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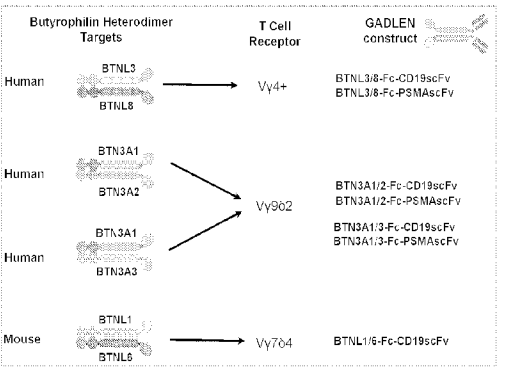
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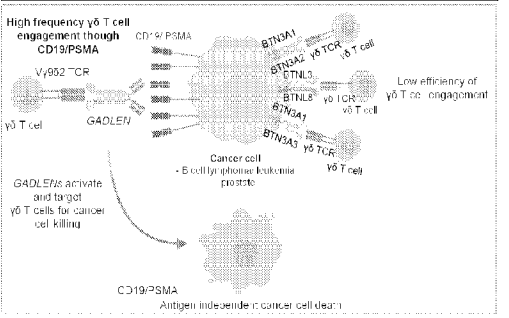
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(54) Title: HETERODIMERIC PROTEINS FOR MODULATING GAMMA DELTA T CELLS

FIG. 1



Other Human Butyrophilin Targets	Potential Cancer Antigens Targeted with scFv Scaffolds
BTN1A1, BTN2A1, BTN2A2, BTN2A3, BTN3A3, BTNL2, BTNL9, BTNL10, SKINT	GD2, PSCA, BCMA, CD123, B7-H3, CD20, CD30, CD33, CD38, CEA, CLEC12A, DLL3, EGFRvIII, EpCAM, CD307, FLT3, GPC3, gpA33, GPCR5D, HER2, MUC16, P-cadherin, SSTR2, mesothelin, etc.



(57) Abrégé/Abstract:
The present technology relates, inter alia, to compositions and methods, including heterodimeric proteins that find use in the treatment of disease, such as immunotherapies for cancer and autoimmunity.

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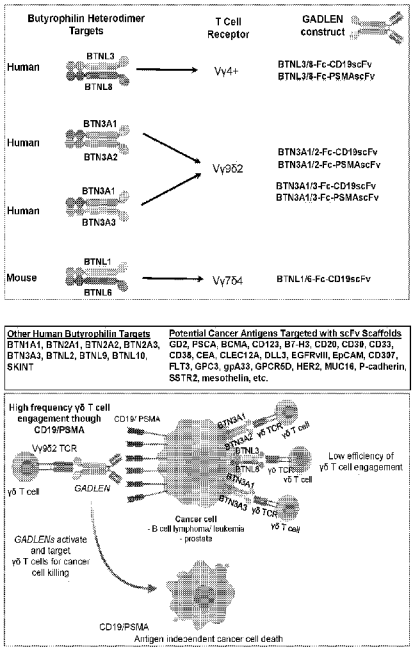
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(54) Title: HETERODIMERIC PROTEINS FOR MODULATING GAMMA DELTA T CELLS

FIG. 1



(57) Abstract: The present technology relates, inter alia, to compositions and methods, including heterodimeric proteins that find use in the treatment of disease, such as immunotherapies for cancer and autoimmunity.

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HETERODIMERIC PROTEINS FOR MODULATING GAMMA DELTA T CELLS

PRIORITY

This Application claims the benefit of, and priority to, US Application Nos. 62/941,176, filed November 27, 2019, 62/876,346, filed July 19, 2019, and 62/789,344, filed January 7, 2019, each of which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

The present technology relates to heterodimeric proteins that find use in the treatment of diseases, such as immunotherapies for cancer and autoimmunity.

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The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: SHK-029PC_116981-5029_ST25; date created: January 7, 2020; file size: 375,970 bytes).

BACKGROUND

The modulation of protein-protein interactions is a useful mechanism for therapeutic intervention in various diseases and pathologies. Soluble binding proteins which interact with ligands can potentially sequester the ligand away from the receptor, thereby reducing the activation of that particular receptor pathway. Alternatively, sequestration of the ligand may delay its elimination or degradation, thereby increasing its duration of effect and biological activity. Additionally, soluble ligands may be utilized to either activate or inhibit specific receptors.

Gamma delta T cells amount to up to 5% of all T cells in a human, but they play an important role against cancer. Recent research has indicated that the amount of gamma delta T cells that infiltrate a tumor is an excellent predictor of a favorable outcome for the patient. Further, unlike the alpha beta T cells commonly used in CAR-T therapy, gamma delta T cells play a role in the innate immune response. The prognostic significance of gamma delta T cells in cancer has prompted an effort to manipulate gamma delta T cells as a therapeutic strategy for cancer. Current approaches are limited to ex vivo strategies, where a patients gamma delta T cells are either harvested and modified to express a chimeric antigen receptor, and/or expanded to greater numbers in cell culture, followed by infusion of the modified gamma delta T cells back into the cancer patient (Front Immunol. 2018 Jun 26;9:1409). Strategies to manipulate gamma delta T cells directly in cancer patients have been hampered by an inability to conclusively identify the molecular entities directly recognized by the gamma delta T cell receptor (Nat Immunol. 2019 Feb;20(2):121-128). In fact, the most widely accepted activators of gamma delta T cells include largely intracellular molecules such as heat shock proteins, intermediates of the non-mevalonate

pathway of isopentyl pyrophosphate (IPP) biosynthesis (including HMB-PP), intracellular bacteria (eg. mycobacteria and listeria), viruses (eg. cytomegalovirus), and other lipid antigens.

Accordingly, there remains a need for novel compositions and methods gamma-delta T cell engagement that do not require use of the above molecules.

SUMMARY

Accordingly, in one aspect, the present technology provides a heterodimeric protein comprising (a) a first domain comprising one or more butyrophilin family proteins, or a fragment thereof; (b) a second domain comprising a targeting domain, the targeting domain being selected from an (i) antibody, antibody-like molecule, or antigen binding fragment thereof, and (ii) a extracellular domain; and (c) a linker that adjoins the first and second domain and which facilitates heterodimerization. In some embodiments, the heterodimeric protein comprises two of the same butyrophilin family proteins or two different butyrophilin family proteins. In some embodiments, the heterodimeric protein comprises two individual polypeptide chains which self-associate. In some embodiments, the butyrophilin family proteins comprise a V-type domain and/or a B30.2 domain. In some embodiments, the first domain is a butyrophilin-like (BTNL) family protein, such as BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT.

In some embodiments, the targeting domain is an antibody, an antibody-like molecule, or antigen binding fragment thereof. The antigen-like molecule may be selected from a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), a DARPin; a Tetranectin; an Affibody; a Transbody; an Anticalin; an AdNectin; an Affilin; an Affimer, a Microbody; an aptamer; an alterase; a plastic antibody; a phylomer; a stradobody; a maxibody; an evibody; a fynomer, an armadillo repeat protein, a Kunitz domain, an avimer, an atrimer, a probody, an immunobody, a triomab, a troybody; a pepbody; a vaccibody, a UniBody; a DuoBody, a Fv, a Fab, a Fab′, and a $F(ab')_2$.

In some embodiments, the targeting domain is an extracellular domain. In some embodiments, the targeting domain is capable of binding an antigen on the surface of a cancer cell. Exemplary the targeting domain specifically binds one of CD19, PSMA, GD2, PSCA, BCMA, CD123, B7-H3, CD20, CD30, CD33, CD38, CEA, CLEC12A, DLL3, EGFRvIII, EpCAM, CD307, FLT3, GPC3, gpA33, HER2, MUC16, P-cadherin, SSTR2, and mesothelin.

In some embodiments, the first polypeptide chain and the second polypeptide chain heterodimers through electrostatic interactions between positively charged amino acid residues and negatively charged amino acid residues on the first and second charge polarized core domains. In some embodiments, the positively charged amino acid residues may include one

or more of amino acids selected from His, Lys, and Arg. In some embodiments, the negatively charged amino acid residues may include one or more amino acids selected from Asp and Glu.

Accordingly, in some embodiments, each of the first and/or second charge polarized core domains comprises proteins having positively or negatively charged amino acid residues at the amino and carboxy terminus of the core domain. In an illustrative embodiment, the first charge polarized core domain may comprise a protein having positively charged amino acids at the amino terminus which are adjoined by a linker (*e.g.*, a stabilizing domain) to a protein having negatively charged amino acid residues at the carboxy terminus. In such an embodiment, the second charge polarized core domain may comprise a protein having negatively charged amino acids at the amino terminus which are adjoined by a linker (*e.g.*, a stabilizing domain) to a protein having positively charged amino acid residues at the carboxy terminus. In another illustrative embodiment, the first charge polarized core domain may comprise a protein having negatively charged amino acids at the amino terminus which are adjoined by a linker (*e.g.*, a stabilizing domain) to a protein having positively charged amino acid residues at the carboxy terminus. In such an embodiment, the second charge polarized core domain may comprise proteins having positively charged amino acids at the amino terminus which are adjoined by a linker (*e.g.*, a stabilizing domain) to a protein having negatively charged amino acid residues at the carboxy terminus.

In various embodiments, each of the first and/or second charge polarized core domains further comprise a linker (*e.g.*, a stabilizing domain) which adjoins the proteins having positively or negatively charged amino acids. In some embodiments, the linker (*e.g.*, a stabilizing domain) is optionally selected from a flexible amino acid sequence, IgG hinge region, or antibody sequence. In an embodiment, the linker (*e.g.*, a stabilizing domain) comprises the hinge-CH2-CH3 Fc domain derived from IgG1, optionally human IgG1. In another embodiment, the linker (*e.g.*, a stabilizing domain) comprises the hinge-CH2-CH3 Fc domain derived from IgG4, optionally human IgG4.

In some embodiments, the heterodimeric protein is capable of engaging gamma-delta T cells.

In embodiments, in a heterochimeric protein, the first domain is selected from Table 1, e.g. one or more ECDs from Table 1, e.g. 2 ECDs of Table 1.

In embodiments, the first domain comprises a portion of an extracellular domain of a Type 1 transmembrane protein, wherein the portion of the extracellular domain is capable of binding the Type 1 transmembrane protein's receptor/ligand. In embodiments, the Type 1 transmembrane protein is selected from lymphocyte-activation gene 3 (LAG-3), programmed cell death protein 1 (PD-1), and T cell immunoreceptor with Ig and ITIM domains (TIGIT).

In embodiments, the protein modulates the function of gamma delta T cells.

In embodiments, the protein on the amino- or carboxy-terminus is natively heterodimeric, and wherein the protein on the opposite terminus is not natively heterodimeric.

In embodiments the heterodimeric protein is a complex of two polypeptide chains.

In embodiments the heterodimeric protein comprises an alpha chain and a beta chain wherein the alpha chain and the beta chain each independently comprise (a) a first domain comprising a butyrophilin family protein, or fragment thereof; (b) a second domain comprising a targeting domain, the targeting domain being selected from an (i) antibody, antibody-like molecule, or antigen binding fragment thereof, and (ii) a extracellular domain; and (c) a linker that adjoins the first and second domain.

In embodiments the alpha chain and the beta chain self-associate to form the heterodimer.

In various aspects, the heterodimeric protein of the present technology is used for contemporaneous activation and targeting of gamma delta T cells to tumor cells, modulating a patient's immune response, and/or stimulating proliferation of gamma delta T cells in vivo. Accordingly, in various aspects, the heterodimeric protein of the present technology is used in a method for treating cancer, infectious, or autoimmune diseases comprising administering an effective amount of a pharmaceutical composition comprising the heterodimeric protein to a patient in need thereof.

In various aspects, the heterodimeric protein of the present technology is used for stimulating proliferation of gamma delta T cells by administering an effective amount of a pharmaceutical composition of the present technology to a subject in need thereof thereby causing an in vivo proliferation of gamma delta T cells and/or contacting an effective amount of a pharmaceutical composition of the present technology with a cell derived from a subject in need thereof thereby causing an ex vivo proliferation of gamma delta T cells.

In various aspects, the heterodimeric protein of the present technology is used for stimulating proliferation of gamma delta T cells in the absence of heat shock proteins, intermediates of the non-mevalonate pathway of isopentyl pyrophosphate (IPP) biosynthesis (including HMB-PP), intracellular bacteria (eg. mycobacteria and listeria), viruses (eg. cytomegalovirus), and other lipid antigens.

Also in various aspects, the present heterodimeric protein is used in a method for treating autoimmune diseases comprising administering an effective amount of a pharmaceutical composition comprising the heterodimeric protein to a patient in need thereof. In further aspects, the present heterodimeric protein is used in a method for treating infections, including without limitation, viral infections or other intracellular pathogens. In still further aspects, the present heterodimeric protein is used in a method for treating cancers.

Also provided in various aspects are pharmaceutical compositions comprising the heterodimeric protein of any of the embodiments disclosed herein, expression vectors comprising a nucleic acids encoding the heterodimeric protein of any of the embodiments disclosed herein, or host cells comprising expression vectors comprising a nucleic acids encoding the heterodimeric protein of any of the embodiments disclosed herein. Any aspect or embodiment disclosed herein can be combined with any other aspect or embodiment as disclosed herein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the illustrations for various non-limiting protein engineering embodiments of the present technology. Top panel shows an illustrative heterodimeric protein of the present technology comprising i) a human butyrophilin 3/8 heterodimer adjoined to either a human CD19-specific or PSMA-specific scFv, ii) a human butyrophilin 3A1/3A2 heterodimer adjoined to either a human CD19-specific or PSMA-specific scFv, iii) a human butyrophilin 3A1/3A3 heterodimer adjoined to either a human CD19-specific or PSMA-specific scFv, or iv) a mouse butyrophilin 1/6 heterodimer adjoined to a mouse CD19-specific scFv. As shown in the middle panel, the butyrophilin family members contemplated in a heterodimeric construct include but are not limited to: BTN1A1, BTN2A1, BTN2A2, BTN2A3, BTN3A3, BTNL2, BTNL9, BTNL10, SKINT, *etc*. Other antigen-targets for a butyrophilin heterodimeric construct include but are not limited to: GD2, PSCA, BCMA, CD123, B7-H3, CD20, CD30, CD33, CD38, CEA, CLEC12A, DLL3, EGFRvIII, EpCAM, CD307, FLT3, GPC3, gpA33, HER2, MUC16, P-cadherin, SSTR2, mesothelin, *etc* (middle panel). Without wishing to be bound by theory, the proposed mechanism of action for a butyrophilin heterodimer construct targeting either CD19 or PSMA as illustrated in the bottom panel. In this example, engagement of gamma delta T cells to CD19 or PSMA positive tumor cells is enhanced due to the butyrophilin heterodimer simultaneously engaging a tumor antigen and the gamma delta T cell receptor. The contemplated GAmma DELta T cell ENgager constructs are referred to herein as 'GADLEN' fusion proteins.

FIG. 2 shows the Western blot analysis of a purified mouse BTNL1/6-CD19 scFv GADLEN protein. The protein was generated by dual-transfection of CHO cells with both a BTNL1-CD19 scFv ('alpha', predicted MW = 77.8 kDa) and a BTNL6-CD19 scFv ('beta', predicted MW = 71.2 kDa) construct, in which the so-called alpha and beta constructs contained linker domains which facilitated heterodimerization of the desired BTNL1/6-CD19 scFv GADLEN protein. The purified protein was analyzed by Western blot using non-reduced (left lane), reduced (middle lane) and both reduced and deglycosylated (right lane) conditions, following detection with an anti-mouse Fc antibody. The results indicate the presence of a disulfide-linked protein that reduces to two individual proteins (following disruption of the interchain disulfide bonds

with β-mercaptoethanol) with molecular weights consistent with the predicted molecular weights for the alpha and beta chains. Based on the similarity between the reduced and both reduced and deglycosylated lanes, the GADLEN construct appears to have few glycosylations.

FIG. 3 depicts the quantitation of the purified mouse BTNL 1/6-CD19 scFv GADLEN using an Fc-specific ELISA method.

FIG. 4 illustrates exemplary flow cytometry profiles for two different mouse tumor cell lines (A20, mouse lymphoma) and WEHI-3 (mouse leukemia), which express CD19 at different densities (A20 at a higher density than WEHI-3).

FIG. 5 provides an example of purified mouse BTNL 1/6-CD19 scFv GADLEN cell surface binding to A20 cells as detected by flow cytometry. In the assay, the purified mouse BTNL 1/6-CD19 scFv GADLEN construct was used to stain cells, and was then detected with an APC-conjugated anti-mouse Fc antibody.

FIG. 6 provides an example of purified mouse BTNL 1/6-CD19 scFv GADLEN cell surface binding to WEHI-3 cells as detected by flow cytometry. In the assay, the purified mouse BTNL 1/6-CD19 scFv GADLEN construct was used to stain cells, and was then detected with an APC-conjugated anti-mouse Fc antibody.

FIG. 7 provides representative results of a mouse gamma delta T cell/mouse tumor cell killing assay mediated by a CD19-specific antibody. In these studies, purified mouse gamma delta T cells (isolated from mouse intestinal epithelium) were co-cultured with mouse A20 lymphoma cells alone (triangles) or together with an anti-mouse CD19 specific antibody (squares, clone 1D3) at a gamma delta T cells:A20 tumor cell ratio of 1:1, for 18 hours with live imaging every hour in an Incucyte imager. A20 cells alone (circles) and gamma delta T cells alone (diamonds) were included as negative controls. The A20 tumor cells were labeled with green fluorescence, and the assay included a red-fluorescent dye specific for Annexin-5, as an indicator of cell death. The y-axis in the figure indicates the overlap between green and red fluorescence, which occurs when green-labeled A20 tumor cells undergo cell death and stain dual-positive for red-labeled Annexin-5.

FIG. 8 provides the representative results from the same gamma delta based A20 killing assay shown in **FIG. 7**, mediated by the BTNL1/6-CD19scFv GADLEN construct of the present technology. Purified mouse gamma delta T cells (isolated from mouse intestinal epithelium) were co-cultured with mouse A20 lymphoma cells alone (triangles) or together with BTNL1/6-CD19scFv GADLEN construct (circles). A20 cells alone (inverted triangles) and gamma delta T cells alone (diamonds) were included as negative controls. The mean maximum A20 killing activity from **FIG. 7** is indicated with the dashed line, and the killing activity in the presence of the GADLEN construct was observed to be superior to that observed with the CD19 specific

antibody. In both **FIGs. 7** and **8**, the elevated reading at time 0 hours suggests that rapid A20 cell killing occurs, since there is a ~30 minute delay between the addition of the cells and reagents and the initial image captured by the Incucyte reader and indicated as 0 hours.

