protein to provide an activated cell composition, wherein the activated cell composition comprises a first therapeutic cell,

wherein the first GNC protein comprising a first cytotoxic binding moiety and a first cancer targeting moiety, wherein the first cytotoxic binding moiety has a specificity to a first cytotoxic cell receptor and is configured to activate the first cytotoxic cell through the binding with the first cytotoxic cell receptor, and wherein the first cancer targeting moiety has a specificity to a first cancer cell receptor, and

wherein the first therapeutic cell comprises the first GNC protein bound to the cytotoxic cell through the binding interaction with the first cytotoxic cell receptor, and

formulating the activated cell composition to provide a therapeutic composition, wherein the therapeutic composition is substantially free of exogenous viral and non-viral DNA or RNA.

2. The method of Claim 1, wherein the incubating step is repeated by incubating a second GNC protein with the activated cell composition,

wherein the second GNC protein comprising a second cytotoxic binding moiety and a second cancer targeting moiety, wherein the second cytotoxic binding moiety has a specificity to a second cytotoxic cell receptor, and wherein the second cancer targeting moiety has a specificity to a second cancer cell receptor,

wherein the activated cell composition further comprises a second therapeutic cell, and

wherein the second therapeutic cell comprises the second GNC protein bound to the cytotoxic cell or the first therapeutic cell through the binding interaction with the second cytotoxic cell receptor.

3. The method of Claim 2, wherein the second GNC protein is the same as the first GNC protein.

4. The method of Claim 2, wherein the second GNC protein is different from the first GNC protein.

5. The method of Claim 1 or 2, wherein the first or the second cancer targeting moiety has the specificity against B cell, and wherein the therapeutic composition is substantially free of B cell.

6. The method of Claim 1, wherein the cytotoxic cell receptor comprises a T-cell receptor, a NK cell receptor, a macrophage receptor, a dendritic cell receptor, or a combination thereof.

7. The method of Claim 1, wherein the molar to cell ratio between the first GNC protein and the cytotoxic cell is at least 30 to 1 when incubating the cell material with the first GNC protein.

8. The method of Claim 1, wherein the therapeutic composition comprises at least 10^6 cells per ml.

9. The method of Claim 1, wherein the therapeutic composition comprises the first therapeutic cell, the first GNC protein, the cytotoxic cell, or a combination thereof.

10. The method of Claim 2, wherein the therapeutic composition comprises the second therapeutic cell, the second GNC protein, comprises the first therapeutic cell, the first GNC protein, the cytotoxic cell, or a combination thereof.

11. The method of Claim 1, wherein the cell material comprises PBMC.

12. The method of Claim 1, wherein the first and the second cancer-targeting moiety independently has a specificity for CD19, PDL1, or a combination thereof.

13. The method of Claim 1, wherein the first and the second cytotoxic binding moiety independently has a specificity for CD3, PDL1, 41BB, or a combination thereof.

14. A method of treating a subject having a cancer, comprising

providing a cell material comprising a cytotoxic cell,

incubating the cell material with a first GNC protein to provide an activated cell composition, wherein the activated cell composition comprises a first therapeutic cell,

wherein the first GNC protein comprising a first cytotoxic binding moiety and a first cancer targeting moiety, wherein the first cytotoxic binding moiety has a specificity to a first cytotoxic cell receptor and is configured to activate the first cytotoxic cell through the binding with the first cytotoxic cell receptor, and wherein the first cancer targeting moiety has a specificity to a first cancer cell receptor, and

wherein the first therapeutic cell comprises the first GNC protein bound to the cytotoxic cell through the binding interaction with the first cytotoxic cell receptor, and

formulating the activated cell composition to provide a therapeutic composition, wherein the therapeutic composition is substantially free of exogenous viral and non-viral DNA or RNA, and

administering the therapeutic composition to the subject.

15. The method of Claim 14, wherein the incubating step is repeated by incubating a second GNC protein with the activated cell composition,

wherein the second GNC protein comprising a second cytotoxic binding moiety and a second cancer targeting moiety, wherein the second cytotoxic binding moiety has a specificity to a second cytotoxic cell receptor, and wherein the second cancer targeting moiety has a specificity to a second cancer cell receptor,

wherein the activated cell composition further comprises a second therapeutic cell, and

wherein the second therapeutic cell comprises the second GNC protein bound to the cytotoxic cell or the first therapeutic cell through the binding interaction with the second cytotoxic cell receptor.

16. The method of Claim 14, wherein the second GNC protein is the same as the first GNC protein.

17. The method of Claim 14, wherein the second GNC protein is different from the first GNC protein.

18. The method of Claim 14 or 15, wherein the first or the second cancer targeting moiety has the specificity against B cell, and wherein the therapeutic composition is substantially free of B cell.

19. The method of Claim 14, further comprising isolating the cytotoxic cell from peripheral blood mononuclear cells (PBMC) before providing the cell material.

20. The method of Claim 19, further comprising isolating the peripheral blood mononuclear cells (PBMC) from a blood.

21. The method of Claim 20, wherein the blood is from the subject.

22. The method of Claim 20, wherein the blood is not from the subject.

23. The method of Claim 14, further comprising administering an additional GNC protein to the subject after the administering the therapeutic composition to the subject.

24. The method of Claim 14, wherein the cytotoxic cell comprises T cell, NK cell, or a combination thereof.

25. The method of Claim 19, wherein the isolating the cytotoxic cell comprising isolating at least one subpopulation of cytotoxic cell to provide therapeutic T cells.

26. The method of Claim 25, wherein the subpopulation of cytotoxic cell comprises CD3+ cells, CD4+ cells, CD8+ cells, CD56+ cells, CD28+ cells, CD69+ cells, CD107a+ cells, CD45RA+cells, CD45RO+ cells, $\gamma\delta$ TCR+ cells, $\alpha\beta$ TCR+ cells, CD25+ cells, CD127 $^{lo/-}$ cells, CCR7+ cells, PD-1+ cells or a combination thereof.

27. The method of Claim 14, further comprising evaluating therapeutic efficacy after the administering step.

28. The method of Claim 26, wherein the evaluating therapeutic efficacy comprises checking one or more biomarkers of the cancer, monitoring the life span of the therapeutic cells, or a combination thereof.

29. The method of Claim 28, wherein the biomarker comprises a tumor antigen, release of cytokines e.g., gamma interferon, IL-2, IL-8, and/or chemokines, and/or CD markers on the surface of various cell types e.g., CD69, PD-1, TIGIT, and/or mutated nucleic acid released into the bloodstream by tumors upon death, circulating tumor cells and their associated nucleic acid, or exosome associated nucleic acid, host inflammatory mediators, or tumor derived analytes, or a combination thereof.

30. The method of Claim 14, wherein the subject is a human.

31. The method of Claim 14, wherein the cancer comprises cells expressing ROR1, CEA, HER2, EGFR, EGFR VIII, LMP1, LMP2A, Mesothelin, PSMA, EpCAM, glypican-3, gpA33, GD2, TROP2, BCMA, CD19, CD20, CD33, CD123, CD22, CD30, or a combination thereof.

32. The method of Claim 14, wherein the cancer comprises 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.

33. The method of Claim 14, wherein the cancer is CD19 positive.

34. The method of Claim 14, further comprising administering an effective amount of a therapeutic agent after the administering the therapeutic composition to the subject.

35. The method of Claim 34, wherein the therapeutic agent comprises a monoclonal antibody, a multi-specific antibody, a chemotherapy agent, an enzyme, a protein, a co-stimulator, an apoptosis sensitizer, a tumor vascular disruptor, or a combination thereof.

36. The method of Claim 35, wherein the co-stimulator is configured to increase the amount of cytotoxic T cells in the subject.

37. A therapeutic composition, comprising a cytotoxic cell, a GNC protein, and a therapeutic cell,

wherein the GNC protein comprising a cytotoxic binding moiety and a cancer targeting moiety, wherein the cytotoxic binding moiety has a specificity for a cytotoxic cell receptor, wherein the cancer targeting moiety has a specificity for a cancer cell receptor, and wherein the cytotoxic binding moiety is configured to activate the cytotoxic cell through the binding with the cytotoxic cell receptor,

wherein the therapeutic cell comprises the GNC protein bound to the cytotoxic cell through the binding interaction with the cytotoxic cell receptor, and

wherein the therapeutic cell composition is substantially free of exogenous viral and non-viral DNA and RNA.

38. The therapeutic composition of Claim 37, wherein the cancer targeting moiety has a specificity for B cell, and the therapeutic composition is substantially free of B cell.

39. The therapeutic composition of Claim 37, further comprising a second GNC protein, a second therapeutic cell, or a combination thereof, wherein the second therapeutic cell comprises the second GNC protein bound to the cytotoxic cell or the first therapeutic cell.

FIGURE 1. A GNC protein comprising four antigen-specific binding domains in an antibody structure with targeting specificity to CD19 positive cells.

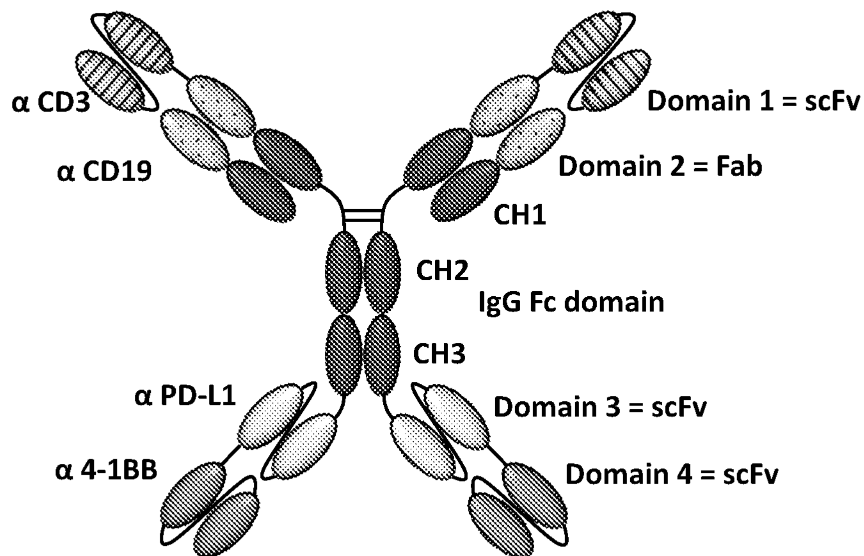


FIGURE 2. An illustration of multi-specific binding mediated by a tetra-specific GNC antibody between a T cell and a tumor cell.