FIGs. 9A-9B provide an example of a different gamma delta T cells:tumor cell killing assay, in which mouse gamma delta T cells (isolated from intestinal epithelium) were co-cultured for 4-5 hours with WEHI-3 tumor cells alone, or in combination with an anti-CD19 antibody or an increasing concentration of the BTNL1/6-CD19scFv GADLEN construct. **FIG. 9A** shows a bar graph of the proportion of gamma delta T cells staining positive for CD107a by flow cytometry following the indicated co-culture. CD107a is a marker of cell degranulation, which occurs when T cells release cytolytic granules containing perforin and granzymes. These data indicate that the GADLEN construct stimulated a dose-dependent CD107a expression in isolated gamma delta T cells. gd = gamma delta T cells. **FIG. 9B** shows a bar graph illustrating dose-dependent upregulation of CD69 (a cell surface marker of activated T cells). Notably, only minor increases in CD107a or CD69 expression were noted when gamma delta T cells were co-cultured with WEHI-3 tumor cells in the absence of the GADLEN construct.

FIGs. 10A-10B provide another example of the CD107a degranulation assay, in which mouse gamma delta T cells (isolated from intestinal epithelium) were co-cultured for 4-5 hours with A20 tumor cells alone, or in combination with an anti-CD19 antibody or an increasing concentration of the BTNL1/6-CD19scFv GADLEN construct. **FIG. 10A** shows a bar graph illustrating the proportion of gamma delta T cells staining positive for CD107a by flow cytometry following co-culture. gd = gamma delta T cells. CD107a is a marker of cell degranulation, which occurs when T cells release cytolytic granules containing perforin and granzymes. These data indicate that the GADLEN construct stimulated dose-dependent CD107a expression in isolated gamma delta T cells (**FIG. 10A**). **FIG. 10B** shows a bar graph illustrating the dose-dependent upregulation of CD69 (a cell surface marker of activated T cells). Notably, only minor increases in CD107a or CD69 expression are noted when gamma delta T cells were co-cultured with A20 tumor cells in the absence of the GADLEN construct.

FIGs. 11A-11B provide a representative series of flow cytometry plots from an *in vivo* study in Balb.c mice treated with a vehicle control, anti-PD1 antibody, anti-CD19 antibody, or the BTNL1/6-CD19scFv GADLEN protein. Briefly, Balb.c mice were implanted in A20 tumor cells in the hind flank and those tumors grew to approximately 40 mm^3 before the first treatment with anti-PD1 antibody, anti-CD19 antibody, or the BTNL1/6-CD19scFv GADLEN protein (all dosed at 100 µg/mouse). 24 hours following the first treatment, peripheral blood was collected from each mouse and analyzed for the proportion of CD20+ B cells (**FIG. 11A**) and CD3+ gamma delta TCR+ T cells (**FIG. 11B**). Each group contained $\geq$ 5 mice, and representative flow cytometry plots using one mouse from each group are shown. **FIG. 11A**

shows the frequency of CD20+ cells in the peripheral blood. These data indicated that 24 hours after treatment, the frequency of CD20+ cells had not significantly changed for the vehicle, anti-PD1 or anti-CD19 antibody treated groups, however, the frequency of CD20+ B cells in the BTNL1/6-CD19scFv GADLEN protein treated group had reduced by > 10 fold. **FIG. 11B** shows the frequency of gamma delta T cells in the peripheral blood of mice out of total CD3+ T cells. These data indicated that whereas <1% of circulating CD3+ T cells expressed the gamma delta TCR in vehicle, anti-PD1 or anti-CD19 antibody treated mice, the proportion of gamma delta T cells has increased >10 fold within 24 hours of treatment with the BTNL1/6-CD19scFv GADLEN protein.

FIGs. 12A-12D provide an expanded a representative expanded series of flow cytometry plots from an *in vivo* study carried out as described in **FIGs. 11A-11B**. Specifically, **FIG. 12A** indicates the frequency of CD19+ B cells (all data shown as mean ± SEM with ≥ 5 mice/group), and **FIG. 12B** shows the frequency of CD20+ B cells 24 hours after the indicated treatment in Balb.c mice with established A20 tumors. Similarly, **FIG. 12C** indicates the frequency of CD19+ B cells (all data shown as mean ± SEM, and **FIG. 12D** shows the frequency of CD20+ B cells 24 hours after the indicated treatment in Balb.c mice with established WEHI-3 tumors. The data illustrate that while the anti-CD19 treatment antibody competed with the CD19 detection antibody, the antibody treatment had not truly depleted B cells in the peripheral blood because the frequenc of CD20+ B cells (where there is no competition between the CD19 treatment antibody and the CD20 detection antibody) had not significantly changed. In contrast, all mice treated with the BTNL1/6-CD19scFv GADLEN protein showed near complete depletion of peripheral blood B cells within 24 hours of treatment.

FIGs. 13A-13B provide an expanded a representative expanded series of flow cytometry plots from an *in vivo* study carried out as described in **FIGs. 11A-11B**. Specifically, **FIG. 13A** indicates the frequency of gamma delta T cells in mice bearing established A20 tumors, and **FIG. 13B** shows the frequency of gamma delta T cells in mice bearing established WEHI-3 tumors 24 hours after the indicated treatment (all data shown as mean ± SEM with ≥ 5 mice/group). These data illustrated that the BTNL1/6-CD19scFv GADLEN protein had stimulated rapid proliferation of mouse gamma delta T cells within 24 hours of treatment.

FIG. 14 provides Western blot analysis of a purified human BTNL3/8-CD19 scFv GADLEN protein. The protein was generated by dual-transfection of CHO cells with both a BTNL3-CD19 scFv ('alpha', predicted MW = 78.4 kDa) and a BTNL8-CD19 scFv ('beta', predicted MW = 78.9 kDa) construct, in which the so-called alpha and beta constructs contained linker domains which facilitated heterodimerization of the desired BTNL 3/8-CD19 scFv GADLEN protein. The purified protein was analyzed by Western blot using non-reducing (left lane), reducing (middle lane) and both reducing and deglycosylating (right lane) conditions, following

detection with an anti-human BTNL8 antibody (left blot) or anti-human Fc antibody (right blot). The results indicated the presence of a disulfide-linked protein that reduces to a single band of roughly half the molecular weight of the non-reduced band (following disruption of the interchain disulfide bonds with β-mercaptoethanol). Because the molecular weights of the alpha and beta chains are so similar, a doublet band could not be detected under reducing conditions in this study. A minor reduction in molecular weight was observed in both reduced and deglycosylated protein (third lane) compared to reduced protein, indicating that the GADLEN construct is likely to be a glycosylated protein.

FIG. 15 depicts quantitation of the purified human BTNL 3/8-CD19 scFv GADLEN using an Fc-specific ELISA method.

FIG. 16 provides Western blot analysis of a purified human BTN3A1/3A2-CD19 scFv GADLEN protein. The protein was generated by dual-transfection of CHO cells with both a BTN3A1-CD19 scFv ('alpha', predicted MW = 77.4 kDa) and a BTN3A2-CD19 scFv ('beta', predicted MW = 77.7 kDa) construct, in which the so-called alpha and beta constructs contained linker domains which facilitated heterodimerization of the desired BTN 3A1/3A2-CD19 scFv GADLEN protein. The purified protein was analyzed by Western blot using non-reducing (left lane), reducing (middle lane) and both reducing and deglycosylating (right lane) conditions, following detection with an anti-human BTN3A1/2 antibody (left blot) or anti-human Fc antibody (right blot). These results indicated the presence of a disulfide-linked protein that reduces to a single band of roughly half the molecular weight of the non- reducing band (following disruption of the interchain disulfide bonds with β-mercaptoethanol). Because the molecular weights of the alpha and beta chains are similar, a doublet band could not be detected under reducing conditions in this study. There was a minor reduction in molecular weight between the reducing and both reducing and deglycosylating conditions lanes, indicating that the GADLEN construct appears to be a glycosylated protein. A series of background bands were also present in the gel, which are likely due to either non-specific binding for each antibody and/or cleaved or incompletely translated proteins which are common in transient transfection productions with multiple plasmids.

FIG. 17 depicts quantitation of the purified human BTNL 3A1/3A2-CD19 scFv GADLEN using an Fc-specific ELISA method. A positive control (recombinant BTN3A1-Fc; squares) was compared to the BTN3A1/3A2-CD19scFv construct (diamonds), as well as to a control BTNL3/8-KIH-CD19scFv protein that includes the 'knob-in-hole' mutations in the linker domain to facilitate heterodimerization (circles).

FIGs. 18A-18B provides an example of CD07a degranulation assay, carried out as described in **FIGs. 10A-10B**, that has been adapted for human gamma delta T cells/tumor cells and the

human GADLEN proteins in this case. Specifically, **FIG. 18A** indicates the frequency of CD107a+ human gamma delta T cells in cell cultures containing human gamma delta T cells isolated from human peripheral blood (total of >11 replicates from >3 human donors) following a 4-5 hour co-culture with human Raji tumor cells (human Burkitt lymphoma cell line) either alone or together with the anti-human CD20 antibody (Rituximab), BTNL3/8-CD19scFv, BTN3A1/3A2-CD19scFv, HMBPP (positive control) or BTN3A1/3A2-CD19scFv in addition to HMBPP. **FIG. 18B** indicates the proportion of human gamma delta T cells expressing the activation marker, CD69, from the same co-cultures as panel A. The data indicate that the BTNL3/8-CD19scFv and BTN3A1/3A2-CD19scFv GADLEN constructs both cause CD107a degranulation and CD69 expression in human gamma delta T cells, similar to what was observed in mouse gamma delta T cells using the species-specific construct. The increased activity of the BTN3A1/3A2 construct in this assay is likely a result of the higher proportion of Vγ9+ gamma delta T cells in peripheral blood, which are the gamma delta T cell population that selectively respond to a BTN3A1/3A2 heterodimer. Gamma delta T cells isolated from human intestinal epithelium would be comparatively enriched for Vγ4+ gamma delta T cells, and therefore be selectively responsive to a BTNL3/8 GADLEN construct.

FIG. 19 shows an ELISA assay that was developed to demonstrate specific detection of the BTNL3/8-Fc-αCD19 heterodimer protein.

FIG. 20 is a graph showing the percentage of binding of the BTNL3/8-Fc-αCD19scFv and BTN3A1/A2-CD19scFv heterodimer proteins to isolated human γδTCR T cells.

FIG. 21 shows graphs illustrating the binding of the BTNL3/8-Fc-CD19scFv and BTN3A1/A2-CD19scFv heterodimer proteins BTNL3/8-Fc-αCD19 heterodimer protein to CD19+ Raji B-cells .

FIG. 22 is a graph showing the ability of the BTNL3/8-Fc-αCD19 heterodimer protein to tether γδ T-cells to CD19+ Raji cells.

FIG. 23 is a graph showing the ability of the BTNL3/8-Fc-αCD19 heterodimer protein to tether γδ T-cells to CD19+ Toledo cells.

FIG. 24 shows an ELISA assay that was developed to demonstrate specific detection of the BTNL3/8-Fc-αPSMA heterodimer protein.

FIG. 25 is a graph showing the percentage of binding of the BTNL3/8-Fc-αPSMA heterodimer protein to isolated human γδTCR T cells.

FIG. 26 shows the flow cytometry profiles and graphs illustrating the binding of the BTNL3/8-Fc-αPSMA heterodimer protein to prostate cancer cell lines.

FIG. 27 shows the flow cytometry profiles and graphs illustrating the binding of the BTNL3/8-Fc-vTIGIT heterodimer protein to isolated human γδTCR T cells.

FIG. 28 are graphs showing binding of the BTNL3/8-Fc-vLAG3 heterodimer proteinto isolated human γδTCR T cells.

FIG. 29 shows Western blots illustrating detection of the TIGIT-Fc-vBTNL3/8 heterodimer protein under different conditions using anti-human TIGIT antibody (left), anti-human Fc antibody (middle), or anti-human BTNL8 antibody (right). "Biol" is boiled, is "DG" is de-glycosylated and "Red" is reducing conditions.

FIG. 30 shows the flow cytometry profiles and graphs illustrating the binding of the TIGIT-Fc-vBTNL3/8 heterodimer protein to isolated human γδTCR T cells.

FIG. 31 shows the flow cytometry profiles and graphs illustrating the binding of the LAG3-Fc-vBTNL3/8 heterodimer protein to isolated human γδTCR T cells.

FIGs. 32A-32C show the *in vivo* anti-tumor activity of BTNL1/6-Fc-CD19scFv GADLEN in aWEHI3 tumor model. **FIG. 32A** shows a line graph of the average tumor growth of a WEHI-3 (leukemia) tumors. WEHI-3 cells were inoculated subcutaneously on the rear flank. When the average starting tumor volume (STV) reached 90.92 mm^3 (day 0), treatments were begun. The treatment groups were anti-PD1 (clone RMP1-14), anti-CD19 (clone 1D3), and BTNL1/6-Fc-CD19scFv were each given in 3 doses IP; 100μg per dose on days 0, 3, and 6. Vehicle alone was used as a negative control. Tumor growth was assessed over a 17-day time course. **FIG. 32B** shows a bar graph of tumor size on day 8. **FIG. 32C** shows a bar graph of tumor size on day 10. Error is SEM and statistical significance was determined using one-way ANOVA.

FIG. 33 shows the Kaplan-Meier curves of mice from **FIGs. 32A-32C**. Survival was assessed over an 18-day time course, following the first treatment on day 0. Significance between survival curves was determined using Mantel-Cox.

FIGs. 34A-34B show the representative results of a human gamma delta T cell/human tumor cell killing assay mediated by BTN3A1/A2-CD19scFv and BTNL3/8-Fc-CD19scFv heterodimer proteins. In these studies, purified human gamma delta T cells (isolated from human peripheral blood mononuclear cells) were co-cultured with human CD19+ Raji cells together with increasing concentrations (1-25μg/ml) of BTN3A1/A2-CD19scFv or BTNL3/8-Fc-CD19scFv heterodimer proteins at a gamma delta T cells:Raji tumor cell ratio of 1:1, for 8 hours with live imaging every hour in an Incucyte imager. Raji cells alone (diamonds) and gamma delta T cells alone (inverted triangle) were included as negative controls. The Raji tumor cells were labeled with green fluorescence, and the assay included a red-fluorescent dye specific for Annexin-5, as an indicator of cell death. The y-axis in the figure indicates the

integrated intensity of red fluorescence, which occurs when Raji tumor cells undergo cell death and stain positive for red-labeled Annexin-5.

DETAILED DESCRIPTION

The present technology is directed to novel chimeric proteins that have the ability to, *inter alia*, target gamma delta T cells and cause their activation, while also forming a synapse with, e.g., tumor cells. Thus, the present multifunctional chimeric proteins provide for unique means to modulate a subject's immune system for therapy.

The Heterodimeric Proteins of the Present Technology

In one aspect, the present technology relates to heterodimeric proteins comprising: (a) a first domain comprising one or more butyrophilin family proteins, or a fragment thereof; (b) a second domain comprising a targeting domain, the targeting domain being selected from an (i) antibody, antibody-like molecule, or antigen binding fragment thereof, and (ii) a extracellular domain; and (c) a linker that adjoins the first and second domain. In some embodiments, the heterodimeric protein of the invention comprises two polypeptide chains, wherein the first polypeptide chain and the second polypeptide chain comprise (a) a first domain comprising one or more butyrophilin family proteins, or a fragment thereof; (b) a second domain comprising a targeting domain, the targeting domain being selected from an (i) antibody, antibody-like molecule, or antigen binding fragment thereof, and (ii) a extracellular domain; and (c) a linker that adjoins the first and second domain. In some embodiments, the heterodimeric protein comprises two individual polypeptide chains which self-associate. In some embodiments, the first domain comprising one or more butyrophilin family proteins, or a fragment thereof of the first and the second polypeptide chain are the same. In some embodiments, the second domain comprising a targeting domain of the first and the second polypeptide chain are the same. In some embodiments, the linker that adjoins the first and second domain are the same.

The Butyrophilin Family Proteins, or Fragments Thereof

The heterodimeric proteins of any of the embodiments disclosed herein comprise a first domain comprising one or more butyrophilin family proteins, or a fragment thereof. In some embodiments, the butyrophilin family proteins are selected from BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT. In some embodiments, the first domain comprises: (a) any one of BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT; and (b) any one of BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT. In some embodiments, the first domain comprises: (a) any one of human BTNL1, human BTN1A1, human BTNL2, human BTN2A1,

human BTN2A2, human BTN2A3, human BTNL3, human BTN3A1, human BTN3A2, human BTN3A3, human BTNL6, human BTNL8, human BTNL9, human BTNL10, and human SKINT, and (b) any one of human BTNL1, human BTN1A1, human BTNL2, human BTN2A1, human BTN2A2, human BTN2A3, human BTNL3, human BTN3A1, human BTN3A2, human BTN3A3, human BTNL6, human BTNL8, human BTNL9, human BTNL10, and human SKINT.

In some embodiments, the first domain comprises a fragment of butyrophilin family proteins, wherein the fragment is capable of binding a gamma delta T cell receptor and is optionally an extracellular domain, optionally comprising one or more of an immunoglobulin V (IgV)- and IgC-like domain. In some embodiments, the first domain comprises a fragment of butyrophilin family proteins, wherein the fragment is capable of binding a gamma delta T cell receptor selected from a Vγ4, Vγ9δ2, or Vγ7δ4 TCR.

In some embodiments, the first domain comprises two of the same butyrophilin family proteins. In some embodiments, wherein the first domain comprises two different butyrophilin family proteins. In some embodiments, the butyrophilin family proteins comprise a V-type domain. Suitable butyrophilin family proteins or fragments thereof are derived from the native butyrophilin family proteins that comprise a B30.2 domain in the cytosolic tail of the full length protein.