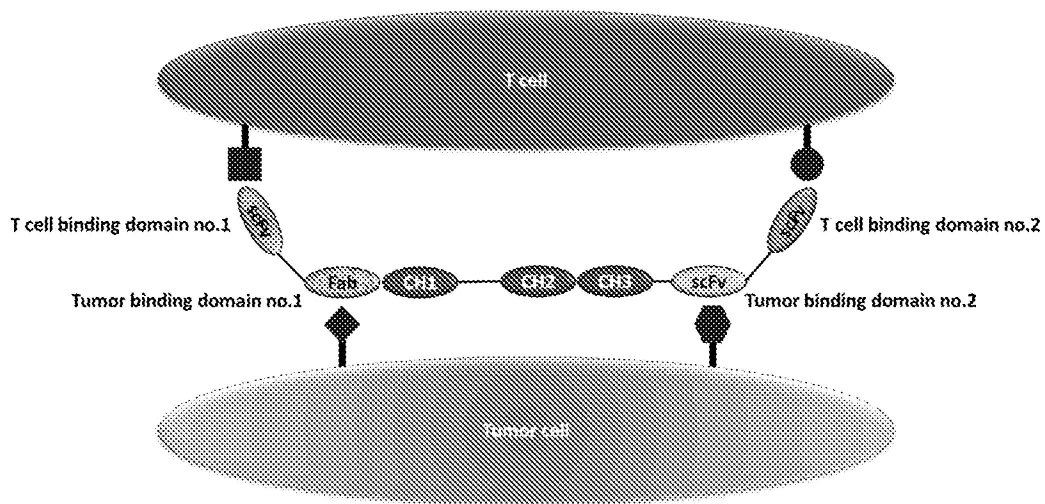


FIGURE 3. A flowchart of manufacturing processes for GNC-T cell therapy (left) and CAR-T cell therapy (right).

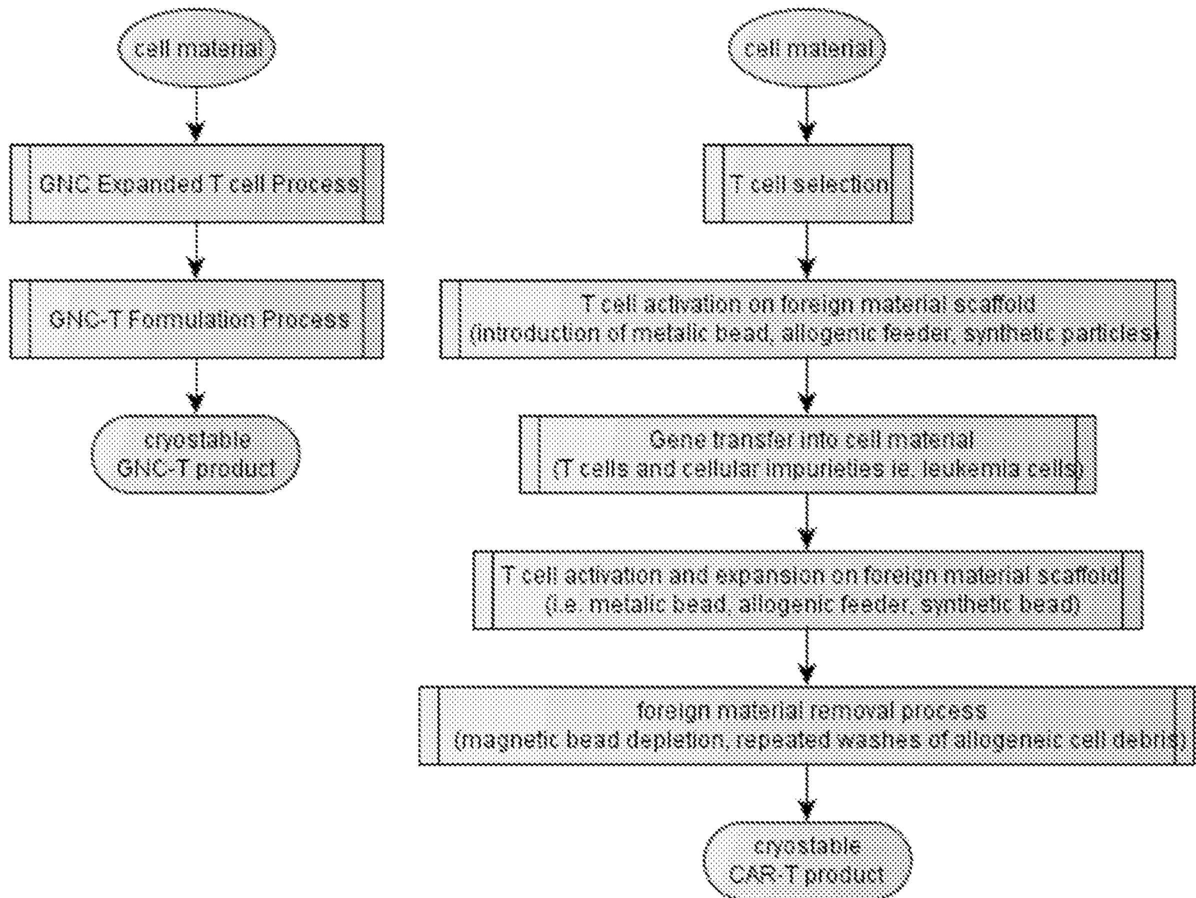


Figure 4. A diagram of sources of cell material for preparing GNC-activated therapeutic cell composition.

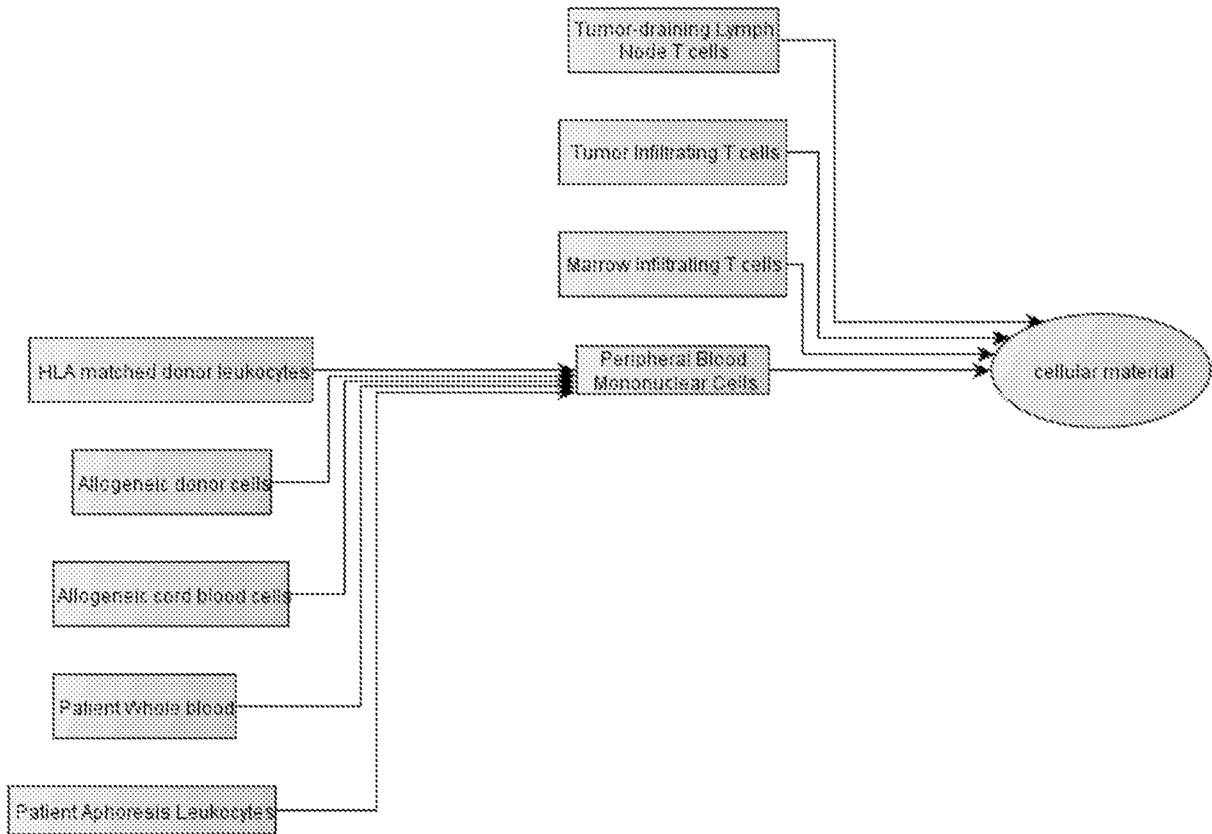


Figure 5. A diagram of sources of selected T cells for preparing GNC-activated therapeutic composition.

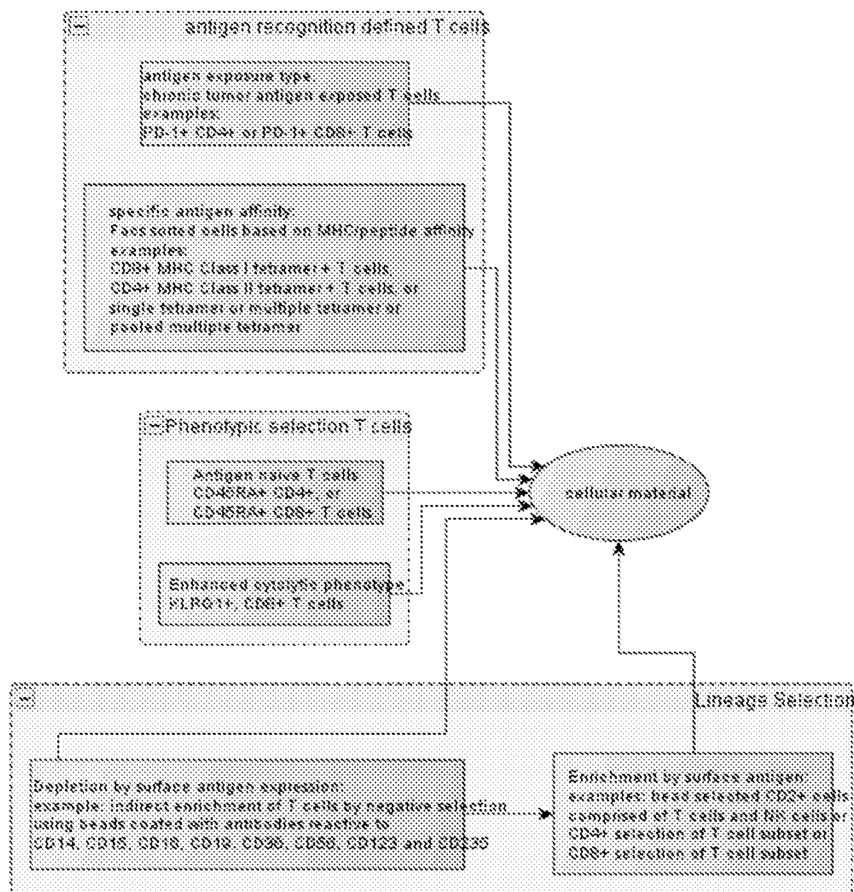


Figure 6. A diagram of preparing GNC-activated therapeutic T cell composition.

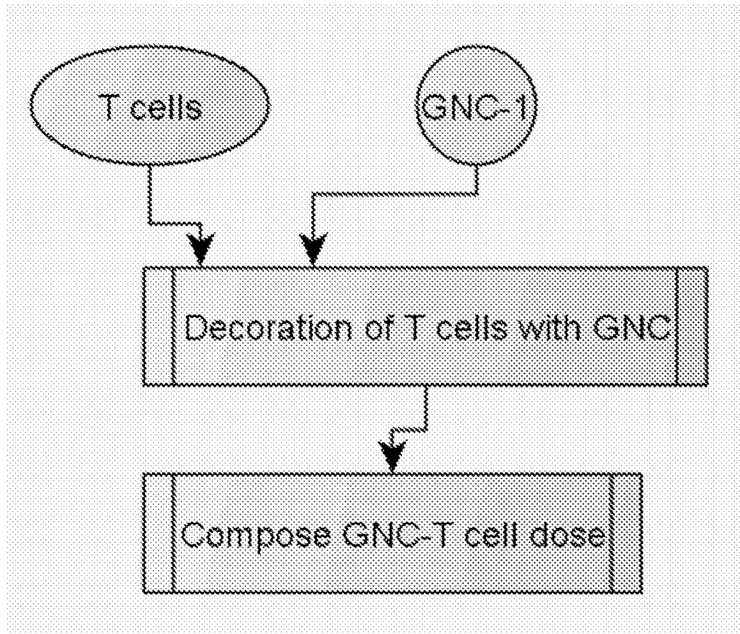


Figure 7. A diagram of incubating and formulating the first GNC-activated T cells for GNC-T cell therapy.

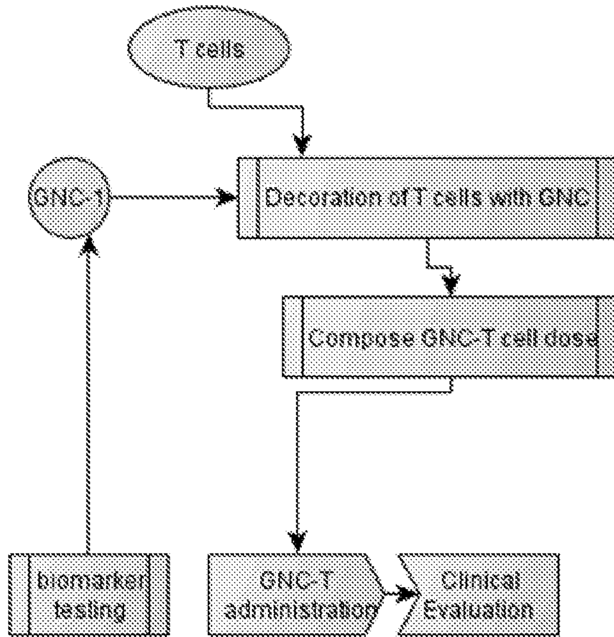


FIGURE 8. GNC proteins induce IL-2 secretion from PBMC.

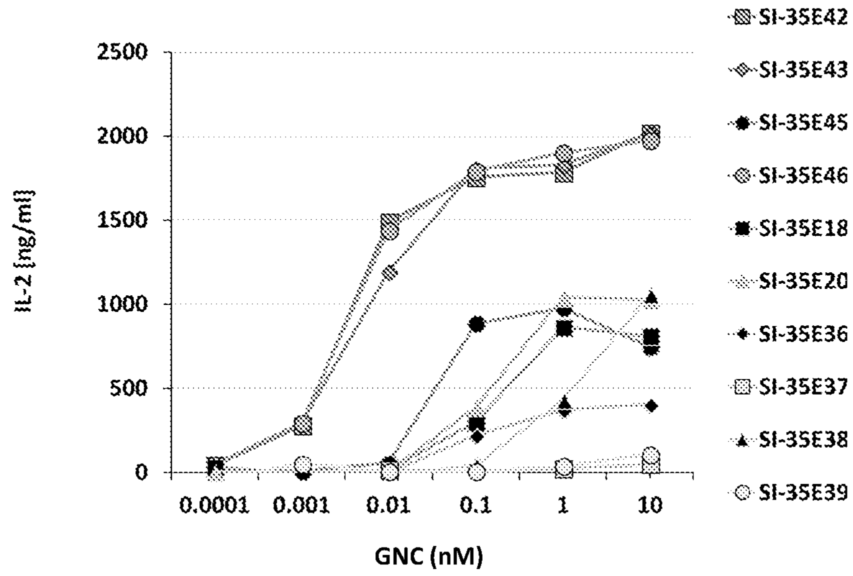


FIGURE 9. GNC proteins induce granzyme B secretion from PBMC.

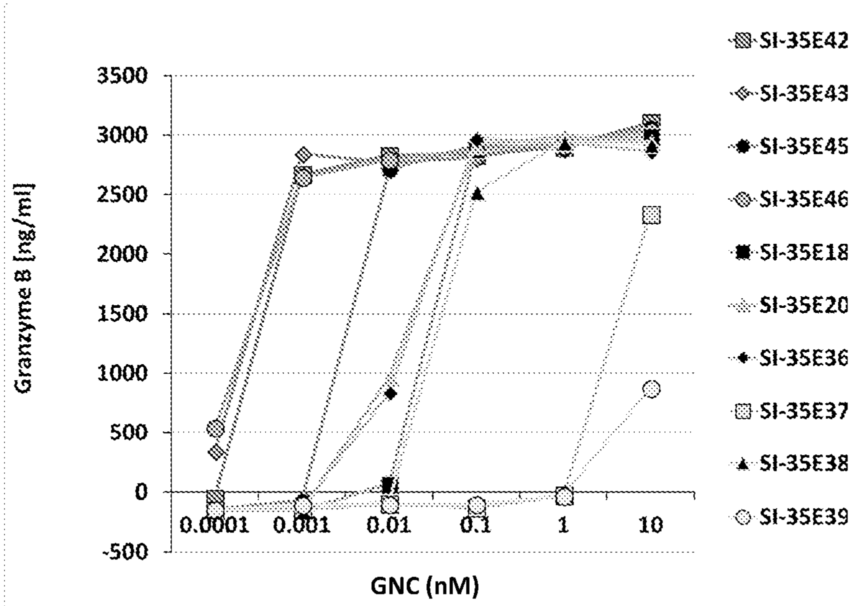


FIGURE 10. GNC proteins induce expression of the activation marker CD69 on CD4+ T cells.

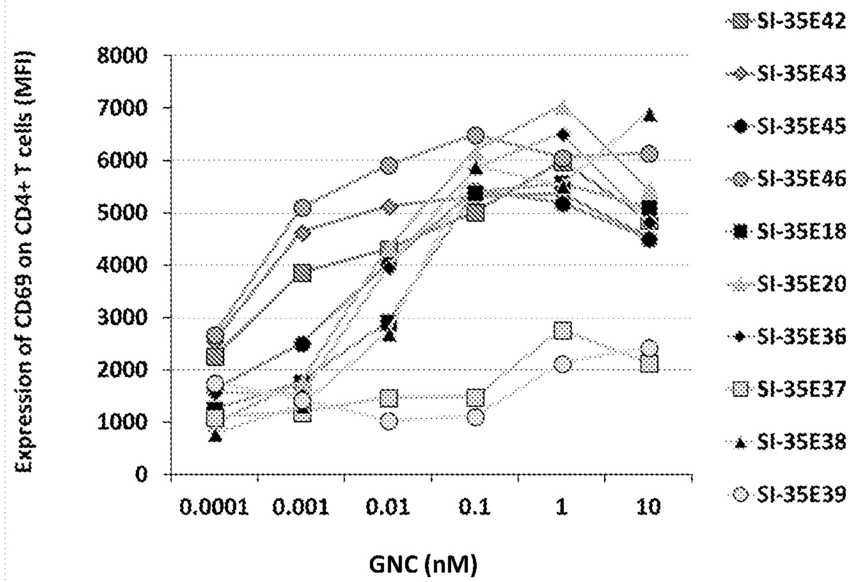


FIGURE 11. GNC proteins induce expression of the activation marker CD69 on CD8+ T cells.

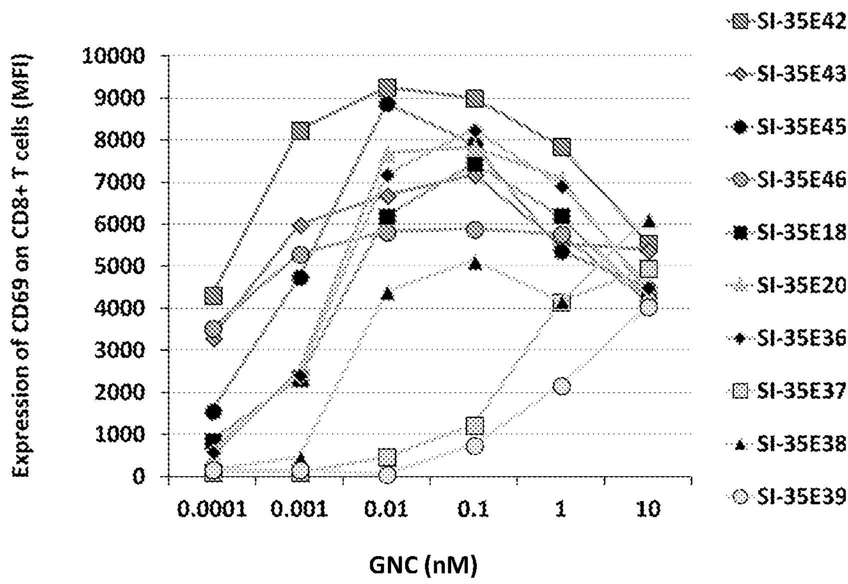


FIGURE 12. GNC proteins induce expression of the activation marker CD69 on CD56+ NK cells.

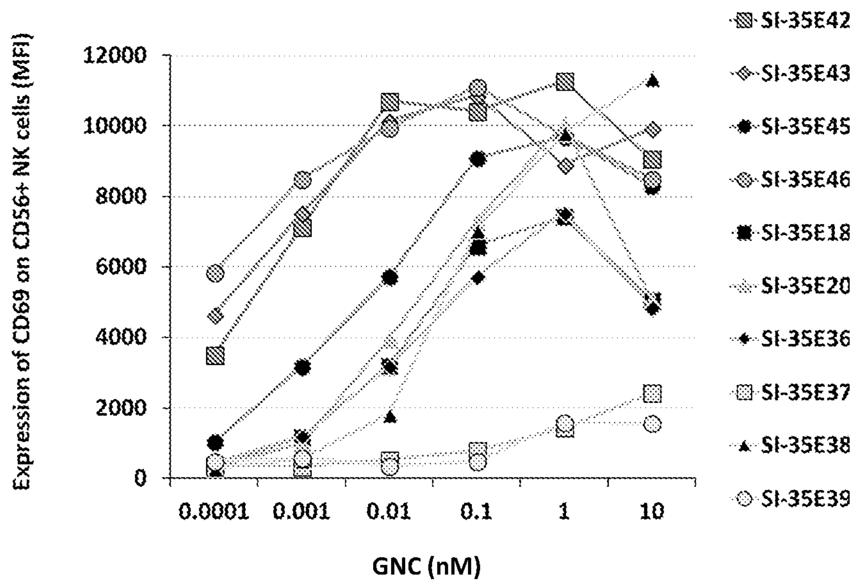


FIGURE 13. GNC proteins induce expression of the marker of cytotoxic degranulation CD107a on CD4+ T cells.