An illustrative amino acid sequence of mouse BTNL1 suitable in the present technology is:

EVSWFSVKGPAEPITVLLGTEATLPCQLSPEQSAARMHIRWYRAQPTPAVLVFHNGQ EQGEVQMPEYRGRTQMVRQAIDMGSVALQIQQVQASDDGLYHCQFTDGFTSQEVS MELRVIGLGSAPLVHMTGPENDGIRVLCSSSGWFPKPKVQWRDTSGNMLLSSSELQT QDREGLFQVEVSLLVTDRAIGNVICSIQNPMYDQEKSKAILLPEPFFPKTCPWK (SEQ ID NO: 47)

An illustrative amino acid sequence of mouse BTNL6 suitable in the present technology (:

EQLPEYSQRTSLVKEQFHQGTAAVRILNVQAPDSGIYICHFKQGVFYEEAILELKVAA MGSVPEVYIKGPEDGGVCVVCITSGWYPEPQVHWKDSRGEKLTASLEIHSEDAQGLF RTETSLVVRDSSVRNVTCSTFNPILGQEKAMAMFLPEPFFPKVSPWKP (SEQ ID NO: 48)

An illustrative amino acid sequence of human BTNL3 suitable in the present technology is the following:

QWQVTGPGKFVQALVGEDAVFSCSLFPETSAEAMEVRFFRNQFHAVVHLYRDGED WESKQMPQYRGRTEFVKDSIAGGRVSLRLKNITPSDIGLYGCWFSSQIYDEEATWEL RVAALGSLPLISIVGYVDGGIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGY

SLYDVEISIIVQENAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLAS (SEQ ID NO: 49)

An illustrative amino acid sequence of human BTN3A1 suitable in the present technology:

QFSVLGPSGPILAMVGEDADLPCHLFPTMSAETMELKWVSSSLRQVVNVYADGKEV
EDRQSAPYRGRTSILRDGITAGKAALRIHNVTASDSGKYLCYFQDGDFYEKALVELK
VAALGSDLHVDVKGYKDGGIHLECRSTGWYPQPQIQWSNNKGENIPTVEAPVVADG
VGLYAVAASVIMRGSSGEGVSCTIRSSLLGLEKTASISIADPFFRSAQRWIAALAG
(SEQ ID NO: 50)

An illustrative amino acid sequence of human BTN3A2 suitable in the present technology

QFSVLGPSGPILAMVGEDADLPCHLFPTMSAETMELKWVSSSLRQVVNVYADGKEV
EDRQSAPYRGRTSILRDGITAGKAALRIHNVTASDSGKYLCYFQDGDFYEKALVELK
VAALGSNLHVEVKGYEDGGIHLECRSTGWYPQPQIQWSNAKGENIPAVEAPVVADG
VGLYEVAASVIMRGGSGEGVSCIIRNSLLGLEKTASISIADPFFRSAQPW (SEQ ID NO: 51)

An illustrative amino acid sequence of human BTNL8 suitable in the present technology is as follows:

QWQVFGPDKPVQALVGEDAAFSCFLSPKTNAEAMEVRFFRGQFSSVVHLYRDGKDQ
PFMQMPQYQGRTKLVKDSIAEGRISLRLENITVLDAGLYGCRISSQSYYQKAIWELQV
SALGSVPLISITGYVDRDIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGL
FDVEISLTVQENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATK (SEQ ID NO: 52)

The Second Domain Comprising a Targeting Domain

The heterodimeris proteins of any of the embodiments disclosed herein comprise a second domain comprising a targeting domain. In some embodiments, the targeting domain is an antibody-like molecule, or antigen binding fragment thereof. In some embodiments, the antibody-like molecule is selected from a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), a DARPin; a Tetranectin; an Affibody; a Transbody; an Anticalin; an AdNectin; an Affilin; an Affimer, a Microbody; an aptamer; an alterase; a plastic antibody; a phylomer; a stradobody; a maxibody; an evibody; a fynomer, an armadillo repeat protein, a Kunitz domain, an avimer, an atrimer, a probody, an immunobody, a triomab, a troybody; a pepbody; a vaccibody, a UniBody; a DuoBody, a Fv, a Fab, a Fab′, and a $F(ab')_2$. In some embodiments, the antibody-like molecule is an scFv. In some embodiments, the targeting domain is an extracellular domain. In some embodiments,

the targeting domain is capable of binding an antigen on the surface of a cancer cell. In some embodiments, the targeting domain specifically binds one of CD19, PSMA, GD2, PSCA, BCMA, CD123, B7-H3, CD20, CD30, CD33, CD38, CEA, CLEC12A, DLL3, EGFRvIII, EpCAM, CD307, FLT3, GPC3, gpA33, HER2, MUC16, P-cadherin, SSTR2, and mesothelin. In some embodiments, the targeting domain comprises a portion of the extracellular domain of LAG-3, PD-1, TIGIT, CD19, or PSMA. In some embodiments, the targeting domain specifically binds CD19. In some embodiments, the targeting domain specifically binds PSMA.

Illustrative sequences of second domain comprising a targeting domain are provided below:

An illustrative targeting domain is scFVh19, which is the heavy chain variable domain of an scFV specific to human CD19, and has the following sequence:

DIQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNL VSGIPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK (SEQ ID NO: 53)

An illustrative targeting domain is scFVlh19, which is light chain variable domain of an scFV specific to human CD19, and has the following sequence:

EVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHWVRQAPGKGLEWVSTINPAGT TDYAESVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCDSYGYRGQGTQVTV (SEQ ID NO: 54)

An illustrative targeting domain is scFVlPSMA, which is light chain variable domain of an scFV specific to human PSMA, and has the following sequence:

RKGGKRGSGSGQTVVTQEPSLTVSPGGTVTLTCASSTGAVTSGNYPNWVQQKPGQA PRGLIGGTKFLVPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVFGG GTKLTVL (SEQ ID NO: 55)

An illustrative targeting domain is scFvCD19, which an scFV specific to human CD19, and has the following sequence:

QVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGD GDTNYNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAM DYWGQGTTVTVSSGGGGSGGGGSGGGGSDIQLTQSPASLAVSLGQRATISCKASQSV DYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSGSGSGTDFTLNIHPVEKVDA ATYHCQQSTEDPWTFGGGTKLEIK (SEQ ID NO: 56)

An illustrative targeting domain is scFvCD19VHVL, which an scFV specific to mouse CD19, and has the following sequence:

EVQLQQSGAELVRPGTSVKLSCKVSGDTITFYYMHFVKQRPGQGLEWIGRIDPEDES
TKYSEKFKNKATLTADTSSNTAYLKLSSLTSEDTATYFCIYGGYYFDYWGQGVMVT
VSSGGGGSGGGGSGGGGSDIQMTQSPASLSTSLGETVTIQCQASEDIYSGLAWYQQK
PGKSPQLLIYGASDLQDGVPSRFSGSGSGTQYSLKITSMQTEDEGVYFCQQGLTYPRT
FGGGTKLELK (SEQ ID NO: 57)

An illustrative targeting domain is scFvCD19VLVH, which an scFV specific to mouse CD19, and has the following sequence:

DIQMTQSPASLSTSLGETVTIQCQASEDIYSGLAWYQQKPGKSPQLLIYGASDLQDGV
PSRFSGSGSGTQYSLKITSMQTEDEGVYFCQQGLTYPRTFGGGTKLELKGGGGSGGG
GSGGGGSEVQLQQSGAELVRPGTSVKLSCKVSGDTITFYYMHFVKQRPGQGLEWIG
RIDPEDESTKYSEKFKNKATLTADTSSNTAYLKLSSLTSEDTATYFCIYGGYYFDYWG
QGVMVTVSS (SEQ ID NO: 58)

An illustrative targeting domain is 19scFv3, which an scFV specific to human CD19, and has the following sequence:

DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGV
PSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGGGSGGGSGG
GSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSE
TTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWG
QGTSVTVSS (SEQ ID NO: 59)

An illustrative targeting domain is GD2scFv3, which an scFV specific to human GD2, and has the following sequence

GTDFTLKISRVEAEDLGVYFCSQSTHVPPLTFGAGTKLELKGGGSGGGSGGGSEVQL
LQSGPELEKPGASVMISCKASGSSFTGYNMNWVRQNIGKSLEWIGAIDPYYGGTSYN
QKFKGRATLTVDKSSSTAYMHLKSLTSEDSAVYYCVSGMKYWGQGTSVTVSS (SEQ ID NO: 60)

The Linker Domain that Adjoins the First and the Second Domain

In some embodiments, the linker that adjoins the first and second domain comprises a charge polartized core domain. In various embodiments, each of the first and second charge polarized core domains comprises proteins having positively or negatively charged amino acid residues at the amino and carboxy terminus of the core domain. In an illustrative embodiment, the first charge polarized core domain may comprise a protein having positively charged amino acids at the amino terminus which are adjoined by a linker (*e.g.*, a stabilizing domain) to a protein having negatively charged amino acid residues at the carboxy terminus. The second charge polarized core domain may comprise a protein having negatively charged amino acids at the

amino terminus which are adjoined by a linker (*e.g.*, a stabilizing domain) to a protein having positively charged amino acid residues at the carboxy terminus.

In another illustrative embodiment, the first charge polarized core domain may comprise a protein having negatively charged amino acids at the amino terminus which are adjoined by a linker (*e.g.*, a stabilizing domain) to a protein having positively charged amino acid residues at the carboxy terminus. The second charge polarized core domain may comprise proteins having positively charged amino acids at the amino terminus which are adjoined by a linker (*e.g.*, a stabilizing domain) to a protein having negatively charged amino acid residues at the carboxy terminus.

In various embodiments, formation of heterodimeric proteins is driven by electrostatic interactions between the positively charged and negatively charged amino acid residues located at the amino and carboxy termini of the first and second charge polarized core domains. Further, formation of homodimeric proteins is prevented by the repulsion between the positively charged amino acid residues or negatively charged amino acid residues located at the amino and carboxy termini of the first and second charge polarized core domains.

In various embodiments, the protein comprising positively and/or negatively charged amino acid residues at the amino or carboxy terminus of the charge polarized core domains is about 2 to about 50 amino acids long. For example, the protein comprising positively and/or negatively charged amino acid residues at either terminus of the charge polarized core domain may be about 50, about 45, about 40, about 35, about 30, about 25, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, or about 2 amino acids long.

In various embodiments, the protein comprising positively charged amino acid residues may include one or more of amino acids selected from His, Lys, and Arg. In various embodiments, the protein comprising negatively charged amino acid residues may include one or more amino acids selected from Asp and Glu.

In various embodiments, each of the first and/or second charge polarized core domains may comprise a protein comprising an amino acid sequence as provided in the Table below or an amino acid sequence having at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto.

SEQ ID NO.	Sequence
1	$Y_nX_nY_nX_nY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine)
2	$Y_nZ_nY_nZ_nY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine)

3	$YY_nXX_nYY_nXX_nYY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine)
4	$YY_nZZ_nYY_nZZ_nYY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine)
5	$Y_nX_nCY_nX_nY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine)
6	$Y_nZ_nCY_nZ_nY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine)
7	GSGSRKGGKRGS
8	GSGSRKCGKRGS
9	GSGSDEGGEDGS
10	GSGSDECGEDGS

For example, in an embodiment, each of the first and second charge polarized core domains may comprise a peptide comprising the sequence $YY_nXX_nYY_nXX_nYY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine; SEQ ID NO: 3). Illustrative peptide sequences include, but are not limited to, RKGGKR (SEQ ID NO: 11) or GSGSRKGGKRGS (SEQ ID NO: 12).

In another illustrative embodiment, each of the first and second charge polarized core domains may comprise a peptide comprising the sequence $YY_nZZ_nYY_nZZ_nYY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine). Illustrative peptide sequences include, but are not limited to, DEGGED (SEQ ID NO: 13) or GSGSDEGGEDGS (SEQ ID NO: 14).

In one aspect, the present technology provides a heterodimeric protein comprising (a) a first domain comprising one or more butyrophilin family proteins, or a fragment thereof; (b) a second domain comprising a targeting domain, the targeting domain being selected from an (i) antibody, antibody-like molecule, or antigen binding fragment thereof, and (ii) a extracellular domain; and (c) a linker that adjoins the first and second domain. In some embodiments, the heterodimeric protein comprises two individual polypeptide chains which self-associate. In some embodiments, the linker facilitates heterodimerization. In some embodiments, the heterodimeric protein comprises two of the same butyrophilin family proteins or two different butyrophilin family proteins. In some embodiments, the butyrophilin family proteins comprise a V-type domain and/or a B30.2 domain. In some embodiments, the first domain is a butyrophilin-like (BTNL) family protein, such as BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT.

Illustrative sequences of linkers that adjoins the first and second domain, also referred to herein as a core domain are provided below:

In some embodiments, the core domain has the following sequence:

SKYGPP**CPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQF NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKG LPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYT QKSLSLSLGK**IEGRMD (SEQ ID NO: 15).

In some embodiments, the core domain has the following sequence:

CPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVD GVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEK TISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSL SLGK (SEQ ID NO: 61).

In some embodiments, the core domain is a KIHT22Y protein having the following sequence:

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLYCLVKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS LSPGK (SEQ ID NO: 62).

In some embodiments, the core domain is a KIHY86T protein having the following sequence:

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLTSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK (SEQ ID NO: 63).

In some embodiments, the core domain is a KIHY86T protein having the following sequence:

VPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFV DDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTIS KTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQWNGQPAENYKN TQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSPGI (SEQ ID NO: 64).

The sequence of an illustrative charge polarized core domain (positive – negative) is provided below:

GSGSRKGGKRGSKYGPP***CPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKC***

KVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHY TQKSLSLSLGKDEGGEDGSGS (SEQ ID NO: 16).

The sequence of an illustrative charge polarized core domain (negative - positive) is provided below:

GSGSDEGGEDGSKYGPP***CPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKC KVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHY TQKSLSLSLGK***RKGGKRGSGS (SEQ ID NO: 17).

The sequence of an illustrative charge polarized core domain (negative - positive) is provided below:

CPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGV EVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNAT GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGK (SEQ ID NO: 65).

In various embodiments, the protein comprising the charged amino acid residues may further comprise one or more cysteine residues to facilitate disulfide bonding between the electrostatically charged core domains as an additional method to stabilize the heterodimer.

In various embodiments, each of the first and second charge polarized core domains comprises a linker sequence which may optionally function as a stabilizing domain. In various embodiments, the linker may be derived from naturally-occurring multi-domain proteins or are empirical linkers as described, for example, in Chichili *et al.*, (2013), Protein Sci. 22(2):153-167, Chen *et al.*, (2013), Adv Drug Deliv Rev. 65(10):1357-1369, the entire contents of which are hereby incorporated by reference. In some embodiments, the linker may be designed using linker designing databases and computer programs such as those described in Chen *et al.*, (2013), Adv Drug Deliv Rev. 65(10):1357-1369 and Crasto *et. al.*, (2000), Protein Eng. 13(5):309-312, the entire contents of which are hereby incorporated by reference.

In some embodiments, the linker (*e.g.*, a stabilizing domain) is a synthetic linker such as PEG.

In other embodiments, the linker (*e.g.*, a stabilizing domain) is a polypeptide. In some embodiments, the linker (*e.g.*, a stabilizing domain) is less than about 500 amino acids long, about 450 amino acids long, about 400 amino acids long, about 350 amino acids long, about 300 amino acids long, about 250 amino acids long, about 200 amino acids long, about 150

amino acids long, or about 100 amino acids long. For example, the linker (*e.g.*, a stabilizing domain) may be less than about 100, about 95, about 90, about 85, about 80, about 75, about 70, about 65, about 60, about 55, about 50, about 45, about 40, about 35, about 30, about 25, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, or about 2 amino acids long.

In various embodiments, the linker (*e.g.*, a stabilizing domain) is substantially comprised of glycine and serine residues (*e.g.,* about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 97% glycines and serines).

In various embodiments, the linker (*e.g.*, a stabilizing domain) is a hinge region of an antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.,* IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). The hinge region, found in IgG, IgA, IgD, and IgE class antibodies, acts as a flexible spacer, allowing the Fab portion to move freely in space. In contrast to the constant regions, the hinge domains are structurally diverse, varying in both sequence and length among immunoglobulin classes and subclasses. For example, the length and flexibility of the hinge region varies among the IgG subclasses. The hinge region of IgG1 encompasses amino acids 216-231 and, because it is freely flexible, the Fab fragments can rotate about their axes of symmetry and move within a sphere centered at the first of two inter-heavy chain disulfide bridges. IgG2 has a shorter hinge than IgG1, with 12 amino acid residues and four disulfide bridges. The hinge region of IgG2 lacks a glycine residue, is relatively short, and contains a rigid poly-proline double helix, stabilized by extra inter-heavy chain disulfide bridges. These properties restrict the flexibility of the IgG2 molecule. IgG3 differs from the other subclasses by its unique extended hinge region (about four times as long as the IgG1 hinge), containing 62 amino acids (including 21 prolines and 11 cysteines), forming an inflexible poly-proline double helix. In IgG3, the Fab fragments are relatively far away from the Fc fragment, giving the molecule a greater flexibility. The elongated hinge in IgG3 is also responsible for its higher molecular weight compared to the other subclasses. The hinge region of IgG4 is shorter than that of IgG1 and its flexibility is intermediate between that of IgG1 and IgG2. The flexibility of the hinge regions reportedly decreases in the order IgG3>IgG1>IgG4>IgG2. In other embodiments, the linker may be derived from human IgG4 and contain one or more mutations to enhance dimerization (including S228P) or FcRn binding.

According to crystallographic studies, the immunoglobulin hinge region can be further subdivided functionally into three regions: the upper hinge region, the core region, and the lower hinge region. *See* Shin *et al.*, 1992 *Immunological Reviews* 130:87. The upper hinge region includes amino acids from the carboxyl end of C_{H1} to the first residue in the hinge that restricts motion, generally the first cysteine residue that forms an interchain disulfide bond

between the two heavy chains. The length of the upper hinge region correlates with the segmental flexibility of the antibody. The core hinge region contains the inter-heavy chain disulfide bridges, and the lower hinge region joins the amino terminal end of the C_{H2} domain and includes residues in C_{H2}. *Id.* The core hinge region of wild-type human IgG1 contains the sequence Cys-Pro-Pro-Cys which, when dimerized by disulfide bond formation, results in a cyclic octapeptide believed to act as a pivot, thus conferring flexibility. In various embodiments, the present linker (*e.g.*, a stabilizing domain) comprises, one, or two, or three of the upper hinge region, the core region, and the lower hinge region of any antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.,* IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). The hinge region may also contain one or more glycosylation sites, which include a number of structurally distinct types of sites for carbohydrate attachment. For example, IgA1 contains five glycosylation sites within a 17-amino-acid segment of the hinge region, conferring resistance of the hinge region polypeptide to intestinal proteases, considered an advantageous property for a secretory immunoglobulin. In various embodiments, the linker (*e.g.*, a stabilizing domain) of the present technology comprises one or more glycosylation sites.

In various embodiments, the linker (*e.g.*, a stabilizing domain) comprises an Fc domain of an antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.,* IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). In various embodiments, the linker (*e.g.*, a stabilizing domain) comprises a hinge-CH2-CH3 Fc domain derived from a human IgG4 antibody. In various embodiments, the linker (*e.g.*, a stabilizing domain) comprises a hinge-CH2-CH3 Fc domain derived from a human IgG1 antibody. In some embodiments, the Fc domain exhibits increased affinity for and enhanced binding to the neonatal Fc receptor (FcRn). In some embodiments, the Fc domain includes one or more mutations that increases the affinity and enhances binding to FcRn. Without wishing to be bound by theory, it is believed that increased affinity and enhanced binding to FcRn increases the *in vivo* half-life of the present heterodimeric proteins.