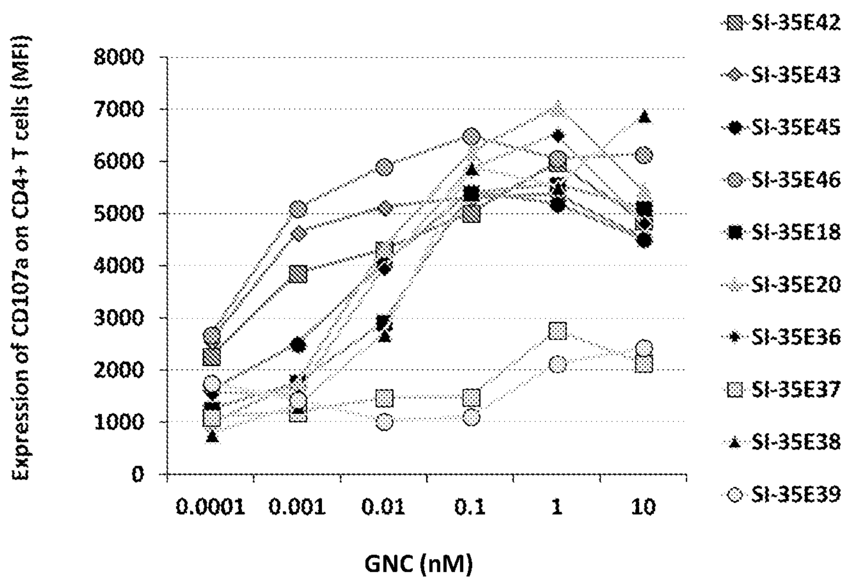


FIGURE 14. GNC proteins induce expression of the marker of cytotoxic degranulation CD107a on CD8+ T cells.

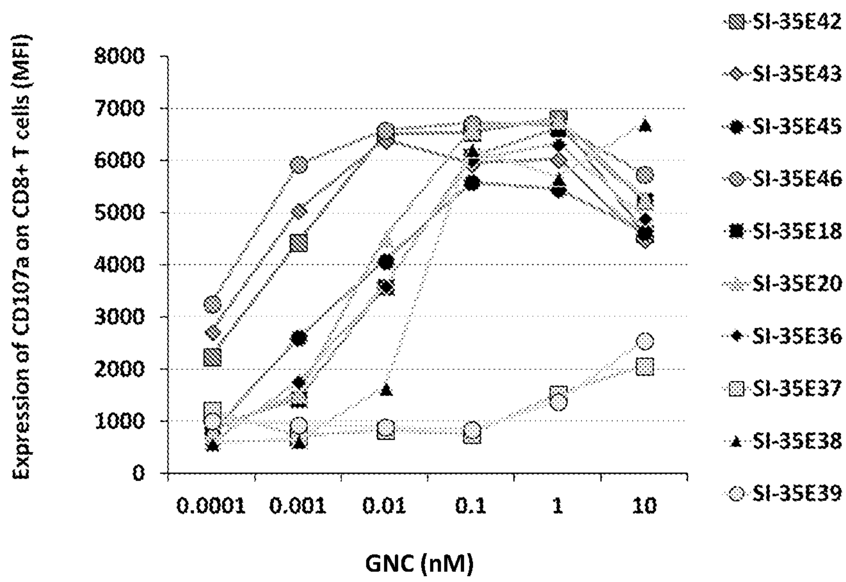


FIGURE 15. GNC proteins induce expression of the marker of cytotoxic degranulation CD107a on CD56+ NK cells.

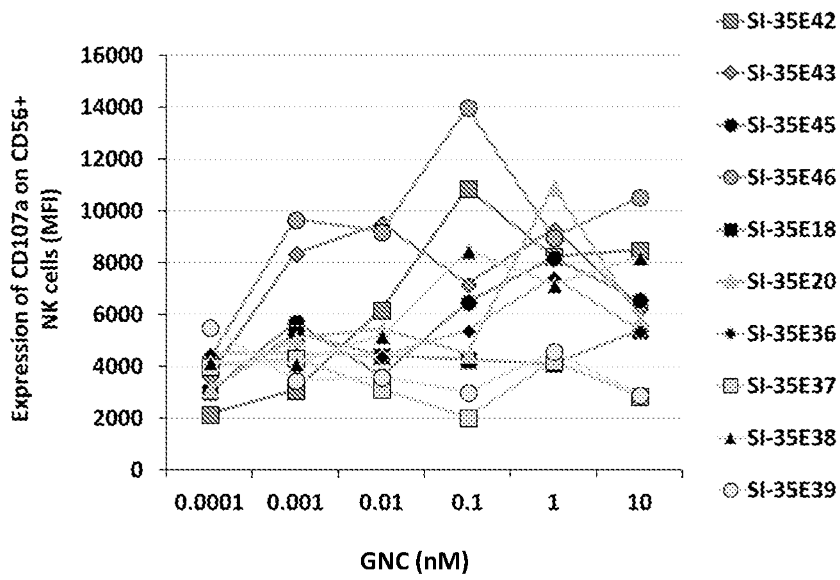
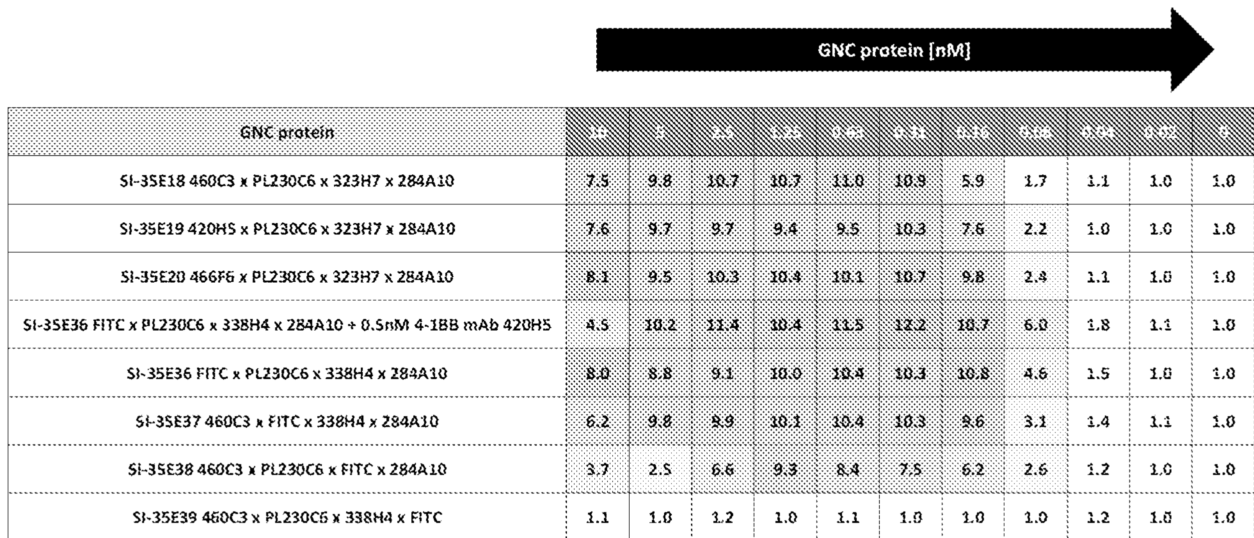


FIGURE 16. GNC proteins activate CD3+ T cells to proliferate.

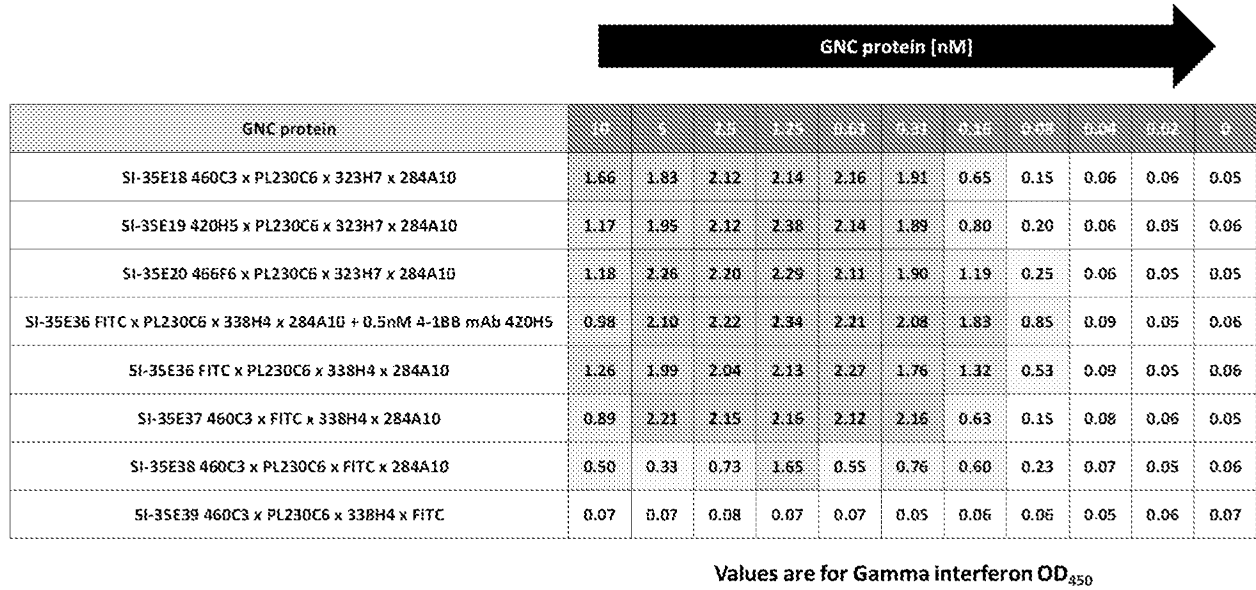


GNC protein [nM]

GNC protein	10	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0
SI-35E18 460C3 x PL230C6 x 323H7 x 284A10	7.5	9.8	10.7	10.7	11.0	10.9	5.9	1.7	1.1	1.0	1.0
SI-35E19 420H5 x PL230C6 x 323H7 x 284A10	7.6	9.7	9.7	9.4	9.5	10.3	7.6	2.2	1.0	1.0	1.0
SI-35E20 466F6 x PL230C6 x 323H7 x 284A10	8.1	9.5	10.3	10.4	10.1	10.7	9.8	2.4	1.1	1.0	1.0
SI-35E36 FITC x PL230C6 x 338H4 x 284A10 + 0.5nM 4-1BB mAb 420H5	4.5	10.2	11.4	10.4	11.5	12.2	10.7	6.0	1.8	1.1	1.0
SI-35E36 FITC x PL230C6 x 338H4 x 284A10	8.0	8.8	9.1	10.0	10.4	10.3	10.8	4.6	1.5	1.0	1.0
SI-35E37 460C3 x FITC x 338H4 x 284A10	6.2	9.8	9.9	10.1	10.4	10.3	9.6	3.1	1.4	1.1	1.0
SI-35E38 460C3 x PL230C6 x FITC x 284A10	3.7	2.5	6.6	9.3	8.4	7.5	6.2	2.6	1.2	1.0	1.0
SI-35E39 460C3 x PL230C6 x 338H4 x FITC	1.1	1.0	1.2	1.0	1.1	1.0	1.0	1.0	1.2	1.0	1.0

Values are for fold-over background proliferation

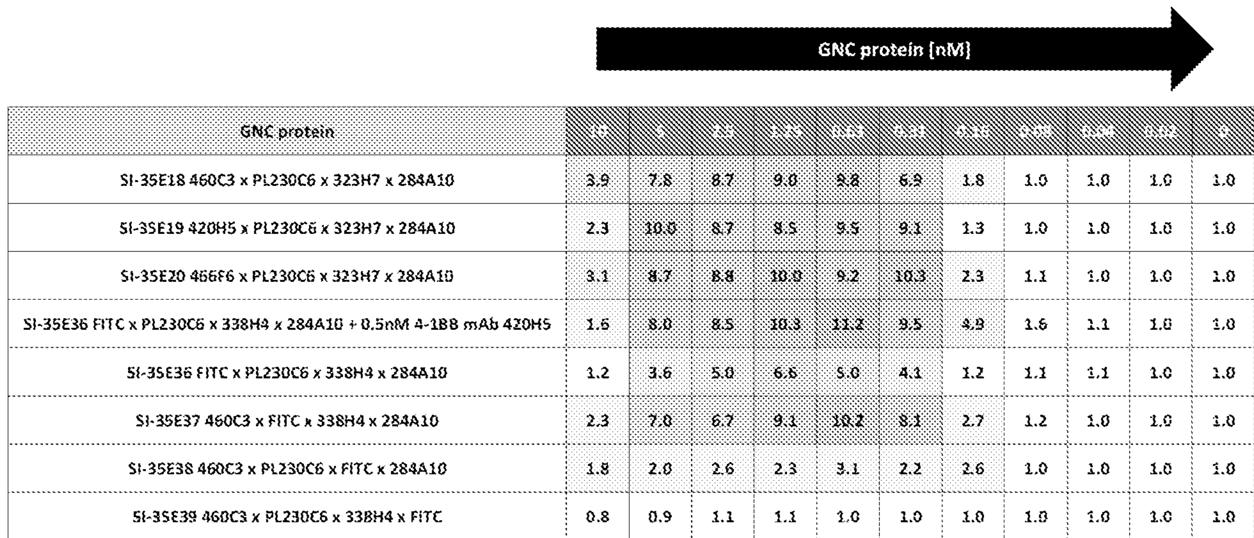
FIGURE 17. GNC proteins activate CD3+ T cells to secrete gamma interferon.