In some embodiments, the Fc domain contains one or more amino acid substitutions at amino acid residue 250, 252, 254, 256, 308, 309, 311, 428, 433 or 434 (in accordance with Kabat numbering), or equivalents thereof. In an embodiment, the amino acid substitution at amino acid residue 250 is a substitution with glutamine. In an embodiment, the amino acid substitution at amino acid residue 252 is a substitution with tyrosine, phenylalanine, tryptophan or threonine. In an embodiment, the amino acid substitution at amino acid residue 254 is a substitution with threonine. In an embodiment, the amino acid substitution at amino acid residue 256 is a substitution with serine, arginine, glutamine, glutamic acid, aspartic acid, or threonine. In an embodiment, the amino acid substitution at amino acid residue 308 is a substitution with threonine. In an embodiment, the amino acid substitution at amino acid residue 309 is a substitution with proline. In an embodiment, the amino acid substitution at

amino acid residue 311 is a substitution with serine. In an embodiment, the amino acid substitution at amino acid residue 385 is a substitution with arginine, aspartic acid, serine, threonine, histidine, lysine, alanine or glycine. In an embodiment, the amino acid substitution at amino acid residue 386 is a substitution with threonine, proline, aspartic acid, serine, lysine, arginine, isoleucine, or methionine. In an embodiment, the amino acid substitution at amino acid residue 387 is a substitution with arginine, proline, histidine, serine, threonine, or alanine. In an embodiment, the amino acid substitution at amino acid residue 389 is a substitution with proline, serine or asparagine. In an embodiment, the amino acid substitution at amino acid residue 428 is a substitution with leucine. In an embodiment, the amino acid substitution at amino acid residue 433 is a substitution with arginine, serine, isoleucine, proline, or glutamine. In an embodiment, the amino acid substitution at amino acid residue 434 is a substitution with histidine, phenylalanine, or tyrosine.

In some embodiments, the Fc domain (*e.g.*, comprising an IgG constant region) comprises one or more mutations such as substitutions at amino acid residue 252, 254, 256, 433, 434, or 436 (in accordance with Kabat numbering). In an embodiment, the IgG constant region includes a triple M252Y/S254T/T256E mutation or YTE mutation. In another embodiment, the IgG constant region includes a triple H433K/N434F/Y436H mutation or KFH mutation. In a further embodiment, the IgG constant region includes an YTE and KFH mutation in combination.

In some embodiments, the modified humanized antibodies of the invention comprise an IgG constant region that contains one or more mutations at amino acid residues 250, 253, 307, 310, 380, 428, 433, 434, and 435. Illustrative mutations include T250Q, M428L, T307A, E380A, I253A, H310A, M428L, H433K, N434A, N434F, N434S, and H435A. In an embodiment, the IgG constant region comprises a M428L/N434S mutation or LS mutation. In another embodiment, the IgG constant region comprises a T250Q/M428L mutation or QL mutation. In another embodiment, the IgG constant region comprises an N434A mutation. In another embodiment, the IgG constant region comprises a T307A/E380A/N434A mutation or AAA mutation. In another embodiment, the IgG constant region comprises an I253A/H310A/H435A mutation or IHH mutation. In another embodiment, the IgG constant region comprises a H433K/N434F mutation. In another embodiment, the IgG constant region comprises a M252Y/S254T/T256E and a H433K/N434F mutation in combination.

In various embodiments, mutations are introduced to increase stability and/or half-life of the Fc domain. An illustrative Fc stabilizing mutant is S228P. Additional illustrative Fc half-life extending mutants are T250Q, M428L, V308T, L309P, and Q311S and the present linkers (*e.g.*, stabilizing domains) may comprise 1, or 2, or 3, or 4, or 5 of these mutants.

Additional illustrative mutations in the IgG constant region are described, for example, in Robbie, *et al.*, Antimicrobial Agents and Chemotherapy (2013), 57(12):6147-6153, Dall'Acqua *et al.*, JBC (2006), 281(33):23514-24, Dall'Acqua *et al.*, Journal of Immunology (2002), 169:5171-80, Ko *et al.,* Nature (2014) 514:642-645, Grevys *et al.,* Journal of Immunology. (2015), 194(11):5497-508, and U.S. Patent No. 7,083,784, the entire contents of which are hereby incorporated by reference.

In various embodiments, the linker may be flexible, including without limitation highly flexible. In various embodiments, the linker may be rigid, including without limitation a rigid alpha helix.

In various embodiments, the linker may be functional. For example, without limitation, the linker may function to improve the folding and/or stability, improve the expression, improve the pharmacokinetics, and/or improve the bioactivity of the present heterodimeric protein. In another example, the linker may function to target the heterodimeric protein to a particular cell type or location.

The Heterodimeric Proteins

In one aspect, the present technology provides a heterodimeric protein comprising: (a) a first domain comprising one or more butyrophilin family proteins, or a fragment thereof; (b) a second domain comprising a targeting domain, the targeting domain being selected from an (i) antibody, antibody-like molecule, or antigen binding fragment thereof, and (ii) a extracellular domain; and (c) a linker that adjoins the first and second domain.

In embodiments the heterodimeric protein is a complex of two polypeptide chains.

In embodiments the heterodimeric protein comprises an alpha chain and a beta chain wherein the alpha chain and the beta chain each independently comprise (a) a first domain comprising a butyrophilin family protein, or fragment thereof; (b) a second domain comprising a targeting domain, the targeting domain being selected from an (i) antibody, antibody-like molecule, or antigen binding fragment thereof, and (ii) a extracellular domain; and (c) a linker that adjoins the first and second domain.

In embodiments the alpha chain and the beta chain self-associate to form the heterodimer.

In some embodiments, the first domain comprises two of the same butyrophilin family proteins. In some embodiments, wherein the first domain comprises two different butyrophilin family proteins. In some embodiments, the butyrophilin family proteins comprise a V-type domain. In some embodiments, the butyrophilin family proteins or fragments thereof are derived from the native butyrophilin family proteins that comprise a B30.2 domain in the cytosolic tail.

In some embodiments, the butyrophilin family proteins are selected from BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT. In some embodiments, the first domain comprises: (a) any one of BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT; and (b) any one of BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT. In some embodiments, the first domain comprises: (a) any one of human BTNL1, human BTN1A1, human BTNL2, human BTN2A1, human BTN2A2, human BTN2A3, human BTNL3, human BTN3A1, human BTN3A2, human BTN3A3, human BTNL6, human BTNL8, human BTNL9, human BTNL10, and human SKINT, and (b) any one of human BTNL1, human BTN1A1, human BTNL2, human BTN2A1, human BTN2A2, human BTN2A3, human BTNL3, human BTN3A1, human BTN3A2, human BTN3A3, human BTNL6, human BTNL8, human BTNL9, human BTNL10, and human SKINT.

In some embodiments, the first domain comprises a fragment of butyrophilin family proteins, wherein the fragment is capable of binding a gamma delta T cell receptor and is optionally an extracellular domain, optionally comprising one or more of an immunoglobulin V (IgV)- and IgC-like domain. In some embodiments, the first domain comprises a fragment of butyrophilin family proteins, wherein the fragment is capable of binding a gamma delta T cell receptor selected from a Vγ4, Vγ9δ2, or Vγ7δ4 TCR.

In some embodiments, the first domain comprises a polypeptide having an amino acid sequence of: (a) any one of SEQ ID NOs: 49-52; and (b) any one of SEQ ID NOs: 49-52. In some embodiments, the first domain comprises: (a) BTNL3 and BTNL8; (b) BTN3A1 and BTN3A2; (c) BTN3A1 and BTN3A3; or (d) BTNL1 and BTNL6. In some embodiments, the first domain comprises: (a) human BTNL3 and human BTNL8; (b) human BTN3A1 and human BTN3A2; or (c) human BTN3A1 and human BTN3A3. In some embodiments, the first domain comprises a polypeptide having (a) an amino acid sequence having at least 90%, or 95%, or 97%, or 98%, or 99% identity with SEQ ID NO: 49, and an amino acid sequence having at least 90%, or 95%, or 97%, or 98%, or 99% identity with SEQ ID NO: 52; or (b) an amino acid sequence having at least 90%, or 95%, or 97%, or 98%, or 99% identity with SEQ ID NO: 50, and an amino acid sequence having at least 90%, or 95%, or 97%, or 98%, or 99% identity with SEQ ID NO: 51. In some embodiments, the first domain comprises a polypeptide having an amino acid sequence of (a) SEQ ID NO: 49 and SEQ ID NO: 52; or (b) SEQ ID NO: 50 and SEQ ID NO: 51.

Additionally, or alternatively, in any of the embodiments disclosed herein, in the targeting domain is an antibody, or antigen binding fragment thereof. In some embodiments, the

targeting domain is an antibody-like molecule, or antigen binding fragment thereof. In some embodiments, the antibody-like molecule is selected from a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), a DARPin; a Tetranectin; an Affibody; a Transbody; an Anticalin; an AdNectin; an Affilin; an Affimer, a Microbody; an aptamer; an alterase; a plastic antibody; a phylomer; a stradobody; a maxibody; an evibody; a fynomer, an armadillo repeat protein, a Kunitz domain, an avimer, an atrimer, a probody, an immunobody, a triomab, a troybody; a pepbody; a vaccibody, a UniBody; a DuoBody, a Fv, a Fab, a Fab′, and a $F(ab')_2$. In some embodiments, the antibody-like molecule is an scFv. In some embodiments, the targeting domain is an extracellular domain. In some embodiments, the targeting domain is capable of binding an antigen on the surface of a cancer cell. In some embodiments, the targeting domain specifically binds one of CD19, PSMA, GD2, PSCA, BCMA, CD123, B7-H3, CD20, CD30, CD33, CD38, CEA, CLEC12A, DLL3, EGFRvIII, EpCAM, CD307, FLT3, GPC3, gpA33, HER2, MUC16, P-cadherin, SSTR2, and mesothelin. In some embodiments, the targeting domain comprises a portion of the extracellular domain of LAG-3, PD-1, TIGIT, CD19, or PSMA. In some embodiments, the targeting domain specifically binds CD19. In some embodiments, the targeting domain specifically binds PSMA. Additionally or alternatively, in some embodiments, the targeting domain is a polypeptide having an amino acid sequence with at least 90%, or 95%, or 97%, or 98%, or 99% identity with a polypeptide selected from SEQ ID NOs: 53-60. In some embodiments, the targeting domain is a polypeptide having an amino acid sequence of selected from SEQ ID NOs: 53-60.

Additionally or alternatively, in some embodiments, the linker comprises (a) a first charge polarized core domain adjoined to a butyrophilin family protein, optionally at the carboxy terminus, and (b) a second charge polarized core domain adjoined to a butyrophilin family protein, optionally at the carboxy terminus. In some embodiments, the linker forms a heterodimer through electrostatic interactions between positively charged amino acid residues and negatively charged amino acid residues on the first and second charge polarized core domains. In some embodiments, the first and/or second charge polarized core domain comprises a polypeptide linker, optionally selected from a flexible amino acid sequence, IgG hinge region, or antibody sequence. In some embodiments, the linker is a synthetic linker, optionally PEG. In some embodiments, the linker comprises the hinge-CH2-CH3 Fc domain derived from IgG1, optionally human IgG1. In some embodiments, the linker comprises the hinge-CH2-CH3 Fc domain derived from IgG4, optionally human IgG4. In some embodiments, the first and/or second charge polarized core domain further comprise peptides having positively and/or negatively charged amino acid residues at the amino and/or carboxy

terminus of the charge polarized core domain. In some embodiments, the positively charged amino acid residues include one or more of amino acids selected from His, Lys, and Arg. In some embodiments, the positively charged amino acid residues are present in a peptide comprising positively charged amino acid residues in the first and/or the second charge polarized core domains.

In some embodiments, the peptide comprising positively charged amino acid residues comprises a sequence selected from $Y_nX_nY_nX_nY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 1), $YY_nXX_nYY_nXX_nYY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 3), and $Y_nX_nCY_nX_nY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 5). In some embodiments, the peptide comprising positively charged amino acid residues comprises the sequence RKGGKR (SEQ ID NO: 11) or GSGSRKGGKRGS (SEQ ID NO: 12). In some embodiments, the negatively charged amino acid residues may include one or more amino acids selected from Asp and Glu. In some embodiments, the negatively charged amino acid residues are present in a peptide comprising negatively charged amino acid residues in the first and/or the second charge polarized core domains. In some embodiments, the peptide comprising negatively charged amino acid residues comprises a sequence selected from $Y_nZ_nY_nZ_nY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 2), $YY_nZZ_nYY_nZZ_nYY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 4), and $Y_nZ_nCY_nZ_nY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 6). In some embodiments, the peptide comprising negatively charged amino acid residues comprises the sequence DEGGED (SEQ ID NO: 13) or GSGSDEGGEDGS (SEQ ID NO: 14).

Additionally or alternatively, in some embodiments, the first domain and/or the heterodimeric protein modulates or is capable of modulating a γδ (gamma delta) T cell. In some embodiments, the gamma delta T cell is selected from a cell expressing Vγ4, Vγ9δ2, or Vγ7δ4. In some embodiments, the first domain comprises BTNL3 and BTNL8 and it modulates a Vγ4-expressing T cell. In some embodiments, the first domain comprises BTNL3A1 and BTNL3A2 and it modulates a Vγ9δ2-expressing T cell. In some embodiments, the first domain comprises BTNL3A1 and BTNLA3 and it modulates a Vγ9δ2-expressing T cell. In some embodiments, the first domain comprises BTNL1 and BTNL6 and it modulates a Vγ7δ4-

expressing T cell. In some embodiments, the modulation of a gamma delta T cell is activation of a gamma delta T cell.

Additionally or alternatively, in some embodiments, the heterodimeric protein is capable of forming a synapse between a gamma delta T cell and a tumor cell. In some embodiments, the heterodimeric protein is capable of contemporaneous activation and targeting of gamma delta T cells to tumor cells.

In some embodiments, the heterodimeric protein comprises an amino acid sequence having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 98%, or at least 99% sequence identity to one or more of SEQ ID NOs: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32. SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, each optionally with a leader sequence omitted.

In embodiments, the present heterodimeric proteins comprise a portion of a butyrophilin-like (BTNL) proteins. In an illustrative embodiment, the first domain is a butyrophilin-like (BTNL) family protein. Examples of BTNL family proteins include BTNL1, BTNL3, BTNL6, BTNL8, BTN3A1, BTN3A2, and BTN3A3. In embodiments, the heterotrimeric protein modulates the function of gamma delta T cells. In embodiments, in addition to the BTNL family protein, the heterodimeric proteins further comprise a portion of the extracellular domain of LAG-3, PD-1, or TIGIT and which is capable of binding its receptor/ligand on the surface of a cancer cell. In embodiments, in addition to the BTNL family protein, the heterodimeric proteins further comprise an antibody or fragment thereof (*e.g.,* comprising a portion of the antigen-binding domain of an antibody and/or a CDR3 that binds a tumor epitope) and which is capable of binding an antigen on the surface of a cancer cell.

In embodiments, the present heterodimeric proteins comprise a portion of a butyrophilin-like (BTNL) proteins. In an illustrative embodiment, the first domain is a butyrophilin-like (BTNL) family protein. Examples of BTNL family proteins include BTNL1, BTNL3, BTNL6, BTNL8, BTN3A1, BTN3A2, and BTN3A3. In embodiments, the heterotrimeric protein modulates the function of gamma delta T cells. In embodiments, in addition to the BTNL family protein, the

heterodimeric proteins further comprise a portion of the extracellular domain of LAG-3, PD-1, TIGIT, CD19, PSMA, or antibody-derived binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*) targeting a tumor antigen (such as CD19 or PSMA) and which is capable of binding its receptor/ligand on the surface of a cancer cell. In embodiments, in addition to the BTNL family protein, the heterodimeric proteins further comprise an antibody or fragment thereof (*e.g.,* comprising a portion of the antigen-binding domain of an antibody) and which is capable of binding an antigen on the surface of a cancer cell.

In embodiments, the second domain is a LAG-3 protein.

In embodiments, the second domain is a PD-1 protein.

In embodiments, the second domain is a TIGIT protein.

In embodiments, the second domain is a CD19 protein binding domain, such as an scFv, CDR3, or Fab. In embodiments, the second domain is a CD19 protein and the heterodimeric protein further comprise an antibody or fragment thereof (*e.g.,* comprising a portion of the antigen-binding domain of an antibody) and which is capable of binding an antigen on the surface of a cancer cell.

In embodiments, the second domain is a PSMA protein binding domain, such as an scFv, CDR3, or Fab.In embodiments, the second domain is a PSMA protein and the heterodimeric protein further comprise an antibody or fragment thereof (*e.g.,* comprising a portion of the antigen-binding domain of an antibody) and which is capable of binding an antigen on the surface of a cancer cell.

In an illustrative embodiment, the second domain is a receptor for EGP such as EGFR (ErbB1), ErbB2, ErbB3 and ErbB4.

In an illustrative embodiment, the second domain is a receptor for insulin or an insulin analog such as the insulin receptor and/or IGF1 or IGF2 receptor.

In an illustrative embodiment, the second domain is a receptor for EPO such as the EPO receptor (EPOR) receptor and/or the ephrin receptor (EphR)

In various embodiments, the heterodimeric protein may comprise a domain of a soluble (*e.g.,* non-membrane associated) protein. In various embodiments, the heterodimeric protein may comprise a fragment of the soluble protein which is involved in signaling (*e.g.,* a portion of the soluble protein which interacts with a receptor).

In various embodiments, the heterodimeric protein may comprise the extracellular domain of a transmembrane protein. In various embodiments, one of the extracellular domains transduces

an immune inhibitory signal and one of the extracellular domains transduces an immune stimulatory signal.

In some embodiments, an extracellular domain refers to a portion of a transmembrane protein which is capable of interacting with the extracellular environment. In various embodiments, an extracellular domain refers to a portion of a transmembrane protein which is sufficient to bind to a ligand or receptor and effective transmit a signal to a cell. In various embodiments, an extracellular domain is the entire amino acid sequence of a transmembrane protein which is external of a cell or the cell membrane. In various embodiments, an extracellular domain is the that portion of an amino acid sequence of a transmembrane protein which is external of a cell or the cell membrane and is needed for signal transduction and/or ligand binding as may be assayed using methods know in the art (*e.g., in vitro* ligand binding and/or cellular activation assays).

In various embodiments, the heterodimeric protein may comprise an antibody binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*). In various embodiments, one of the antibody binding domains transduces an immune inhibitory signal and one of the antibody binding domains transduces an immune stimulatory signal.

In some embodiments, an immune inhibitory signal refers to a signal that diminishes or eliminates an immune response. For example, in the context of oncology, such signals may diminish or eliminate antitumor immunity. Under normal physiological conditions, inhibitory signals are useful in the maintenance of self-tolerance (*e.g.,* prevention of autoimmunity) and also to protect tissues from damage when the immune system is responding to pathogenic infection. For instance, without limitation, immune inhibitory signal may be identified by detecting an increase in cellular proliferation, cytokine production, cell killing activity or phagocytic activity when such an inhibitory signal is blocked.