GNC protein	10	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0
SI-35E18 460C3 x PL230C6 x 323H7 x 284A10	1.66	1.83	2.12	2.14	2.16	1.91	0.65	0.15	0.06	0.06	0.05
SI-35E19 420H5 x PL230C6 x 323H7 x 284A10	1.17	1.95	2.12	2.38	2.14	1.89	0.80	0.20	0.06	0.05	0.06
SI-35E20 466F6 x PL230C6 x 323H7 x 284A10	1.18	2.26	2.20	2.29	2.11	1.90	1.19	0.25	0.06	0.05	0.05
SI-35E36 FITC x PL230C6 x 338H4 x 284A10 + 0.5nM 4-1BB mAb 420H5	0.98	2.10	2.22	2.34	2.21	2.08	1.83	0.85	0.09	0.05	0.06
SI-35E36 FITC x PL230C6 x 338H4 x 284A10	1.26	1.99	2.04	2.13	2.27	1.76	1.32	0.53	0.09	0.05	0.06
SI-35E37 460C3 x FITC x 338H4 x 284A10	0.89	2.21	2.15	2.16	2.12	2.16	0.63	0.15	0.08	0.06	0.05
SI-35E38 460C3 x PL230C6 x FITC x 284A10	0.50	0.33	0.73	1.65	0.55	0.76	0.60	0.23	0.07	0.05	0.06
SI-35E39 460C3 x PL230C6 x 338H4 x FITC	0.07	0.07	0.08	0.07	0.07	0.05	0.06	0.06	0.05	0.06	0.07

Values are for Gamma interferon OD_{450}

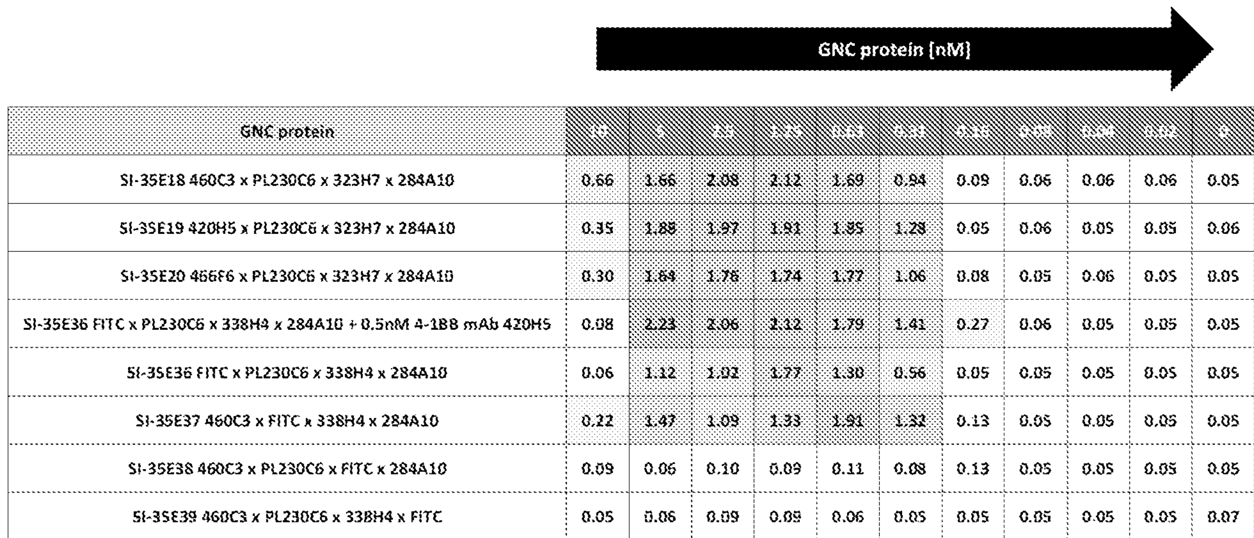
FIGURE 18. GNC proteins activate naïve CD8+/CD45RA+ T cells to proliferate.



GNC protein	10	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0
SI-35E18 460C3 x PL230C6 x 323H7 x 284A10	3.9	7.8	8.7	9.0	9.8	6.9	1.8	1.0	1.0	1.0	1.0
SI-35E19 420H5 x PL230C6 x 323H7 x 284A10	2.3	10.0	8.7	8.5	9.5	9.1	1.3	1.0	1.0	1.0	1.0
SI-35E20 466F6 x PL230C6 x 323H7 x 284A10	3.1	8.7	8.8	10.0	9.2	10.3	2.3	1.1	1.0	1.0	1.0
SI-35E36 FITC x PL230C6 x 338H4 x 284A10 + 0.5nM 4-1BB mAb 420H5	1.6	8.0	8.5	10.3	11.2	9.5	4.9	1.6	1.1	1.0	1.0
SI-35E36 FITC x PL230C6 x 338H4 x 284A10	1.2	3.6	5.0	6.6	5.0	4.1	1.2	1.1	1.1	1.0	1.0
SI-35E37 460C3 x FITC x 338H4 x 284A10	2.3	7.0	6.7	9.1	10.2	8.1	2.7	1.2	1.0	1.0	1.0
SI-35E38 460C3 x PL230C6 x FITC x 284A10	1.8	2.0	2.6	2.3	3.1	2.2	2.6	1.0	1.0	1.0	1.0
SI-35E39 460C3 x PL230C6 x 338H4 x FITC	0.8	0.9	1.1	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Values are for fold-over background proliferation

FIGURE 19. GNC proteins activate naïve CD8+/CD45RA+ T cells to secrete gamma interferon.



GNC protein	10	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0
SI-35E18 460C3 x PL230C6 x 323H7 x 284A10	0.66	1.66	2.08	2.12	1.69	0.94	0.09	0.06	0.06	0.06	0.05
SI-35E19 420H5 x PL230C6 x 323H7 x 284A10	0.35	1.88	1.97	1.91	1.85	1.28	0.05	0.06	0.05	0.05	0.06
SI-35E20 466F6 x PL230C6 x 323H7 x 284A10	0.30	1.64	1.78	1.74	1.77	1.06	0.08	0.05	0.06	0.05	0.05
SI-35E36 FITC x PL230C6 x 338H4 x 284A10 + 0.5nM 4-1BB mAb 420H5	0.08	2.23	2.06	2.12	1.79	1.41	0.27	0.06	0.05	0.05	0.05
SI-35E36 FITC x PL230C6 x 338H4 x 284A10	0.06	1.12	1.02	1.77	1.30	0.56	0.05	0.05	0.05	0.05	0.05
SI-35E37 460C3 x FITC x 338H4 x 284A10	0.22	1.47	1.09	1.33	1.91	1.32	0.13	0.05	0.05	0.05	0.05
SI-35E38 460C3 x PL230C6 x FITC x 284A10	0.09	0.06	0.10	0.09	0.11	0.08	0.13	0.05	0.05	0.05	0.05
SI-35E39 460C3 x PL230C6 x 338H4 x FITC	0.05	0.06	0.09	0.09	0.06	0.05	0.05	0.05	0.05	0.05	0.07

Values are for Gamma interferon OD_{450}

Figure 20. Images of GET cell growth in 6-well G-Rex plates over time

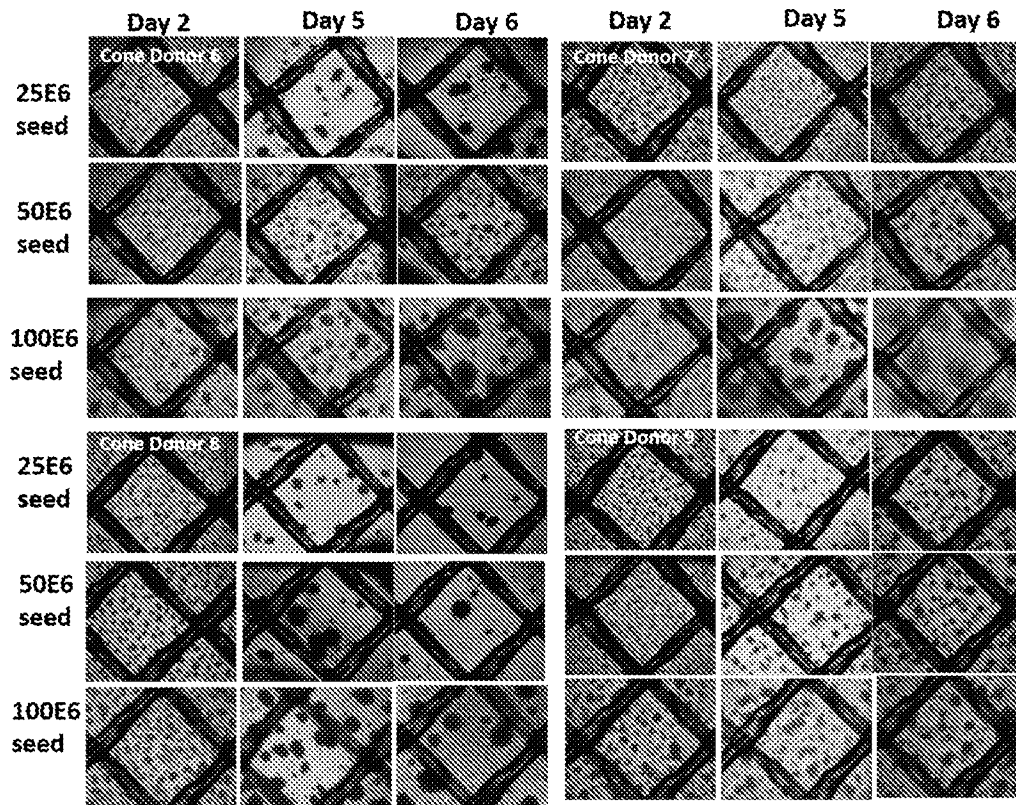
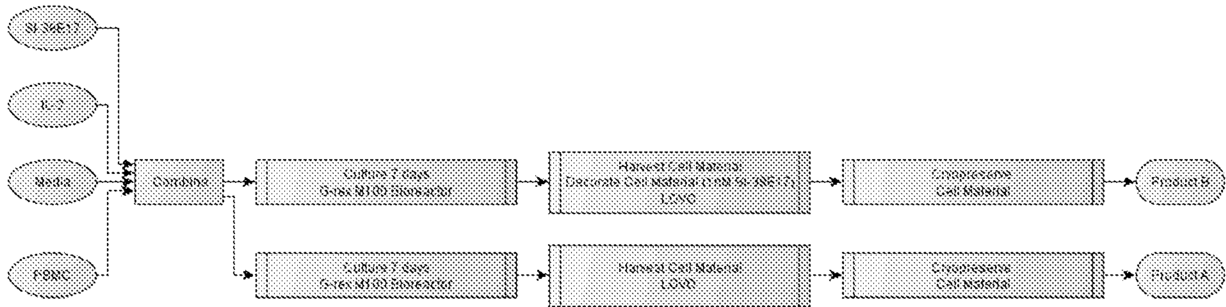
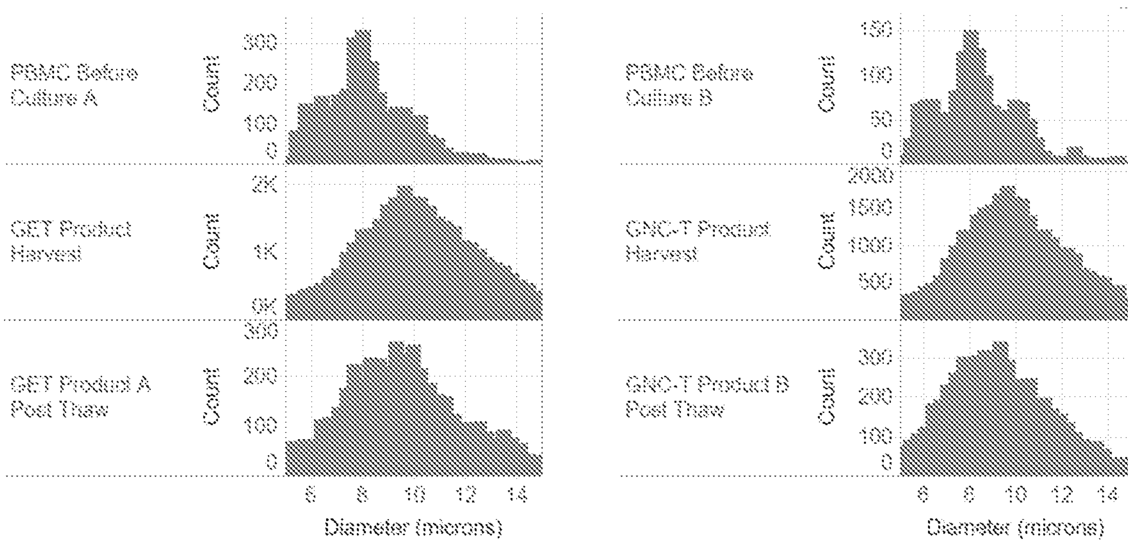


FIGURE 21. A, an example process of making the therapeutic composition as disclosed thereof, and B, cell viability of PBMC, GET, and GNC-T cells after thawing.

A.



B.



Sample ID	Avg. Viability (%)	Avg. Average Diameter (microns)
Donor12 post-thaw PBMC A	86.35	8.56
Donor12 post-thaw PBMC B	88.52	8.83
Donor12 post-thaw GET A	78.43	9.93
Donor12 post-thaw GNC-T B	77.74	9.50

FIGURE 22. A. Flow cytometry analysis of PBMC-derived, the first GNC (SI-38E17)-activated therapeutic cell composition (Product A).

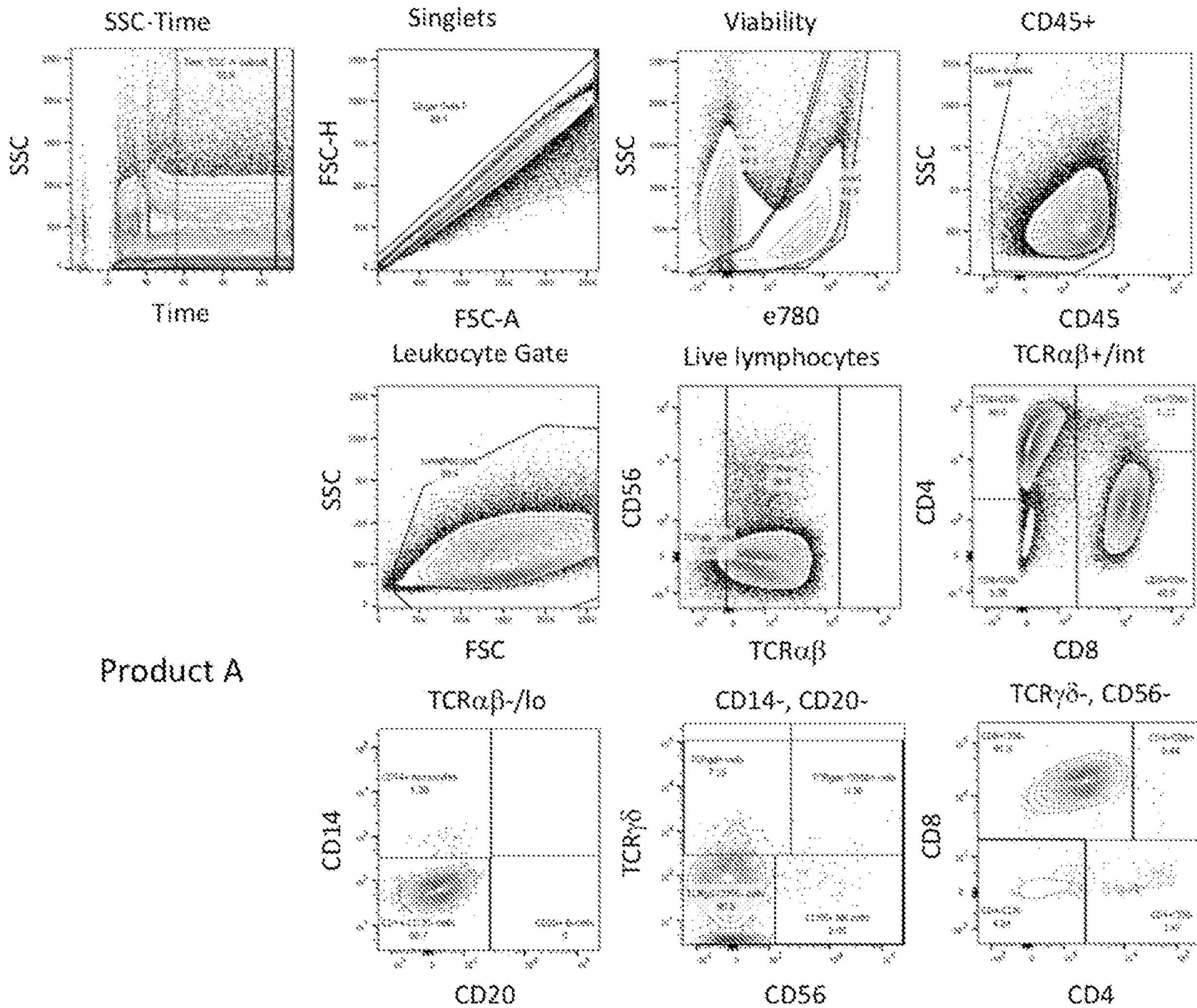


FIGURE 22. B. Flow cytometry analysis of PBMC-derived, the second GNC (SI-38E17)-decorated therapeutic cell composition (Product B).

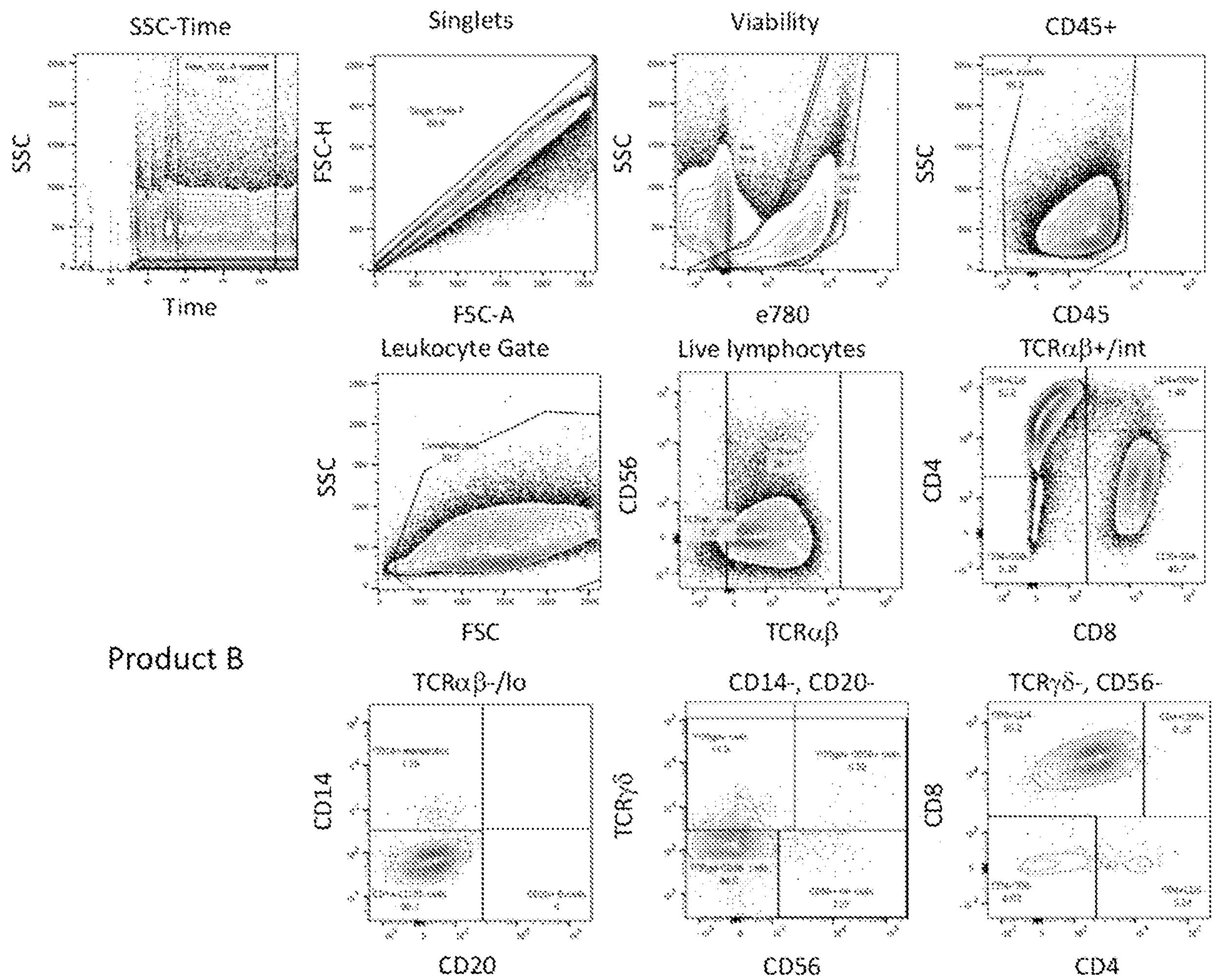


FIGURE 22. C. Flow cytometry analysis of input PBMC cell material.