In some embodiments, an immune stimulatory signal refers to a signal that enhances an immune response. For example, in the context of oncology, such signals may enhance antitumor immunity. For instance, without limitation, immune stimulatory signal may be identified by directly stimulating proliferation, cytokine production, killing activity or phagocytic activity of leukocytes. Specific examples include direct stimulation of cytokine receptors such as IL-2R, IL-7R, IL-15R, IL-17R or IL-21R using fusion proteins encoding the ligands for such receptors (IL-2, IL-7, IL-15, IL-17 or IL-21, respectively). Stimulation from any one of these receptors may directly stimulate the proliferation and cytokine production of individual T cell subsets.

In some embodiments, the extracellular domain or antibody binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*) may be used to produce a soluble protein to competitively inhibit signaling by that receptor's ligand. For instance, without limitation, competitive inhibition of PD-L1 or

PD-L2 could be achieved using PD-1, or competitive inhibition of PVR could be achieved using TIGIT. In some embodiments, the extracellular domain or antibody binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*) may be used to provide artificial signaling.

In some embodiments, the present heterodimeric proteins deliver or mask an immune inhibitory signal. In some embodiments, the present heterodimeric proteins deliver or mask an immune stimulatory signal.

In various embodiments, the present heterodimeric proteins comprise two independent binding domains, each from one subunit of a heterodimeric human protein. Illustrative proteins that may be formed as part of the heterodimeric protein of the invention are provided in Table 1. In various embodiments, the present heterodimeric proteins have one of the illustrative proteins provided in Table 1. In various embodiments, the present heterodimeric proteins have two of the illustrative proteins provided in Table 1.

TABLE 1

Illustrative butyrophilin-like (BTNL) family protein which may be incorporated into the present compositions and methods include the following proteins (as used herein, "Entry" refers to the protein entry in the Uniprot database and "Entry name" refers to the protein entry in the Uniprot database):

Entry/ Name	**Protein names Gene names**	**ECD Sequence**	**SEQ ID NO**
Q13410 BT1A1_HUMAN	Butyrophilin subfamily 1 member A1 Butyrophilin subfamily 1 member A1; BTN1A1 BTN	APFDVIGPPEPILAVVGEDAELPCRLSPNASAEHL ELRWFRKKVSPAVLVHRDGREQEAEQMPEYRGR ATLVQDGIAKGRVALRIRGVRVSDDGEYTCFFRE DGSYEEALVHLKVAALGSDPHISMQVQENGEICL ECTSVGWYPEPQVQWRTSKGEKFPSTSESRNPDE EGLFTVAASVIIRDTSAKNVSCYIQNLLLGQEKKV EISIPASSLPR	99
Q13410 BT1A1_HUMAN	Butyrophilin subfamily 1 member A1 BTN1A1 BTN	APFDVIGPPEPILAVVGEDAELPCRLSPNASAEHL ELRWFRKKVSPAVLVHRDGREQEAEQMPEYRGR ATLVQDGIAKGRVALRIRGVRVSDDGEYTCFFRE DGSYEEALVHLKVAALGSDPHISMQVQENGEICL ECTSVGWYPEPQVQWRTSKGEKFPSTSESRNPDE	100

		EGLFTVAASVIIRDTSAKNVSCYIQNLLLGQEKKV EISIPASSLP	
Q4VAN1Q4VAN1_HUMAN	BTN1A1 protein BTN1A1		
Q4VAN2 Q4VAN2_HUMAN	Butyrophilin, subfamily 1, member A. BTN1A1		
Q9UIR0 BTNL2_HUMAN	Butyrophilin-like protein 2 BTNL2	KQSEDFRVIGPAHPILAGVGEDALLTCQLLPKRTT MHVEVRWYRSEPSTPVFVHRDGVEVTEMQMEE YRGWVEWIENGIAKGNVALKIHNIQPSDNGQYW CHFQDGNYCGETSLLLKVAGLGSAPSIHMEGPGE SGVQLVCTARGWFPEPQVYWEDIRGEKLLAVSE HRIQDKDGLFYAEATLVVRNASAESVSCLVHNPV LTEEKGSVISLPEKLQTELASLKVNGPSQPILVRV GEDIQLTCYLSPKANAQSMEVRWDRSHRYPAVH VYMDGDHVAGEQMAEYRGRTVLVSDAIDEGRLT LQILSARPSDDGQYRCLFEKDDVYQEASLDLKV VSLGSSPLITVEGQEDGEMQPMCSSDGWFPQPHV PWRDMEGKTIPSSSQALTQGSHGLFHVQTLLRVT NISAVDVTCSISIPFLGEEKIATFSLSGW	101
F8WBA1 F8WBA1_HUMAN	Butyrophilin-like protein 2 BTNL2		
F6UPS5	Butyrophilin-like protein 2		

F6UPS5_HUMAN	BTNL2		
F8WDK6 F8WDK6_HUMAN	Butyrophilin-like protein 2 BTNL2		
A0A0G2JJ84 A0A0G2JJ84_HUMAN	Butyrophilin-like protein 2 BTNL2		
X5D146 X5D146_HUMAN	BTNL2 BTNL2		
A0A0G2JPB7 A0A0G2JPB7_HUMAN	BTNL2		
A0PJV4 A0PJV4_HUMAN	BTNL2 protein BTNL2		

I7HPB5 I7HPB5_HUMAN	Butyrophilin-like 2 (MHC class II a... BTNL2 RP5-1077I5.2-002		
A0A1U9X7B7 A0A1U9X7B7_HUMAN	BTNL2		
X5CF33 X5CF33_HUMAN	BTNL2 BTNL2 hCG_43715		
A0A1U9X7C0 A0A1U9X7C0_HUMAN	BTNL2		
A0A1U9X7C3 A0A1U9X7C3_H	Truncated BTNL2		

UMAN			
A0A1U9X7C4 A0A1U9X7C4_HUMAN	Truncated BTNL2		
Q7KYR7 BT2A1_HUMAN	Butyrophilin subfamily 2 member A1 BTN2A1 BT2.1, BTF1	QFIVVGPTDPILATVGENTTLRCHLSPEKNAEDM EVRWFRSQFSPAVFVYKGGRERTEEQMEEYRGRT TFVSKDISRGSVALVIHNITAQENGTYRCYFQEGR SYDEAILHLVVAGLGSKPLISMRGHEDGGIRLECI SRGWYPKPLTVWRDPYGGVAPALKEVSMPDAD GLFMVTTAVIIRDKSVRNMSCSINNTLLGQKKES VIFIPESFMPSVSPCA	102
H7BYC3 H7BYC3_HUMAN	Butyrophilin subfamily 2 member A1 BTN2A1		
H7C542 H7C542_HUMAN	Butyrophilin subfamily 2 member A1 BTN2A1		
C9JNC3 C9JNC3_HUMAN	Butyrophilin subfamily 2 member A1 BTN2A1		

Q8WVV5 BT2A2_HUMAN	Butyrophilin subfamily 2 member A2 BTN2A2 BT2.2, BTF2	QFTVVGPANPILAMVGENTTLRCHLSPEKNAEDMEVRWFRSQFSPAVFVYKGGRERTEEQMEEYRGRITFVSKDINRGSVALVIHNVTAQENGIYRCYFQEGRSYDEAILRLVVAGLGSKPLIEIKAQEDGSIWLECISGGWYPEPLTVWRDPYGEVVPALKEVSIADADGLFMVTTAVIIRDKYVRNVSCSVNNTLLGQEKETVIFIPESFMPSASPWMVALAVILTASPWM	103
A0A024R038 A0A024R038_HUMAN	Butyrophilin, subfamily 2, member A... BTN2A2 hCG_1980289		
C9J8J5 C9J8J5_HUMAN	Butyrophilin subfamily 2 member A2 BTN2A2		
C9IZY2 C9IZY2_HUMAN	Butyrophilin subfamily 2 member A2 BTN2A2		
B4E3J1 B4E3J1_HUMAN	cDNA FLJ52852, highly similar to Ho...		
C9IY66 C9IY66_HUMAN	Butyrophilin subfamily 2 member A2 BTN2A2		

C9J8R3 C9J8R3_HUMAN	Butyrophilin subfamily 2 member A2 BTN2A2		
C9JAJ6 C9JAJ6_HUMAN	Butyrophilin subfamily 2 member A2 BTN2A2		
C9JWH2 C9JWH2_HUMAN	Butyrophilin subfamily 2 member A2 BTN2A2		
H7C4E8 H7C4E8_HUMAN	Butyrophilin subfamily 2 member A2 BTN2A2		
F8WC65 F8WC65_HUMAN	Butyrophilin subfamily 2 member A2 BTN2A2		
Q96KV6 BT2A3_HUMAN	Putative butyrophilin subfamily 2 m...	QVTVVGPTDPILAMVGENTTLRCCLSPEENAED MEVRWFQSQFSPAVFVYKGGRERTEEQKEEYRG RTTFVSKDSRGSVALIIHNVTAEDNGIYQCYFQEG RSCNEAILHLVVAGLDSEPVIEMRDHEDGGIQLEC ISGGWYPKPLTVWRDPYGEVVPALKEVSTPDAD	104

	BTN2A3P **BT** N2A3	SLFMVTTAVIIRDKSVRNVSCSINDTLLGQKKESV IFIPESFMPSRSPCV	
Q6UX E8 BTNL 3_HU MAN	Butyrophilin-like protein 3 BTNL3 BTN LR, COLF4100, UNQ744/PRO 1472	QWQVTGPGKFVQALVGEDAVFSCSLFPETSAEA MEVRFFRNQFHAVVHLYRDGEDWESKQMPQYR GRTEFVKDSIAGGRVSLRLKNITPSDIGLYGCWFS SQIYDEEATWELRVAALGSLPLISIVGYVDGGIQL LCLSSGWFPQPTAKWKGPQGQDLSSDSRANADG YSLYDVEISIIVQENAGSILCSIHLAEQSHEVESKV LIGETFFQPSPWRLAS	105
L8EA U7 L8EA U7_H UMA N	Alternative protein BTNL3 BTNL3		
O0048 1 BT3A 1_HU MAN	Butyrophilin subfamily 3 member A1 BTN3A1 BTF 5	QFSVLGPSGPILAMVGEDADLPCHLFPTMSAETM ELKWVSSSLRQVVNVYADGKEVEDRQSAPYRGR TSILRDGITAGKAALRIHNVTASDSGKYLCYFQD GDFYEKALVELKVAALGSDLHVDVKGYKDGGIH LECRSTGWYPQPQIQWSNNKGENIPTVEAPVVAD GVGLYAVAASVIMRGSSGEGVSCTIRSSLLGLEKT ASISIADPFFRSAQRWIAALAG	106
E7EPR 2 E7EPR 2_HU MAN	Butyrophilin subfamily 3 member A1 BTN3A1		
E9PFB 8 E9PFB 8_HU MAN	Butyrophilin subfamily 3 member A1 BTN3A1		

A6PVC0 A6PVC0_HUMAN	Butyrophilin subfamily 3 member A1 BTN3A1		
P78410 BT3A2_HUMAN	Butyrophilin subfamily 3 member A2 BTN3A2 BT3.2, BTF3, BTF4	QFSVLGPSGPILAMVGEDADLPCHLFPTMSAETMELKWVSSSLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAALRIHNVTASDSGKYLCYFQDGDFYEKALVELKVAALGSNLHVEVKGYEDGGIHLECRSTGWYPQPQIQWSNAKGENIPAVEAPVVADGVGLYEVAASVIMRGGSGEGVSCIIRNSLLGLEKTASISIADPFFRSAQPW	107
A0A024QZZ1 A0A024QZZ1_HUMAN	Butyrophilin, subfamily 3, member A... BTN3A2 hCG_17993		
S4R3N0 S4R3N0_HUMAN	Butyrophilin subfamily 3 member A2 BTN3A2		
E9PJE9 E9PJE9_HUMAN	Butyrophilin subfamily 3 member A2 BTN3A2		
E9PIU5	Butyrophilin subfamily 3 member A2 BTN3A2		

E9PIU5_HUMAN			
E9PRR1 E9PRR1_HUMAN	Butyrophilin subfamily 3 member A2 BTN3A2		
E9PRX1 E9PRX1_HUMAN	Butyrophilin subfamily 3 member A2 BTN3A2		
O00478 BT3A3_HUMAN	Butyrophilin subfamily 3 member A3 BTN3A3 BTF3	QFSVLGPSGPILAMVGEDADLPCHLFPTMSAETMELRWVSSSLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAALRIHNVTASDSGKYLCYFQDGDFYEKALVELKVAALGSDLHIEVKGYEDGGIHLECRSTGWYPQPQIKWSDTKGENIPAVEAPVVADGVGLYAVAASVIMRGSSGGGVSCIIRNSLLGLEKTASISIADPFFRSAQPW	108
A0A024R042 A0A024R042_HUMAN	Butyrophilin, subfamily 3, member A... BTN3A3 hCG_17992		
A0A089GIA6 A0A089GIA6	Butyrophilin subfamily 3 member A3 ... BTN3A3		

_HUMAN			
C9JUV8 C9JUV8_HUMAN	Butyrophilin subfamily 3 member A3 BTN3A3		
C9JQT8 C9JQT8_HUMAN	Butyrophilin subfamily 3 member A3 BTN3A3		
C9JVU4 C9JVU4_HUMAN	Butyrophilin subfamily 3 member A3 BTN3A3		
C9J3Q8 C9J3Q8_HUMAN	Butyrophilin subfamily 3 member A3 BTN3A3		
C9JZT5 C9JZT5_HUMAN	Butyrophilin subfamily 3 member A3 BTN3A3		
C9J877	Butyrophilin subfamily 3 member A3		

C9J877_HUMAN	BTN3A3		
C9JNZ3 C9JNZ3_HUMAN	Butyrophilin subfamily 3 member A3 BTN3A3		
Q6UX41 BTNL8_HUMAN	Butyrophilin-like protein 8 BTNL8 UNQ702/PRO1347	QWQVFGPDKPVQALVGEDAAFSCFLSPKTNAEA MEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQ GRTKLVKDSIAEGRISLRLENITVLDAGLYGCRISS QSYYQKAIWELQVSALGSVPLISITGYVDRDIQLL CQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMH GLFDVEISLTVQENAGSISCSMRHAHLSREVESRV QIGDTFFEPISWHLATK	109
D6RIR7 D6RIR7_HUMAN	Butyrophilin-like protein 8 BTNL8		
D6R9I8 D6R9I8_HUMAN	Butyrophilin-like protein 8 BTNL8		
Q6UXG8 BTNL9_HUMAN	Butyrophilin-like protein 9 BTNL9 UNQ1900/PRO4346	SSEVKVLGPEYPILALVGEEVEFPCHLWPQLDAQ QMEIRWFRSQTFNVVHLYQEQQELPGRQMPAFR NRTKLVKDDIAYGSVVLQLHSIIPSDKGTYGCRFH SDNFSGEALWELEVAGLGSDPHLSLEGFKEGGIQ LRLRSSGWYPKPKVQWRDHQGQCLPPEFEAIVW DAQDLFSLETSVVVRAGALSNVSVSIQNLLLSQK KELVVQIADVFVPGASAWK	110

A0A1S5UZ21 A0A1S5UZ21_HUMAN	Butyrophilin-like protein 9 BTNL9		
B7Z4Y8 B7Z4Y8_HUMAN	Butyrophilin-like protein 9 BTNL9		
Q8N324 Q8N324_HUMAN	BTNL9 protein BTNL9		
A8MVZ5 BTNLA_HUMAN	Butyrophilin-like protein 10 BTNL10	SIWKADFDVTGPHAPILAMAGGHVELQCQLFPNISAEDMELRWYRCQPSLAVHMHERGMDMDGEQKWQYRGRTTFMSDHVARGKAMVRSHRVTTFDNRTYCCRFKDGVKFGEATVQVQVAGLGREPRIQVTDQQDGVRAECTSAGCFPKSWVERRDFRGQARPAVTNLSASATTRLWAVASSLTLWDRAVEGLSCSISSPLLPERRKVAESHLPATFSRSSQFTAWKA	111

In various embodiments, the present heterodimeric proteins may be engineered to target one or more molecules that reside on human leukocytes including, without limitation, the extracellular domains (where applicable) of SLAMF4, IL-2Rα, IL-2 R β, ALCAM, B7-1, IL-4 R, B7-H3, BLAME/SLAMFS, CEACAM1, IL-6 R, IL-7 Rα, IL-10R α, IL-1 0 R β, IL-12 R β 1, IL-12 R β 2, CD2, IL-13 R α 1, IL-13, CD3, CD4, ILT2/CDS5j, ILT3/CDS5k, ILT4/CDS5d, ILT5/CDS5a, lutegrin α 4/CD49d, CDS, Integrin α E/CD103, CD6, Integrin α M/CD 11 b, CDS, Integrin α X/CD11c, Integrin β 2/CDlS, KIR/CD15S, KIR2DL1, CD2S, KIR2DL3, KIR2DL4/CD15Sd, CD31/PECAM-1, KIR2DS4, LAG-3, CD43, LAIR1, CD45, LAIR2,

CDS3, Leukotriene B4-R1, CDS4/SLAMF5, NCAM-L1, CD94, NKG2A, CD97, NKG2C, CD229/SLAMF3, NKG2D, CD2F-10/SLAMF9, NT-4, CD69, NTB-A/SLAMF6, Common γ Chain/IL-2 R γ, Osteopontin, CRACC/SLAMF7, PD-1, CRTAM, PSGL-1, CTLA-4, CX3CR1, CX3CL1, L-Selectin, SIRP β 1, SLAM, TCCR/WSX-1, DNAM-1, Thymopoietin, EMMPRIN/CD147, TIM-1, EphB6, TIM-2, TIM-3, TIM-4, Fcγ RIII/CD16, TIM-6, Granulysin, ICAM-1/CD54, ICAM-2/CD102, IFN-γR1, IFN-γ R2, TSLP, IL-1 R1 and TSLP R.

In some embodiments, the present heterodimeric proteins may be engineered to target one or more molecules involved in immune inhibition, including for example: CTLA-4, PD-L1, PD-L2, PD-1, BTLA, HVEM, TIM3, GAL9, LAG3, VISTA/VSIG8, KIR, 2B4, TIGIT, CD160 (also referred to as BY55), CHK 1 and CHK2 kinases, A2aR, CEACAM (*e.g.*, CEACAM-1, CEACAM-3 and/or CEACAM-5), and various B-7 family ligands (including, but are not limited to, B7-1, B7-2, B7-DC, B7-H1, B7-H2, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7).

In some embodiments, the present heterodimeric proteins comprise an extracellular domain of an immune inhibitory agent. In some embodiments, the present heterodimeric proteins comprise an antibody binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*) directed against an immune inhibitory agent.

In some embodiments, the present heterodimeric proteins comprise an extracellular domain of a soluble or membrane protein which has immune inhibitory properties. In some embodiments, the present heterodimeric proteins comprise an antibody binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*) which has immune inhibitory properties

In some embodiments, the present heterodimeric proteins simulate binding of an inhibitory signal ligand to its cognate receptor but inhibit the inhibitory signal transmission to an immune cell (*e.g.,* a T cell, macrophage or other leukocyte).