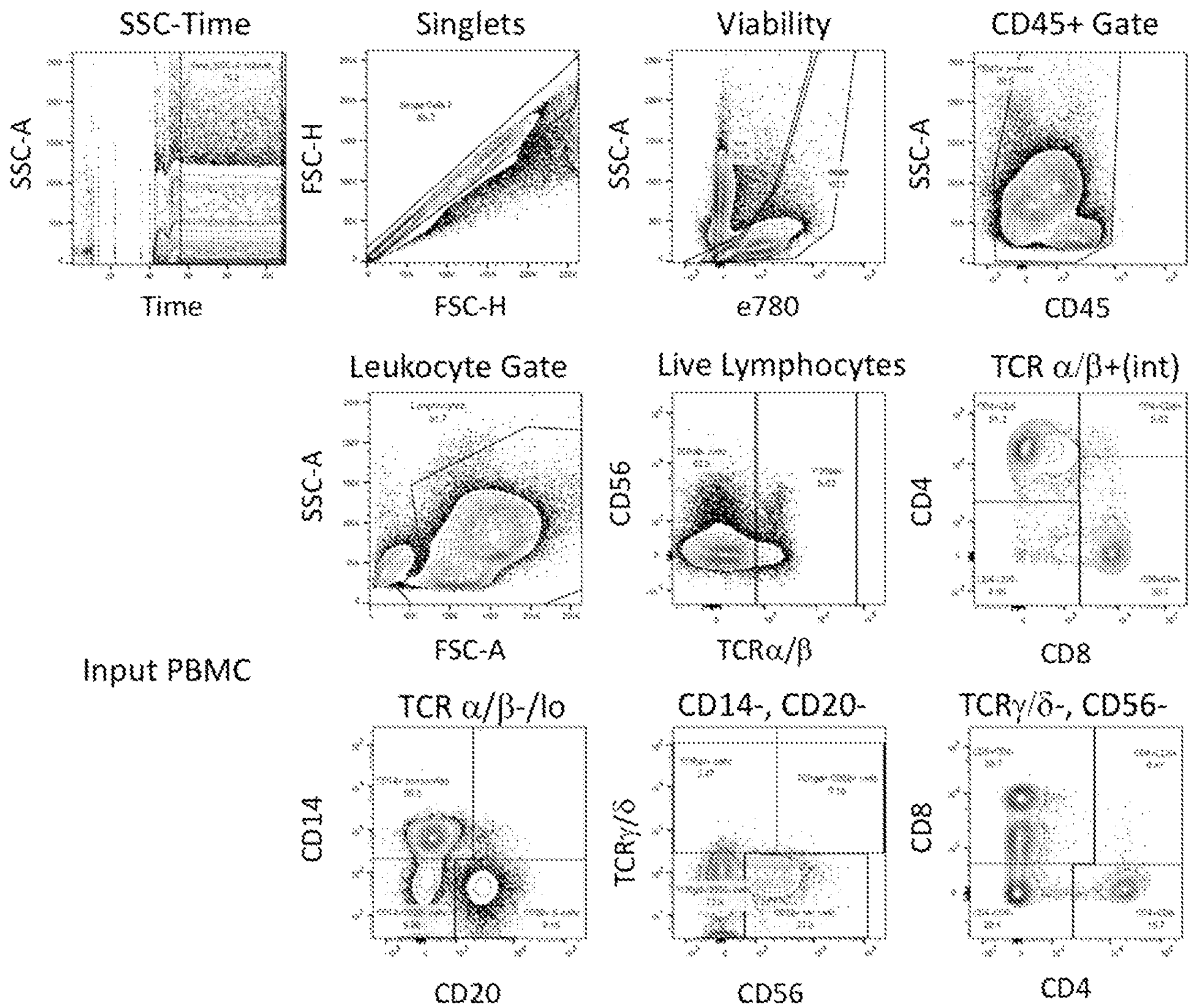


Figure 23. GNC-T therapeutic cell composition of GET cells and formulated GNC-T cells from G-Rex 100M bioreactor after thawing.

Cell Composition	PBMC as input cell material Number of Cells (%)	GNC-activated therapeutic composition-A Number of Cells (%)	GNC-activated therapeutic composition-B Number of Cells (%)
Leukocyte	250,000,000 (100)	1,000,000,000 (100)	1,000,000,000 (100)
TCR α/β T cells	17,375,000 (7)	964,000,000 (96.4)	959,000,000 (95.9)
CD4+ TCR α/β+ T cells	10,225,000 (4.1)	468,000,000 (46.8)	504,000,000 (50.4)
CD8+ TCR α/β+ T cells	5,725,000 (2.3)	451,000,000 (45.1)	409,000,000 (40.9)
TCR α/β-/lo T cells	224,500,000 (89.8)	30,500,000 (3.1)	32,800,000 (3.3)
CD14+ monocytes	179,250,000 (71.7)	380,000 (0)	610,000 (0.1)
CD56+ NK cells	4,825,000 (1.9)	620,000 (0.1)	730,000 (0.1)
TCR γ/δ T cells	450,000 (0.2)	2,200,000 (0.2)	3,800,000 (0.4)
CD20+ B cells	20,350,000 (8.1)	0 (0)	0 (0)

FIGURE 24. RTCC of CHO-ROR1 cells by using GNC (SI-35E class)-treated PBMC cells.

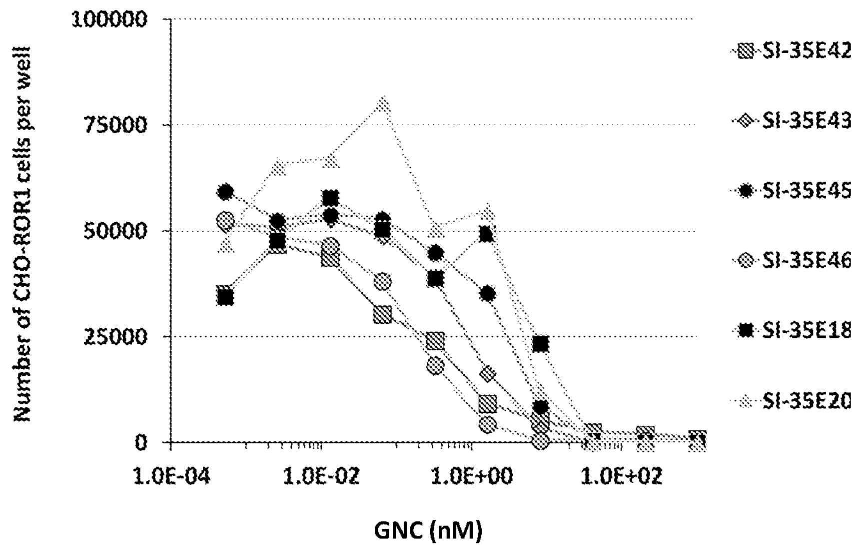


FIGURE 25. Kinetics of PBMC-derived, SI-38E17 GNC-activated therapeutic cells on killing precursor B cell leukemia Kasumi over time.

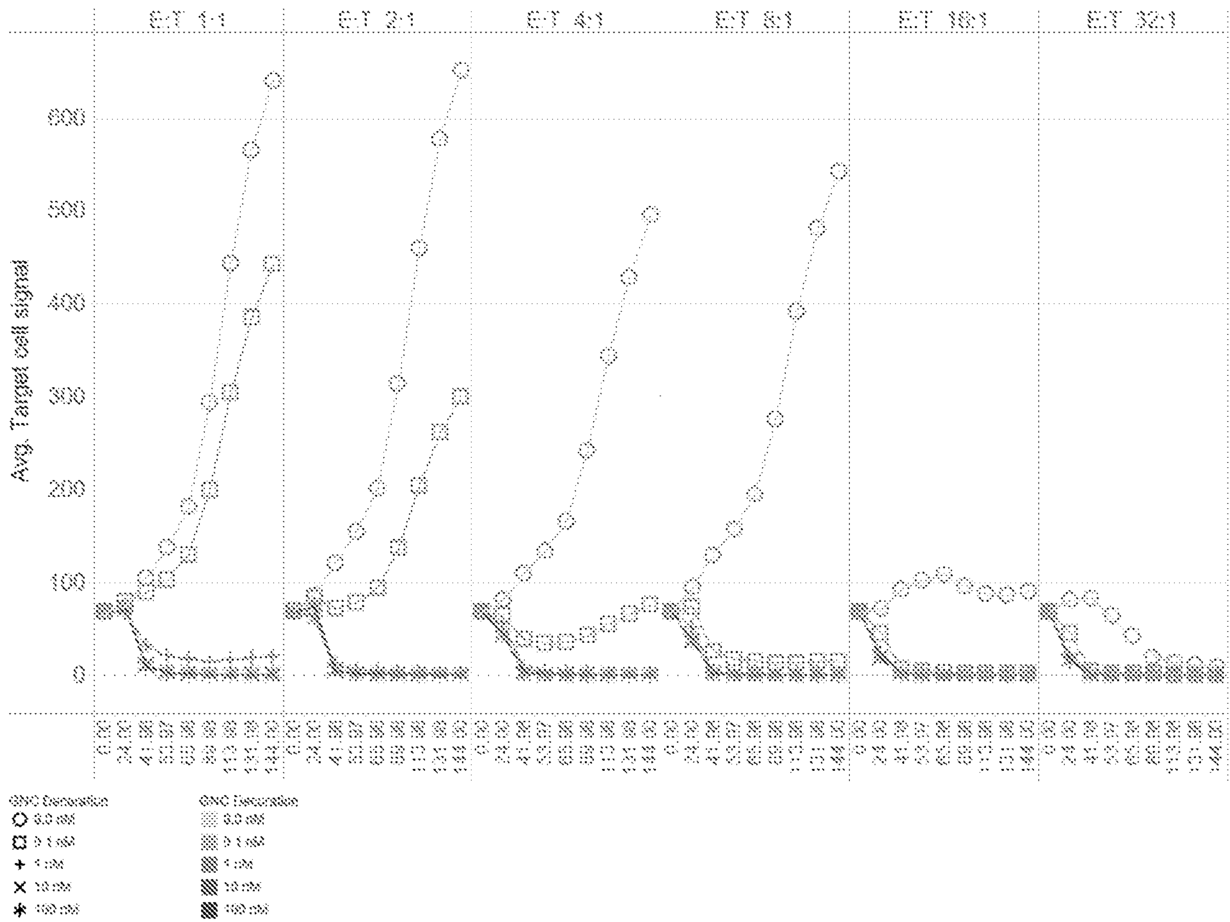


FIGURE 26. Efficacy of killing Nalm-6, MEC-1, Daudi, and Jurkat cells by using PMBC-derived, SI-38E17 GNC-activated therapeutic cells.