In various embodiments, the heterodimeric protein comprises an immune inhibitory receptor extracellular domain or antibody binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*) and an immune stimulatory ligand extracellular domain or antibody binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*) which can, without limitation, deliver an immune stimulation to a T cell while masking a tumor cell's immune inhibitory signals. In various embodiments, the heterodimeric protein delivers a signal that has the net result of T cell activation.

In some embodiments, the present heterodimeric proteins comprise an extracellular domain of a soluble or membrane protein which has immune stimulatory properties. In some embodiments, the present heterodimeric proteins comprise an antibody binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*) which has immune stimulatory properties.

In various embodiments, the present heterodimeric protein may comprise variants of any of the known cytokines, growth factors, and/or hormones. In various embodiments, the present heterodimeric proteins may comprise variants of any of the known receptors for cytokines, growth factors, and/or hormones. In various embodiments, the present heterodimeric proteins may comprises variants of any of the known extracellular domains, for instance, a sequence having at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99%) sequence identity with the known amino acid or nucleic acid sequences.

In various embodiments, the present heterodimeric protein may comprise an amino acid sequence having one or more amino acid mutations relative to any of the known protein sequences. In some embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations.

In some embodiments, the amino acid mutations are amino acid substitutions, and may include conservative and/or non-conservative substitutions.

"Conservative substitutions" may be made, for instance, on the basis of similarity in polarity, charge, size, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the amino acid residues involved. The 20 naturally occurring amino acids can be grouped into the following six standard amino acid groups: (1) hydrophobic: Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr; Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; and (6) aromatic: Trp, Tyr, Phe.

As used herein, "conservative substitutions" are defined as exchanges of an amino acid by another amino acid listed within the same group of the six standard amino acid groups shown above. For example, the exchange of Asp by Glu retains one negative charge in the so modified polypeptide. In addition, glycine and proline may be substituted for one another based on their ability to disrupt α-helices.

As used herein, "non-conservative substitutions" are defined as exchanges of an amino acid by another amino acid listed in a different group of the six standard amino acid groups (1) to (6) shown above.

In various embodiments, the substitutions may also include non-classical amino acids (*e.g.,* selenocysteine, pyrrolysine, *N*-formylmethionine β-alanine, GABA and δ-Aminolevulinic acid, 4-aminobenzoic acid (PABA), D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosme, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α-methyl amino acids, N α-methyl amino acids, and amino acid analogs in general).

Mutations may also be made to the nucleotide sequences of the heterodimeric proteins by reference to the genetic code, including taking into account codon degeneracy.

In various embodiments, the present chimeric protein is or comprises an amino acid sequence having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 98%, or at least 99% (e.g. about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 98%, or about 99%) sequence identity to one or more of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32. SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, each optionally with the leader sequence (as indicated with double underlining elsewhere herein, or, in embodiments: MEFGLSWVFLVAIIKGVQC (SEQ ID NO: 47)) omitted.

In any of these sequence, the core domain having the following amino acid sequence is or comprises an amino acid sequence having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 98%, or at least 99% (e.g. about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 98%, or about 99%) sequence identity to SEQ ID NO: 34.

In various embodiments, the present heterodimeric proteins are capable of, and can be used in methods comprising, promoting immune activation (*e.g.,* against tumors). In various embodiments, the present heterodimeric proteins are capable of, and can be used in methods

comprising, suppressing immune inhibition (*e.g.,* that allows tumors to survive). In various embodiments, the present heterodimeric protein provides improved immune activation and/or improved suppression of immune inhibition.

In various embodiments, the present heterodimeric proteins are capable of, or can be used in methods comprising, modulating the amplitude of an immune response, *e.g.,* modulating the level of effector output. In some embodiments, *e.g.,* when used for the treatment of cancer, the present heterodimeric protein alters the extent of immune stimulation as compared to immune inhibition to increase the amplitude of a T cell response, including, without limitation, stimulating increased levels of cytokine production, proliferation or target killing potential.

In embodiments, a subject is further administered autologous or allogeneic gamma delta T cells that were expanded *ex vivo.*

In embodiments, a subject is further administered autologous or allogeneic T cells that express a Chimeric Antigen Receptor (*i.e.,* CAR-T cells). CAR-T cells are described in, as examples, Eshhar, *et al., PNAS USA*. 90(2):720-724, 1993; Geiger, *et al., J Immunol.* 162(10):5931-5939, 1999; Brentjens, *et al., Nat Med.* 9(3):279-286, 2003; Cooper, *et al., Blood* 101(4):1637-1644, 2003; Imai, *et al., Leukemia.* 18:676-684, 2004, Pang, *et al., Mol Cancer.* 2018; 17:91, and Schmidts, *et al., Front. Immunol* 2018; 9:2593; the entire contents of which are hereby incorporated by reference.

In embodiments, the heterodimeric proteins act synergistically when used in combination with Chimeric Antigen Receptor (CAR) T-cell therapy. In an illustrative embodiment, the heterodimeric proteins act synergistically when used in combination with CAR T-cell therapy in treating a tumor or cancer. In an embodiment, the heterodimeric proteins act synergistically when used in combination with CAR T-cell therapy in treating blood-based tumors. In an embodiment, the heterodimeric proteins act synergistically when used in combination with CAR T-cell therapy in treating solid tumors. For example, use of heterodimeric proteins and CAR T-cells may act synergistically to reduce or eliminate the tumor or cancer, or slow the growth and/or progression and/or metastasis of the tumor or cancer. In various embodiments, the heterodimeric proteins of the invention induce CAR T-cell division. In various embodiments, the heterodimeric proteins of the invention induce CAR T-cell proliferation. In various embodiments, the heterodimeric proteins of the invention prevents anergy of the CAR T cells.

In various embodiments, the CAR T-cell therapy comprises CAR T cells that target antigens (*e.g.*, tumor antigens) such as, but not limited to, carbonic anhydrase IX (CAIX), 5T4, CD19, CD20, CD22, CD30, CD33, CD38, CD47, CS1, CD138, Lewis-Y, L1-CAM, MET, MUC1, MUC16, ROR-1, IL13Rα2, gp100, prostate stem cell antigen (PSCA), prostate-specific

membrane antigen (PSMA), B-cell maturation antigen (BCMA), human papillomavirus type 16 E6 (HPV-16 E6), CD171, folate receptor alpha (FR-α), GD2, GPC3, human epidermal growth factor receptor 2 (HER2), κ light chain, mesothelin, EGFR, EGFRvIII, ErbB, fibroblast activation protein (FAP), carcinoembryonic antigen (CEA), PMSA, Receptor Tyrosine Kinase Like Orphan Receptor 1 (ROR1), TAG72, and vascular endothelial growth factor receptor 2 (VEGF-R2), as well as other tumor antigens well known in the art. Additional illustrative tumor antigens include, but are not limited to MART-1/Melan-A, gp100, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)-0017-1A/GA733, Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (*e.g.*, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5), GAGE-family of tumor antigens (*e.g.*, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin and γ-catenin, p120ctn, gp100 Pmel117, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papilloma virus proteins, Smad family of tumor antigens, lmp-1, NA, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 CT-7, c-erbB-2, CD19, CD37, CD56, CD70, CD74, CD138, AGS16, MUC1 , GPNMB, Ep-CAM, PD-L1, and PD-L2.

Exemplary CAR T-cell therapy include, but are not limited to, JCAR014 (Juno Therapeutics), JCAR015 (Juno Therapeutics), JCAR017 (Juno Therapeutics), JCAR018 (Juno Therapeutics), JCAR020 (Juno Therapeutics), JCAR023 (Juno Therapeutics), JCAR024 (Juno Therapeutics), CTL019 (Novartis), KTE-C19 (Kite Pharma), BPX-401 (Bellicum Pharmaceuticals), BPX-501 (Bellicum Pharmaceuticals), BPX-601 (Bellicum Pharmaceuticals), bb2121 (Bluebird Bio), CD-19 Sleeping Beauty cells (Ziopharm Oncology), UCART19 (Cellectis), UCART123 (Cellectis), UCART38 (Cellectis), UCARTCS1 (Cellectis), OXB-302 (Oxford BioMedica, MB-101 (Mustang Bio) and CAR T-cells developed by Innovative Cellular Therapeutics.

In embodiments, the CAR-T cells are autologous or allogeneic gamma delta T cells.

In various embodiments the present heterodimeric proteins, in some embodiments are capable of, or find use in methods involving, masking an inhibitory ligand on the surface of a tumor cell and replacing that immune inhibitory ligand with an immune stimulatory ligand.

Accordingly, the present heterodimeric proteins, in some embodiments are capable of, or find use in methods involving, reducing or eliminating an inhibitory immune signal and/or increasing or activating an immune stimulatory signal. For example, a tumor cell bearing an inhibitory signal (and thus evading an immune response) may be substituted for a positive signal binding on a T cell that can then attack a tumor cell. Accordingly, in some embodiments, an inhibitory immune signal is masked by the present heterodimeric proteins and a stimulatory immune signal is activated. Such beneficial properties are enhanced by the single construct approach of the present heterodimeric proteins. For instance, the signal replacement can be effected nearly simultaneously and the signal replacement is tailored to be local at a site of clinical importance (*e.g.,* the tumor microenvironment).

In various embodiments, the present heterodimeric proteins are capable of, or find use in methods comprising, stimulating or enhancing the binding of immune stimulatory receptor/ligand pairs.

In other embodiments, the present heterodimeric proteins are capable of, or find use in methods involving, enhancing, restoring, promoting and/or stimulating immune modulation. In some embodiments, the present heterodimeric proteins described herein, restore, promote and/or stimulate the activity or activation of one or more immune cells against tumor cells including, but not limited to: T cells, cytotoxic T lymphocytes, T helper cells, natural killer (NK) cells, natural killer T (NKT) cells, anti-tumor macrophages (*e.g.,* M1 macrophages), B cells, and dendritic cells. In some embodiments, the present heterodimeric proteins enhance, restore, promote and/or stimulate the activity and/or activation of T cells, including, by way of a non-limiting example, activating and/or stimulating one or more T- cell intrinsic signals, including a pro-survival signal; an autocrine or paracrine growth signal; a p38 MAPK-, ERK-, STAT-, JAK-, AKT- or PI3K-mediated signal; an anti-apoptotic signal; and/or a signal promoting and/or necessary for one or more of: proinflammatory cytokine production or T cell migration or T cell tumor infiltration.

In some embodiments, the present heterodimeric proteins are capable of, or find use in methods involving, causing an increase of one or more of T cells (including without limitation cytotoxic T lymphocytes, T helper cells, natural killer T (NKT) cells), B cells, natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells, monocytes, and macrophages (*e.g.,* one or more of M1 and M2) into a tumor or the tumor microenvironment. In some embodiments, the present heterodimeric proteins are capable of, or find use in methods involving, inhibiting and/or causing a decrease in recruitment of immunosuppressive cells (*e.g.,* myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), tumor associated neutrophils (TANs), M2 macrophages, and tumor associated macrophages (TAMs)) to the tumor and/or tumor

microenvironment (TME). In some embodiments, the present therapies may alter the ratio of M1 versus M2 macrophages in the tumor site and/or TME to favor M1 macrophages.

In embodiments, the heterotrimeric protein modulates the function of gamma delta T cells.

In various embodiments, the present heterodimeric proteins are capable of, and can be used in methods comprising, inhibiting and/or reducing T cell inactivation and/or immune tolerance to a tumor, comprising administering an effective amount of a heterodimeric protein described herein to a subject. In some embodiments, the present heterodimeric proteins are able to increase the serum levels of various cytokines including, but not limited to, one or more of IFNγ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, and IL-22. In some embodiments, the present heterodimeric proteins are capable of enhancing IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, IL-22, or IFNγ in the serum of a treated subject.

In various embodiments, the present heterodimeric proteins inhibit, block and/or reduce cell death of an anti-tumor CD8+ and/or CD4+ T cell; or stimulate, induce, and/or increase cell death of a pro-tumor T cell. T cell exhaustion is a state of T cell dysfunction characterized by progressive loss of proliferative and effector functions, culminating in clonal deletion. Accordingly, a pro-tumor T cell refers to a state of T cell dysfunction that arises during many chronic infections and cancer. This dysfunction is defined by poor proliferative and/or effector functions, sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T cells. Exhaustion prevents optimal control of infection and tumors. In addition, an anti-tumor CD8+ and/or CD4+ T cell refers to T cells that can mount an immune response to a tumor. Illustrative pro-tumor T cells include, but are not limited to, Tregs, CD4+ and/or CD8+ T cells expressing one or more checkpoint inhibitory receptors, Th2 cells and Th17 cells. Checkpoint inhibitory receptors refers to receptors (*e.g.,* CTLA-4, B7-H3, B7-H4, TIM-3) expressed on immune cells that prevent or inhibit uncontrolled immune responses.

In various embodiments, the present heterodimeric proteins are capable of, and can be used in methods comprising, increasing a ratio of effector T cells to regulatory T cells. Illustrative effector T cells include $ICOS^+$ effector T cells; cytotoxic T cells (*e.g.,* αβ TCR, $CD3^+$, $CD8^+$, $CD45RO^+$); $CD4^+$ effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD4^+$, $CCR7^+$, CD62Lhi, IL^- $7R/CD127^+$); $CD8^+$ effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD8^+$, $CCR7^+$, CD62Lhi, IL^- $7R/CD127^+$); effector memory T cells (*e.g.,* CD62Llow, $CD44^+$, TCR, $CD3^+$, $IL^-7R/CD127^+$, $IL\text{-}15R^+$, CCR7low); central memory T cells (*e.g.,* $CCR7^+$, $CD62L^+$, $CD27^+$; or CCR7hi, $CD44^+$, CD62Lhi, TCR, $CD3^+$, IL-7R/$CD127^+$, $IL\text{-}15R^+$); $CD62L^+$ effector T cells; $CD8^+$ effector memory T cells (TEM) including early effector memory T cells ($CD27^+$ $CD62L^-$) and late effector memory T cells ($CD27^-$ $CD62L^-$) (TemE and TemL, respectively);

CD127(+)CD25(low/-) effector T cells; CD127(-)CD25(-) effector T cells; $CD8^+$ stem cell memory effector cells (TSCM) (*e.g.,* CD44(low)CD62L(high)CD122(high)sca(+)); TH1 effector T-cells (*e.g.,* $CXCR3^+$, $CXCR6^+$ and $CCR5^+$; or αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}12R^+$, $IFN\gamma R^+$, $CXCR3^+$), TH2 effector T cells (*e.g.,* $CCR3^+$, $CCR4^+$ and $CCR8^+$; or αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}4R^+$, $IL\text{-}33R^+$, $CCR4^+$, $IL\text{-}17RB^+$, $CRTH2^+$); TH9 effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD4^+$); TH17 effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}23R^+$, $CCR6^+$, $IL\text{-}1R^+$); $CD4^+CD45RO^+CCR7^+$ effector T cells, $CD4^+CD45RO^+CCR7^-$ effector T cells; and effector T cells secreting IL-2, IL-4 and/or IFN-γ. Illustrative regulatory T cells include $ICOS^+$ regulatory T cells, $CD4^+CD25^+FOXP3^+$ regulatory T cells, $CD4^+CD25^+$ regulatory T cells, $CD4^+CD25^-$ regulatory T cells, $CD4^+$CD25high regulatory T cells, $TIM\text{-}3^+PD\text{-}1^+$ regulatory T cells, lymphocyte activation gene-3 $(LAG\text{-}3)^+$ regulatory T cells, $CTLA\text{-}4/CD152^+$ regulatory T cells, neuropilin-1 $(Nrp\text{-}1)^+$ regulatory T cells, $CCR4^+CCR8^+$ regulatory T cells, CD62L (L-selectin)$^+$ regulatory T cells, CD45RBlow regulatory T cells, CD127low regulatory T cells, $LRRC32/GARP^+$ regulatory T cells, $CD39^+$ regulatory T cells, $GITR^+$ regulatory T cells, LAP^+ regulatory T cells, $1B11^+$ regulatory T cells, $BTLA^+$ regulatory T cells, type 1 regulatory T cells (Tr1 cells),T helper type 3 (Th3) cells, regulatory cell of natural killer T cell phenotype (NKTregs), $CD8^+$ regulatory T cells, $CD8^+CD28^-$ regulatory T cells and/or regulatory T-cells secreting IL-10, IL-35, TGF-β, TNF-α, Galectin-1, IFN-γ and/or MCP1.

In various embodiments, the present heterodimeric proteins are capable of, and can be used in methods comprising, transiently stimulating effector T cells for no longer than about 12 hours, about 24 hours, about 48 hours, about 72 hours or about 96 hours or about 1 week or about 2 weeks. In various embodiments, the present heterodimeric proteins are capable of, and can be used in methods comprising, transiently depleting or inhibiting regulatory T cells for no longer than about 12 hours, about 24 hours, about 48 hours, about 72 hours or about 96 hours or about 1 week or about 2 weeks. In various embodiments, the transient stimulation of effector T cells and/or transient depletion or inhibition of regulatory T cells occurs substantially in a patient's bloodstream or in a particular tissue/location including lymphoid tissues such as for example, the bone marrow, lymph-node, spleen, thymus, mucosa-associated lymphoid tissue (MALT), non-lymphoid tissues, or in the tumor microenvironment.

In various embodiments, the present heterodimeric proteins provide advantages including, without limitation, ease of use and ease of production. This is because two distinct immunotherapy agents are combined into a single product which allows for a single manufacturing process instead of two independent manufacturing processes. In addition, administration of a single agent instead of two separate agents allows for easier administration and greater patient compliance. Further, in contrast to, for example, monoclonal antibodies, which are large multimeric proteins containing numerous disulfide bonds and post-translational

modifications such as glycosylation, the present heterodimeric proteins are easier and more cost effective to manufacture.

In various embodiments, the present heterodimeric proteins provide synergistic therapeutic effects as it allows for improved site-specific interplay of two immunotherapy agents. In some embodiments, the present heterodimeric proteins provide the potential for reducing off-site and/or systemic toxicity.

Diseases; Methods of Treatment, and Patient Selections

In one aspect, the present technology provides a method of treating cancer, comprising administering to a subject in need thereof an effective amount of a pharmaceutical composition of any of the embodiments disclosed herein to a subject in need thereof. In some embodiments, the cancer is a lymphoma. In some embodiments, the cancer is a leukemia. In some embodiments, the cancer is a Hodgkin's and non-Hodgkin's lymphoma, B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; or chronic myeloblastic leukemia. In some embodiments, the cancer is basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute

lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (*e.g.* that associated with brain tumors), and Meigs' syndrome. In some embodiments, the cancer is prostate cancer. In some embodiments, the cancer is an epithelial-derived carcinoma. In some embodiments, the cancer is known to express the antigenic target of the second domain of the heterodimeric protein. In some embodiments, the cancer is known to contain mutations which limit recognition by alpha beta T cells, including but not limited to mutations in MHC I, beta 2 microglobulin, TAP, etc.

In some embodiments, the subject is further administered autologous or allogeneic gamma delta T cells that were expanded *ex vivo*. In some embodiments, the autologous or allogeneic gamma delta T cells express a Chimeric Antigen Receptor. In some embodiments, the subject is further administered autologous or allogeneic T cells that express a Chimeric Antigen Receptor.

In one aspect, the present technology provides a method of treating an autoimmune disease or disorder, comprising administering an effective amount of a pharmaceutical composition of any of the embodiments disclosed herein to a subject in need thereof, wherein the autoimmune disease or disorder is optionally selected from rheumatoid arthritis, systemic lupus erythematosus, diabetes mellitus, ankylosing spondylitis, Sjögren's syndrome, inflammatory bowel diseases (*e.g.,* colitis ulcerosa, Crohn's disease), multiple sclerosis, sarcoidosis, psoriasis, Grave's disease, Hashimoto's thyroiditis, , psoriasis, hypersensitivity reactions (*e.g.*, allergies, hay fever, asthma, and acute edema cause type I hypersensitivity reactions), and vasculitis.

In various embodiments, the present technology pertains to the use of the heterodimeric proteins for the treatment of one or more autoimmune diseases or disorders. In various embodiments, the treatment of an autoimmune disease or disorder may involve modulating the immune system with the present heterodimeric proteins to favor immune inhibition over immune stimulation. Illustrative autoimmune diseases or disorders treatable with the present heterodimeric proteins include those in which the body's own antigens become targets for an immune response, such as, for example, rheumatoid arthritis, systemic lupus erythematosus, diabetes mellitus, ankylosing spondylitis, Sjögren's syndrome, inflammatory bowel diseases (*e.g.,* colitis ulcerosa, Crohn's disease), multiple sclerosis, sarcoidosis, psoriasis, Grave's disease, Hashimoto's thyroiditis, , psoriasis, hypersensitivity reactions (*e.g.*, allergies, hay fever, asthma, and acute edema cause type I hypersensitivity reactions), and vasculitis.

Illustrative autoimmune diseases or conditions that may be treated or prevented using the heterodimeric protein of the invention include, but are not limited to, multiple sclerosis, diabetes mellitus, lupus, celiac disease, Crohn's disease, ulcerative colitis, Guillain-Barre

syndrome, scleroderms, Goodpasture's syndrome, Wegener's granulomatosis, autoimmune epilepsy, Rasmussen's encephalitis, Primary biliary sclerosis, Sclerosing cholangitis, Autoimmune hepatitis, Addison's disease, Hashimoto's thyroiditis, Fibromyalgia, Menier's syndrome; transplantation rejection (*e.g.*, prevention of allograft rejection), pernicious anemia, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, lupus erythematosus, multiple sclerosis, myasthenia gravis, Reiter's syndrome, Grave's disease, and other autoimmune diseases.

In various embodiments, the present technology pertains to cancers and/or tumors; for example, the treatment or prevention of cancers and/or tumors. As described elsewhere herein, the treatment of cancer may involve in various embodiments, modulating the immune system with the present heterodimeric proteins to favor immune stimulation over immune inhibition.

Cancers or tumors refer to an uncontrolled growth of cells and/or abnormal increased cell survival and/or inhibition of apoptosis which interferes with the normal functioning of the bodily organs and systems. Included are benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases. Also, included are cells having abnormal proliferation that is not impeded by the immune system (*e.g.*, virus infected cells). The cancer may be a primary cancer or a metastatic cancer. The primary cancer may be an area of cancer cells at an originating site that becomes clinically detectable, and may be a primary tumor. In contrast, the metastatic cancer may be the spread of a disease from one organ or part to another non-adjacent organ or part. The metastatic cancer may be caused by a cancer cell that acquires the ability to penetrate and infiltrate surrounding normal tissues in a local area, forming a new tumor, which may be a local metastasis. The cancer may also be caused by a cancer cell that acquires the ability to penetrate the walls of lymphatic and/or blood vessels, after which the cancer cell is able to circulate through the bloodstream (thereby being a circulating tumor cell) to other sites and tissues in the body. The cancer may be due to a process such as lymphatic or hematogeneous spread. The cancer may also be caused by a tumor cell that comes to rest at another site, re-penetrates through the vessel or walls, continues to multiply, and eventually forms another clinically detectable tumor. The cancer may be this new tumor, which may be a metastatic (or secondary) tumor.

The cancer may be caused by tumor cells that have metastasized, which may be a secondary or metastatic tumor. The cells of the tumor may be like those in the original tumor. As an example, if a breast cancer or colon cancer metastasizes to the liver, the secondary tumor, while present in the liver, is made up of abnormal breast or colon cells, not of abnormal liver cells. The tumor in the liver may thus be a metastatic breast cancer or a metastatic colon cancer, not liver cancer.

The cancer may have an origin from any tissue. The cancer may originate from melanoma, colon, breast, or prostate, and thus may be made up of cells that were originally skin, colon, breast, or prostate, respectively. The cancer may also be a hematological malignancy, which may be leukemia or lymphoma. The cancer may invade a tissue such as liver, lung, bladder, or intestinal.

Representative cancers and/or tumors of the present technology include, but are not limited to, a basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

In embodiments, the cancer is an epithelial-derived carcinoma.

In some embodiments, the heterodimeric protein is used to treat a subject that has a treatment-refractory cancer. In some embodiments, the heterodimeric protein is used to treat a subject that is refractory to one or more immune-modulating agents. For example, in some embodiments, the heterodimeric protein is used to treat a subject that presents no response to treatment, or even progress, after 12 weeks or so of treatment. For instance, in some embodiments, the subject is refractory to a PD-1 and/or PD-L1 and/or PD-L2 agent, including, for example, nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB),

pembrolizumab (KEYTRUDA, MERCK), pidilizumab (CT-011, CURE TECH), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and/or MPDL328OA (ROCHE)-refractory patients. For instance, in some embodiments, the subject is refractory to an anti-CTLA-4 agent, *e.g.,* ipilimumab (YERVOY)-refractory patients (*e.g.,* melanoma patients). Accordingly, in various embodiments the present technology provides methods of cancer treatment that rescue patients that are non-responsive to various therapies, including monotherapy of one or more immune-modulating agents.

In various embodiments, the present technology provides heterodimeric proteins which target a cell or tissue within the tumor microenvironment. In some embodiments, the cell or tissue within the tumor microenvironment expresses one or more targets or binding partners of the heterodimeric protein. The tumor microenvironment refers to the cellular milieu, including cells, secreted proteins, physiological small molecules, and blood vessels in which the tumor exists. In some embodiments, the cells or tissue within the tumor microenvironment are one or more of: tumor vasculature; tumor-infiltrating lymphocytes; fibroblast reticular cells; endothelial progenitor cells (EPC); cancer-associated fibroblasts; pericytes; other stromal cells; components of the extracellular matrix (ECM); dendritic cells; antigen presenting cells; T-cells; regulatory T cells; macrophages; neutrophils; and other immune cells located proximal to a tumor. In various embodiments, the present heterodimeric protein targets a cancer cell. In some embodiments, the cancer cell expresses one or more of targets or binding partners of the heterodimeric protein.

In various embodiments, the heterodimeric protein of the invention may target a cell (*e.g.,* cancer cell or immune cell) that expresses any of the receptors as described herein. For example, the heterodimeric protein of the invention may target a cell that expresses any of the receptors for a cytokine, growth factor, and/or hormone as described herein.

In some embodiments, the present methods provide treatment with the heterodimeric protein in a patient who is refractory to an additional agent, such "additional agents" being described elsewhere herein, inclusive, without limitation, of the various chemotherapeutic agents described herein.

In some aspects, the present chimeric agents are used to eliminate intracellular pathogens. In some aspects, the present chimeric agents are used to treat one or more infections. In some embodiments, the present heterodimeric proteins are used in methods of treating viral infections (including, for example, HIV and HCV), parasitic infections (including, for example, malaria), and bacterial infections. In various embodiments, the infections induce immunosuppression. For example, HIV infections often result in immunosuppression in the infected subjects.

Accordingly, as described elsewhere herein, the treatment of such infections may involve, in various embodiments, modulating the immune system with the present heterodimeric proteins to favor immune stimulation over immune inhibition. Alternatively, the present technology provides methods for treating infections that induce immunoactivation. For example, intestinal helminth infections have been associated with chronic immune activation. In these embodiments, the treatment of such infections may involve modulating the immune system with the present heterodimeric proteins to favor immune inhibition over immune stimulation.

In various embodiments, the present technology provides methods of treating viral infections including, without limitation, acute or chronic viral infections, for example, of the respiratory tract, of papilloma virus infections, of herpes simplex virus (HSV) infection, of human immunodeficiency virus (HIV) infection, and of viral infection of internal organs such as infection with hepatitis viruses. In some embodiments, the viral infection is caused by a virus of family Flaviviridae. In some embodiments, the virus of family Flaviviridae is selected from Yellow Fever Virus, West Nile virus, Dengue virus, Japanese Encephalitis Virus, St. Louis Encephalitis Virus, and Hepatitis C Virus. In other embodiments, the viral infection is caused by a virus of family Picornaviridae, *e.g.*, poliovirus, rhinovirus, coxsackievirus. In other embodiments, the viral infection is caused by a member of Orthomyxoviridae, *e.g.*, an influenza virus. In other embodiments, the viral infection is caused by a member of Retroviridae, *e.g.*, a lentivirus. In other embodiments, the viral infection is caused by a member of Paramyxoviridae, *e.g.*, respiratory syncytial virus, a human parainfluenza virus, rubulavirus (*e.g.*, mumps virus), measles virus, and human metapneumovirus. In other embodiments, the viral infection is caused by a member of Bunyaviridae, *e.g.*, hantavirus. In other embodiments, the viral infection is caused by a member of Reoviridae, *e.g.*, a rotavirus.

In various embodiments, the present technology provides methods of treating parasitic infections such as protozoan or helminths infections. In some embodiments, the parasitic infection is by a protozoan parasite. In some embodiments, the oritiziab parasite is selected from intestinal protozoa, tissue protozoa, or blood protozoa. Illustrative protozoan parasites include, but are not limited to, *Entamoeba hystolytica, Giardia lamblia, Cryptosporidium muris, Trypanosomatida gambiense, Trypanosomatida rhodesiense, Trypanosomatida crusi, Leishmania mexicana, Leishmania braziliensis, Leishmania tropica, Leishmania donovani, Toxoplasma gondii, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium falciparum, Trichomonas vaginalis*, and *Histomonas meleagridis.* In some embodiments, the parasitic infection is by a helminthic parasite such as nematodes (*e.g.*, Adenophorea). In some embodiments, the parasite is selected from Secementea (*e.g.*, *Trichuris trichiura, Ascaris lumbricoides, Enterobius vermicularis, Ancylostoma duodenale, Necator americanus, Strongyloides stercoralis, Wuchereria bancrofti, Dracunculus medinensis*). In

some embodiments, the parasite is selected from trematodes (*e.g.*, blood flukes, liver flukes, intestinal flukes, and lung flukes). In some embodiments, the parasite is selected from: *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Fasciola hepatica, Fasciola gigantica, Heterophyes heterophyes, Paragonimus westermani.* In some embodiments, the parasite is selected from cestodes (*e.g.*, *Taenia solium, Taenia saginata, Hymenolepis nana, Echinococcus granulosus*).

In various embodiments, the present technology provides methods of treating bacterial infections. In various embodiments, the bacterial infection is by gram-positive bacteria, gram-negative bacteria, aerobic and/or anaerobic bacteria. In various embodiments, the bacteria are selected from, but not limited to, *Staphylococcus, Lactobacillus, Streptococcus, Sarcina, Escherichia, Enterobacter, Klebsiella, Pseudomonas, Acinetobacter, Mycobacterium, Proteus, Campylobacter, Citrobacter, Nisseria, Baccillus, Bacteroides, Peptococcus, Clostridium, Salmonella, Shigella, Serratia, Haemophilus, Brucella* and other organisms. In some embodiments, the bacteria is selected from, but not limited to, *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas acidovorans, Pseudomonas alcaligenes, Pseudomonas putida, Stenotrophomonas maltophilia, Burkholderia cepacia, Aeromonas hydrophilia, Escherichia coli, Citrobacter freundii, Salmonella typhimurium, Salmonella typhi, Salmonella paratyphi, Salmonella enteritidis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Enterobacter cloacae, Enterobacter aerogenes, Klebsiella pneumoniae, Klebsiella oxytoca, Serratia marcescens, Francisella tularensis, Morganella morganii, Proteus mirabilis, Proteus vulgaris, Providencia alcalifaciens, Providencia rettgeri, Providencia stuartii, Acinetobacter baumannii, Acinetobacter calcoaceticus, Acinetobacter haemolyticus, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Yersinia intermedia, Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Haemophilus influenzae, Haemophilus parainfluenzae, Haemophilus haemolyticus, Haemophilus parahaemolyticus, Haemophilus ducreyi, Pasteurella multocida, Pasteurella haemolytica, Branhamella catarrhalis, Helicobacter pylori, Campylobacter fetus, Campylobacter jejuni, Campylobacter coli, Borrelia burgdorferi, Vibrio cholerae, Vibrio parahaemolyticus, Legionella pneumophila, Listeria monocytogenes, Neisseria gonorrhoeae, Neisseria meningitidis, Kingella, Moraxella, Gardnerella vaginalis, Bacteroides fragilis, Bacteroides distasonis, Bacteroides* 3452A homology group, *Bacteroides vulgatus, Bacteroides ovalus, Bacteroides thetaiotaomicron, Bacteroides uniformis, Bacteroides eggerthii, Bacteroides splanchnicus, Clostridium difficile, Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium leprae, Corynebacterium diphtheriae, Corynebacterium ulcerans, Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus pyogenes, Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Staphylococcus epidermidis,*

Staphylococcus saprophyticus, Staphylococcus intermedius, Staphylococcus hyicus subsp. *hyicus, Staphylococcus haemolyticus, Staphylococcus hominis*, or *Staphylococcus saccharolyticus*.

In still another other aspect, the present technology is directed toward methods of treating and preventing T cell-mediated diseases and disorders, such as, but not limited to diseases or disorders described elsewhere herein and inflammatory disease or disorder, graft-versus-host disease (GVHD), transplant rejection, and T cell proliferative disorder.

In some aspects, the present chimeric agents are used in methods of activating a T cell, *e.g., via* the extracellular domain having an immune stimulatory signal or antibody binding domain (*e.g.* CDR3, Fab, scFv domain, *etc.*) having an immune stimulatory signal.

In some aspects, the present chimeric agents are used in methods of preventing the cellular transmission of an immunosuppressive signal.

Combination Therapies and Conjugation

In some embodiments, the invention provides for heterodimeric proteins and methods that further comprise administering an additional agent to a subject. In some embodiments, the invention pertains to co-administration and/or co-formulation. Any of the compositions described herein may be co-formulated and/or co-administered.

In some embodiments, any heterodimeric protein described herein acts synergistically when co-administered with another agent and is administered at doses that are lower than the doses commonly employed when such agents are used as monotherapy. In various embodiments, any agent referenced herein may be used in combination with any of the heterodimeric proteins described herein.

In various embodiments, any of the heterodimeric proteins disclosed herein may be co-administered with another heterodimeric protein disclosed herein. Without wishing to be bound by theory, it is believed that a combined regimen involving the administration of one or more heterodimeric proteins which induce an innate immune response and one or more heterodimeric proteins which induce an adaptive immune response may provide synergistic effects (*e.g.*, synergistic anti-tumor effects).

In various embodiments, any heterodimeric protein which induces an innate immune response may be utilized in the present technology. In various embodiments, any heterodimeric protein which induces an adaptive immune response may be utilized in the present technology.

In some embodiments, inclusive of, without limitation, cancer applications, the present technology pertains to chemotherapeutic agents as additional agents. Examples of chemotherapeutic agents include, but are not limited to, alkylating agents such as thiotepa and

CYTOXAN cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (*e.g.*, bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; cally statin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (*e.g.*, cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB 1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammall and calicheamicin omegall (*see*, *e.g.*, Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN doxorubicin (including morpholino- doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as minoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-

trichlorotriethylamine; trichothecenes (*e.g.*, T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, TAXOL paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, 111.), and TAXOTERE doxetaxel (Rhone-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE. vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (TYKERB); inhibitors of PKC-α, Raf, H-Ras, EGFR (*e.g.*, erlotinib (Tarceva)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above. In addition, the methods of treatment can further include the use of radiation. In addition, the methods of treatment can further include the use of photodynamic therapy.

In various embodiments, inclusive of, without limitation, cancer applications, the present additional agent is one or more immune-modulating agents selected from an agent that blocks, reduces and/or inhibits PD-1 and PD-L1 or PD-L2 and/or the binding of PD-1 with PD-L1 or PD-L2 (by way of non-limiting example, one or more of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, Merck), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), atezolizumab (TECENTRIQ, GENENTECH), MPDL328OA (ROCHE)), an agent that increases and/or stimulates CD137 (4-1BB) and/or the binding of CD137 (4-1BB) with one or more of 4-1BB ligand (by way of non-limiting example, urelumab (BMS-663513 and anti-4-1BB antibody), and an agent that blocks, reduces and/or inhibits the activity of CTLA-4 and/or the binding of CTLA-4 with one or more of AP2M1, CD80, CD86, SHP-2, and PPP2R5A and/or the binding of OX40 with OX40L (by way of non-limiting example GBR 830 (GLENMARK), MEDI6469 (MEDIMMUNE).

In some embodiments, inclusive of, without limitation, infectious disease applications, the present technology pertains to anti-infectives as additional agents. In some embodiments, the anti-infective is an anti-viral agent including, but not limited to, Abacavir, Acyclovir, Adefovir, Amprenavir, Atazanavir, Cidofovir, Darunavir, Delavirdine, Didanosine, Docosanol, Efavirenz, Elvitegravir, Emtricitabine, Enfuvirtide, Etravirine, Famciclovir, and Foscarnet. In

some embodiments, the anti-infective is an anti-bacterial agent including, but not limited to, cephalosporin antibiotics (cephalexin, cefuroxime, cefadroxil, cefazolin, cephalothin, cefaclor, cefamandole, cefoxitin, cefprozil, and ceftobiprole); fluoroquinolone antibiotics (cipro, Levaquin, floxin, tequin, avelox, and norflox); tetracycline antibiotics (tetracycline, minocycline, oxytetracycline, and doxycycline); penicillin antibiotics (amoxicillin, ampicillin, penicillin V, dicloxacillin, carbenicillin, vancomycin, and methicillin); monobactam antibiotics (aztreonam); and carbapenem antibiotics (ertapenem, doripenem, imipenem/cilastatin, and meropenem). In some embodiments, the anti-infectives include anti-malarial agents (*e.g.*, chloroquine, quinine, mefloquine, primaquine, doxycycline, artemether/lumefantrine, atovaquone/proguanil and sulfadoxine/pyrimethamine), metronidazole, tinidazole, ivermectin, pyrantel pamoate, and albendazole.