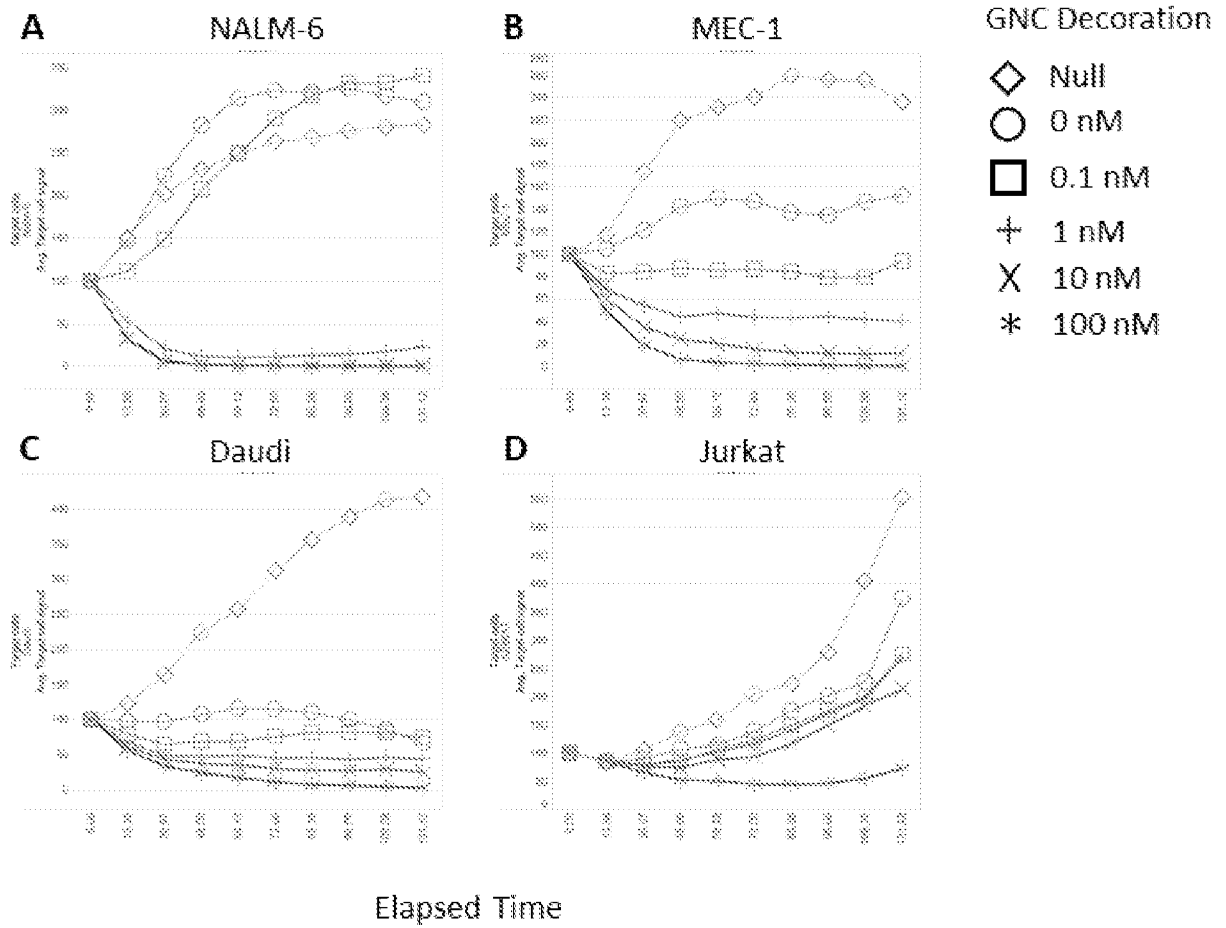
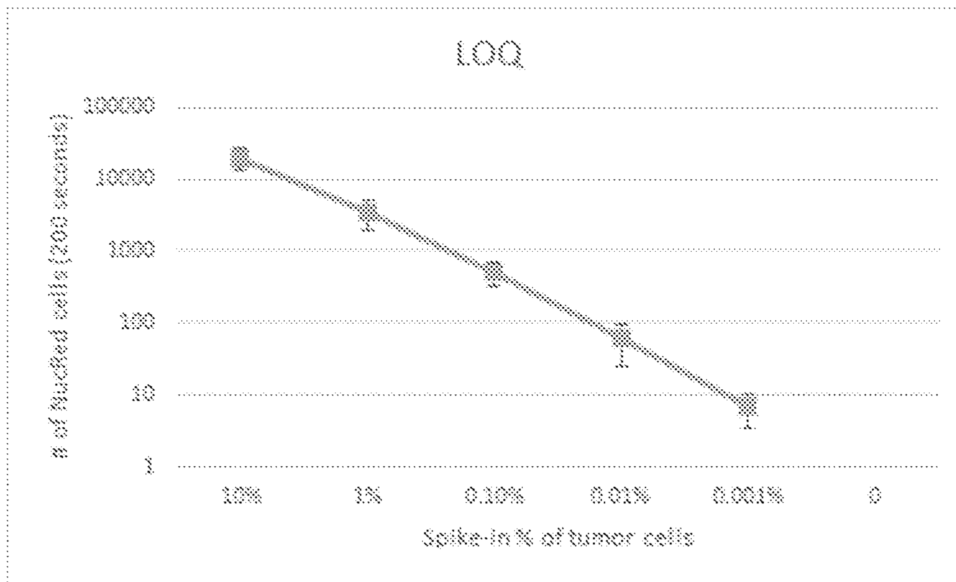


FIGURE 27. Killing of Nalm-6, MEC-1, Daudi, and Jurkat leukemic cells by PBMC-derived, SI-38E17 GNC-activated therapeutic cells in a spike-in model.



Spike-in	Nalm-6	Mec-1	Jurkat	Daudi
10%	1	44	0	0
1%	0	0	0	0
0.10%	1	0	0	0

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/24111

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 16/28, A61K 39/395, C07K 16/28, C07K 16/30 (2019.01)
CPC - C07K 16/2887, A61K 39/3955, C07K 16/2809, C07K 16/2818, C07K 16/3061

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2017/0210819 A1 (MEMORIAL SLOAN KETTERING CANCER CENTER) 27 July 2017 (27.07.2017) Especially para [0003], [0028], [0172], [0173], [0189], [0249], [0292]	1, 6-9, 11 --------------- 2-5, 10, 12, 13
Y	BOHLEN et al. Cytolysis of Leukemic B-Cells by T-Cells Activated via Two Bispecific Antibodies" Cancer Research, 15 September 1993, Vol 53, No 18, pp 4310-4314 Especially Abstract, pg 4313	2-5, 10, 12, 13
Y	GRZYWNOWICZ et al. Expression of Programmed Death 1 Ligand in Different Compartments of Chronic Lymphocytic Leukemia, Acta Haematologica, November 2015, Vol 134, No 4, pp 255 -262 Especially Abstract, pg 254	12
Y	WO 2017/064221 A1 (AFFIMED GMBH) 20 April 2017 (20.04.2017) Especially pg 17	13

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 July 2019	24 JUL 2019

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/24111

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

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

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

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: claims 1-13, drawn to a method for generating a therapeutic composition.

Group II: claims 14-36, drawn to a method of treating a subject having a cancer.

Group III: claims 37-39, drawn to a therapeutic composition.

-----please see continuation in extra sheet------

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-13

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box No. III Observations where unity of invention is lacking

The inventions listed as Groups I, II and III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I includes the special technical feature of a method for generating a therapeutic composition, not required by Groups II and III.

Group II includes the special technical feature of a method of treating a subject having a cancer, not required by Groups I and III.

Group III includes the special technical feature of a therapeutic composition, not required by Groups I and II.

Common Technical Features

The inventions of Groups I-III share the technical feature of a cytotoxic cell, a GNC protein, and a therapeutic cell.

However, these shared technical features do not represent a contribution over prior art in view of US 2017/0210819 A1 to Memoral Sloan Kettering Cancer Center (hereinafter 'MSKCC').

MSKCC discloses (instant claim 1) a method for generating a therapeutic composition (para [0028] - "provided herein is a pharmaceutical composition"), comprising
providing a cell material comprising a cytotoxic cell,
incubating the cell material with a first GNC protein to provide an activated cell composition,
wherein the activated cell composition comprises a first therapeutic cell,
wherein the first GNC protein comprising a first cytotoxic binding moiety and a first cancer targeting moiety,
wherein the first cytotoxic binding moiety has a specificity to a first cytotoxic cell receptor and is configured to activate the first cytotoxic cell through the binding with the first cytotoxic cell receptor, and
wherein the first cancer targeting moiety has a specificity to a first cancer cell receptor (para [0028] - "provided herein is a pharmaceutical composition comprising a therapeutically effective amount of the bispecific binding molecule, a pharmaceutically acceptable carrier, and T cells"; para [0249] - "This example describes a HER2/CD3 bi-specific binding molecule (herein referred to as "HER2-BsAb")...the effectiveness of this BsAb centers on the exploitation of the cytotoxic potential of polyclonal T cells"; para [0172]-[0173] - "when the bispecific binding molecules provided herein are bound to T cells,...., an anti-CD3 scFv of the bispecific binding molecule binds to CD3 on the surface of the T cell...it is believed that binding of the bispecific binding molecule to the T cell (i.e., binding of an anti-CD3 scFv to CD3 expressed on the T cell) activates the T cell, and consequently, allows for the T cell receptor-based cytotoxicity to be redirected to desired tumor targets...Thus, the invention also provides T cells which are bound to a bispecific binding molecule of the invention"; para [0247] - "a bispecific binding molecule provided herein, polynucleotide, vector, or cell encoding the bispecific binding molecule, or a pharmaceutical composition comprising the bispecific binding molecule, is administered in combination with T cell infusion.), and
wherein the first therapeutic cell comprises the first GNC protein bound to the cytotoxic cell through the binding interaction with the first cytotoxic cell receptor (para [0058] - "provided herein are bispecific binding molecules that specifically bind to HER2 and to CD3, and invoke T cell cytotoxicity for treating cancer."), and
formulating the activated cell composition to provide a therapeutic composition (para [0189] - "In specific embodiments involving combination therapy with infusion of T cells, provided herein is a pharmaceutical composition comprising (a) a bispecific binding molecule described herein....; (b) T cells; and/or (c) a pharmaceutically effective carrier...the T cells are bound to the bispecific binding molecule.").

MSKCC does not teach that the therapeutic composition is substantially free of exogenous viral and non-viral DNA or RNA. Since it was commonly known in the art to eliminate the presence of contaminants, it would have been obvious to one of ordinary skill in the art that the therapeutic composition is substantially free of exogenous viral and non-viral DNA or RNA in order to ensure safety upon administration.

As said technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Groups I, II and III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

NOTE, claim 5 depends from claim 1 or 2, as drafted, is objected to, because claim 1 lacks antecedent "the second cancer targeting moiety" required in claim 5. For this International Search and Opinion, claim 5 is reconstrued as dependent claim of claim 2.

NOTE, claim 12 depends from claim 1, as drafted, is objected to, because claim 1 lacks antecedent "the second cancer-targeting moiety" required in claim 12. For this International Search and Opinion, claim 12 is reconstrued as dependent claim of claim 2.

NOTE, claim 13 depends from claim 1, as drafted, is objected to, because claim 1 lacks antecedent "the second cytotoxic binding moiety" required in claim 13. For this International Search and Opinion, claim 13 is reconstrued as dependent claim of claim 2.