In some embodiments, inclusive, without limitation, of autoimmune applications, the additional agent is an immunosuppressive agent. In some embodiments, the immunosuppressive agent is an anti-inflammatory agent such as a steroidal anti-inflammatory agent or a non-steroidal anti-inflammatory agent (NSAID). Steroids, particularly the adrenal corticosteroids and their synthetic analogues, are well known in the art. Examples of corticosteroids useful in the present technology include, without limitation, hydroxyltriamcinolone, alpha-methyl dexamethasone, beta-methyl betamethasone, beclomethasone dipropionate, betamethasone benzoate, betamethasone dipropionate, betamethasone valerate, clobetasol valerate, desonide, desoxymethasone, dexamethasone, diflorasone diacetate, diflucortolone valerate, fluadrenolone, fluclorolone acetonide, flumethasone pivalate, fluosinolone acetonide, fluocinonide, flucortine butylester, fluocortolone, fluprednidene (fluprednylidene) acetate, flurandrenolone, halcinonide, hydrocortisone acetate, hydrocortisone butyrate, methylprednisolone, triamcinolone acetonide, cortisone, cortodoxone, flucetonide, fludrocortisone, difluorosone diacetate, fluradrenolone acetonide, medrysone, amcinafel, amcinafide, betamethasone and the balance of its esters, chloroprednisone, clocortelone, clescinolone, dichlorisone, difluprednate, flucloronide, flunisolide, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone, meprednisone, paramethasone, prednisolone, prednisone, beclomethasone dipropionate. (NSAIDS) that may be used in the present technology, include but are not limited to, salicylic acid, acetyl salicylic acid, methyl salicylate, glycol salicylate, salicylmides, benzyl-2,5-diacetoxybenzoic acid, ibuprofen, fulindac, naproxen, ketoprofen, etofenamate, phenylbutazone, and indomethacin. In some embodiments, the immunosupressive agent may be cytostatics such as alkylating agents, antimetabolites (*e.g.*, azathioprine, methotrexate), cytotoxic antibiotics, antibodies (*e.g.*, basiliximab, daclizumab, and muromonab), anti-immunophilins (*e.g.*, cyclosporine, tacrolimus, sirolimus), inteferons,

opioids, TNF binding proteins, mycophenolates, and small biological agents (*e.g.*, fingolimod, myriocin).

In some embodiments, the heterodimeric proteins (and/or additional agents) described herein, include derivatives that are modified, *i.e.,* by the covalent attachment of any type of molecule to the composition such that covalent attachment does not prevent the activity of the composition. For example, but not by way of limitation, derivatives include composition that have been modified by, *inter alia*, glycosylation, lipidation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc*. Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of turicamycin, *etc.* Additionally, the derivative can contain one or more non-classical amino acids. In still other embodiments, the heterodimeric proteins (and/or additional agents) described herein further comprise a cytotoxic agent, comprising, in illustrative embodiments, a toxin, a chemotherapeutic agent, a radioisotope, and an agent that causes apoptosis or cell death. Such agents may be conjugated to a composition described herein.

The heterodimeric proteins (and/or additional agents) described herein may thus be modified post-translationally to add effector moieties such as chemical linkers, detectable moieties such as for example fluorescent dyes, enzymes, substrates, bioluminescent materials, radioactive materials, and chemiluminescent moieties, or functional moieties such as for example streptavidin, avidin, biotin, a cytotoxin, a cytotoxic agent, and radioactive materials.

Formulations

In one aspect, the present technology provides a pharmaceutical composition, comprising the heterodimeric protein of any of the embodiments disclosed herein.

The heterodimeric proteins (and/or additional agents) described herein can possess a sufficiently basic functional group, which can react with an inorganic or organic acid, or a carboxyl group, which can react with an inorganic or organic base, to form a pharmaceutically acceptable salt. A pharmaceutically acceptable acid addition salt is formed from a pharmaceutically acceptable acid, as is well known in the art. Such salts include the pharmaceutically acceptable salts listed in, for example, *Journal of Pharmaceutical Science*, 66, 2-19 (1977) and *The Handbook of Pharmaceutical Salts; Properties, Selection, and Use*. P. H. Stahl and C. G. Wermuth (eds.), Verlag, Zurich (Switzerland) 2002, which are hereby incorporated by reference in their entirety.

In some embodiments, the compositions described herein are in the form of a pharmaceutically acceptable salt.

Further, any heterodimeric protein (and/or additional agents) described herein can be administered to a subject as a component of a composition that comprises a pharmaceutically acceptable carrier or vehicle. Such compositions can optionally comprise a suitable amount of a pharmaceutically acceptable excipient so as to provide the form for proper administration. Pharmaceutical excipients can be liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical excipients can be, for example, saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea and the like. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents can be used. In one embodiment, the pharmaceutically acceptable excipients are sterile when administered to a subject. Water is a useful excipient when any agent described herein is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid excipients, specifically for injectable solutions. Suitable pharmaceutical excipients also include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Any agent described herein, if desired, can also comprise minor amounts of wetting or emulsifying agents, or pH buffering agents.

In some embodiments, the compositions described herein are resuspended in a saline buffer (including, without limitation TBS, PBS, and the like).

In various embodiments, the heterodimeric proteins may by conjugated and/or fused with another agent to extend half-life or otherwise improve pharmacodynamic and pharmacokinetic properties. In some embodiments, the heterodimeric proteins may be fused or conjugated with one or more of PEG, XTEN (*e.g.*, as rPEG), polysialic acid (POLYXEN), albumin (*e.g.*, human serum albumin or HAS), elastin-like protein (ELP), PAS, HAP, GLK, CTP, transferrin, and the like. In various embodiments, each of the individual heterodimeric proteins is fused to one or more of the agents described in BioDrugs (2015) 29:215–239, the entire contents of which are hereby incorporated by reference.

Administration, Dosing, and Treatment Regimens

The present technology includes the described heterodimeric protein (and/or additional agents) in various formulations. Any heterodimeric protein (and/or additional agents) described herein can take the form of solutions, suspensions, emulsion, drops, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. DNA or RNA constructs encoding the protein sequences may also be used. In one embodiment, the composition is in the form of a capsule (*see, e.g.*, U.S. Patent No. 5,698,155). Other examples of suitable

pharmaceutical excipients are described in *Remington's Pharmaceutical Sciences* 1447-1676 (Alfonso R. Gennaro eds., 19th ed. 1995), incorporated herein by reference.

Where necessary, the formulations comprising the heterodimeric protein (and/or additional agents) can also include a solubilizing agent. Also, the agents can be delivered with a suitable vehicle or delivery device as known in the art. Combination therapies outlined herein can be co-delivered in a single delivery vehicle or delivery device. Compositions for administration can optionally include a local anesthetic such as, for example, lignocaine to lessen pain at the site of the injection.

The formulations comprising the heterodimeric protein (and/or additional agents) of the present technology may conveniently be presented in unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods generally include the step of bringing the therapeutic agents into association with a carrier, which constitutes one or more accessory ingredients. Typically, the formulations are prepared by uniformly and intimately bringing the therapeutic agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into dosage forms of the desired formulation (*e.g.*, wet or dry granulation, powder blends, *etc.*, followed by tableting using conventional methods known in the art)

In one embodiment, any heterodimeric protein (and/or additional agents) described herein is formulated in accordance with routine procedures as a composition adapted for a mode of administration described herein.

Routes of administration include, for example: intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically, particularly to the ears, nose, eyes, or skin. In some embodiments, the administering is effected orally or by parenteral injection. In most instances, administration results in the release of any agent described herein into the bloodstream.

Any heterodimeric protein (and/or additional agents) described herein can be administered orally. Such heterodimeric proteins (and/or additional agents) can also be administered by any other convenient route, for example, by intravenous infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and can be administered together with another biologically active agent. Administration can be systemic or local. Various delivery systems are known, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, capsules, *etc.*, and can be used to administer.

In specific embodiments, it may be desirable to administer locally to the area in need of treatment. In one embodiment, for instance in the treatment of cancer, the heterodimeric protein

(and/or additional agents) are administered in the tumor microenvironment (*e.g.,* cells, molecules, extracellular matrix and/or blood vessels that surround and/or feed a tumor cell, inclusive of, for example, tumor vasculature; tumor-infiltrating lymphocytes; fibroblast reticular cells; endothelial progenitor cells (EPC); cancer-associated fibroblasts; pericytes; other stromal cells; components of the extracellular matrix (ECM); dendritic cells; antigen presenting cells; T-cells; regulatory T cells; macrophages; neutrophils; and other immune cells located proximal to a tumor) or lymph node and/or targeted to the tumor microenvironment or lymph node. In various embodiments, for instance in the treatment of cancer, the heterodimeric protein (and/or additional agents) are administered intratumorally.

In the various embodiments, the present heterodimeric protein allows for a dual effect that provides less side effects than are seen in conventional immunotherapy (*e.g.,* treatments with one or more of OPDIVO, KEYTRUDA, YERVOY, and TECENTRIQ). For example, the present heterodimeric proteins reduce or prevent commonly observed immune-related adverse events that affect various tissues and organs including the skin, the gastrointestinal tract, the kidneys, peripheral and central nervous system, liver, lymph nodes, eyes, pancreas, and the endocrine system; such as hypophysitis, colitis, hepatitis, pneumonitis, rash, and rheumatic disease. Further, the present local administration, *e.g.,* intratumorally, obviate adverse event seen with standard systemic administration, *e.g.,* IV infusions, as are used with conventional immunotherapy (*e.g.,* treatments with one or more of OPDIVO, KEYTRUDA, YERVOY, and TECENTRIQ).

Dosage forms suitable for parenteral administration (*e.g.,* intravenous, intramuscular, intraperitoneal, subcutaneous and intra-articular injection and infusion) include, for example, solutions, suspensions, dispersions, emulsions, and the like. They may also be manufactured in the form of sterile solid compositions (*e.g.,* lyophilized composition), which can be dissolved or suspended in sterile injectable medium immediately before use. They may contain, for example, suspending or dispersing agents known in the art.

The dosage of any heterodimeric protein (and/or additional agents) described herein as well as the dosing schedule can depend on various parameters, including, but not limited to, the disease being treated, the subject's general health, and the administering physician's discretion. Any heterodimeric protein described herein, can be administered prior to (*e.g.,* 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concurrently with, or subsequent to (*e.g.,* 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of an additional agent, to a subject in need thereof. In various embodiments any

heterodimeric protein and additional agent described herein are administered 1 minute apart, 10 minutes apart, 30 minutes apart, less than 1 hour apart, 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, 1 day apart, 2 days apart, 3 days apart, 4 days apart, 5 days apart, 6 days apart, 1 week apart, 2 weeks apart, 3 weeks apart, or 4 weeks apart.

In various embodiments, the present technology relates to the co-administration of a heterodimeric protein which induces an innate immune response and another heterodimeric protein which induces an adaptive immune response. In such embodiments, the heterodimeric protein which induces an innate immune response may be administered before, concurrently with, or subsequent to administration of the heterodimeric protein which induces an adaptive immune response. For example, the heterodimeric proteins may be administered 1 minute apart, 10 minutes apart, 30 minutes apart, less than 1 hour apart, 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, 1 day apart, 2 days apart, 3 days apart, 4 days apart, 5 days apart, 6 days apart, 1 week apart, 2 weeks apart, 3 weeks apart, or 4 weeks apart. In an illustrative embodiment, the heterodimeric protein which induces an innate immune response and the heterodimeric protein which induces an adaptive response are administered 1 week apart, or administered on alternate weeks (*i.e.*, administration of the heterodimeric protein inducing an innate immune response is followed 1 week later with administration of the heterodimeric protein which induces an adaptive immune response and so forth).

The dosage of any heterodimeric protein (and/or additional agents) described herein can depend on several factors including the severity of the condition, whether the condition is to be treated or prevented, and the age, weight, and health of the subject to be treated. Additionally, pharmacogenomic (the effect of genotype on the pharmacokinetic, pharmacodynamic or efficacy profile of a therapeutic) information about a particular subject may affect dosage used. Furthermore, the exact individual dosages can be adjusted somewhat depending on a variety of factors, including the specific combination of the agents being administered, the time of administration, the route of administration, the nature of the formulation, the rate of excretion, the particular disease being treated, the severity of the disorder, and the anatomical location of the disorder. Some variations in the dosage can be expected.

For administration of any heterodimeric protein (and/or additional agents) described herein by parenteral injection, the dosage may be about 0.1 mg to about 250 mg per day, about 1 mg to

about 20 mg per day, or about 3 mg to about 5 mg per day. Generally, when orally or parenterally administered, the dosage of any agent described herein may be about 0.1 mg to about 1500 mg per day, or about 0.5 mg to about 10 mg per day, or about 0.5 mg to about 5 mg per day, or about 200 to about 1,200 mg per day (*e.g.*, about 200 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, about 1,000 mg, about 1,100 mg, about 1,200 mg per day).

In some embodiments, administration of the heterodimeric protein (and/or additional agents) described herein is by parenteral injection at a dosage of about 0.1 mg to about 1500 mg per treatment, or about 0.5 mg to about 10 mg per treatment, or about 0.5 mg to about 5 mg per treatment, or about 200 to about 1,200 mg per treatment (*e.g.*, about 200 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, about 1,000 mg, about 1,100 mg, about 1,200 mg per treatment).

In some embodiments, a suitable dosage of the heterodimeric protein (and/or additional agents) is in a range of about 0.01 mg/kg to about 100 mg/kg of body weight ,or about 0.01 mg/kg to about 10 mg/kg of body weight of the subject, for example, about 0.01 mg/kg, about 0.02 mg/kg, about 0.03 mg/kg, about 0.04 mg/kg, about 0.05 mg/kg, about 0.06 mg/kg, about 0.07 mg/kg, about 0.08 mg/kg, about 0.09 mg/kg, about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1 mg/kg, about 1.1 mg/kg, about 1.2 mg/kg, about 1.3 mg/kg, about 1.4 mg/kg, about 1.5 mg/kg, about 1.6 mg/kg, about 1.7 mg/kg, about 1.8 mg/kg, 1.9 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6 mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg, about 10 mg/kg body weight, inclusive of all values and ranges therebetween.

In another embodiment, delivery can be in a vesicle, in particular a liposome (*see* Langer, 1990, *Science* 249:1527-1533; Treat *et al.*, in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989).

Any heterodimeric protein (and/or additional agents) described herein can be administered by controlled-release or sustained-release means or by delivery devices that are well known to those of ordinary skill in the art. Examples include, but are not limited to, those described in U.S. Patent Nos. 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5,674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; and 5,733,556, each of which is incorporated herein by reference in its entirety. Such dosage forms can be useful for providing controlled- or sustained-release of one or more active ingredients using, for example, hydropropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or a combination

thereof to provide the desired release profile in varying proportions. Controlled- or sustained-release of an active ingredient can be stimulated by various conditions, including but not limited to, changes in pH, changes in temperature, stimulation by an appropriate wavelength of light, concentration or availability of enzymes, concentration or availability of water, or other physiological conditions or compounds.

In another embodiment, polymeric materials can be used (*see Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, 1983, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61; *see also* Levy *et al.*, 1985, *Science* 228:190; During *et al.*, 1989, *Ann. Neurol.* 25:351; Howard *et al.*, 1989, *J. Neurosurg.* 71:105).

In another embodiment, a controlled-release system can be placed in proximity of the target area to be treated, thus requiring only a fraction of the systemic dose (*see, e.g.,* Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in the review by Langer, 1990, *Science* 249:1527-1533) may be used.

Administration of any heterodimeric protein (and/or additional agents) described herein can, independently, be one to four times daily or one to four times per month or one to six times per year or once every two, three, four or five years. Administration can be for the duration of one day or one month, two months, three months, six months, one year, two years, three years, and may even be for the life of the subject.

The dosage regimen utilizing any heterodimeric protein (and/or additional agents) described herein can be selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the subject; the severity of the condition to be treated; the route of administration; the renal or hepatic function of the subject; the pharmacogenomic makeup of the individual; and the specific compound of the invention employed. Any heterodimeric protein (and/or additional agents) described herein can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, any heterodimeric protein (and/or additional agents) described herein can be administered continuously rather than intermittently throughout the dosage regimen.

Cells and Nucleic Acids

In one aspect, the present technology provides an expression vector, comprising a nucleic acid encoding the first and/or second polypeptide chains of the heterodimeric protein of any of any of the embodiments disclosed herein. In some embodiments, the expression vector is a

mammalian expression vector. In some embodiments, the expression vector comprises DNA or RNA. In some embodiments, In one aspect, the present technology provides a host cell comprising the expression vector of any one of the embodiments disclosed herein.

In various embodiments, the present technology provides an expression vector, comprising a nucleic acid encoding the heterodimeric protein (*e.g.*, a heterodimeric protein comprising a first and second polypeptide chains) described herein. In various embodiments, the expression vector comprises DNA or RNA. In various embodiments, the expression vector is a mammalian expression vector.

Both prokaryotic and eukaryotic vectors can be used for expression of the heterodimeric protein. Prokaryotic vectors include constructs based on *E. coli* sequences (*see, e.g.,* Makrides, *Microbiol Rev* 1996, 60:512-538). Non-limiting examples of regulatory regions that can be used for expression in *E. coli* include lac, trp, lpp, phoA, recA, tac, T3, T7 and λP_L. Non-limiting examples of prokaryotic expression vectors may include the λgt vector series such as λgt11 (Huynh *et al.*, in "DNA Cloning Techniques, Vol. I: A Practical Approach," 1984, (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier *et al.*, *Methods Enzymol* 1990, 185:60-89). Prokaryotic host-vector systems cannot perform much of the post-translational processing of mammalian cells, however. Thus, eukaryotic host- vector systems may be particularly useful. A variety of regulatory regions can be used for expression of the heterodimeric proteins in mammalian host cells. For example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter can be used. Inducible promoters that may be useful in mammalian cells include, without limitation, promoters associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the β-interferon gene, and the hsp70 gene (*see*, Williams *et al.*, *Cancer Res* 1989, 49:2735-42; and Taylor *et al.*, *Mol Cell Biol* 1990, 10:165-75). Heat shock promoters or stress promoters also may be advantageous for driving expression of the fusion proteins in recombinant host cells.

In some embodiments, expression vectors of the invention comprise a nucleic acid encoding at least the first and/or second polypeptide chains of the heterodimeric proteins (and/or additional agents), or a complement thereof, operably linked to an expression control region, or complement thereof, that is functional in a mammalian cell. The expression control region is capable of driving expression of the operably linked blocking and/or stimulating agent encoding nucleic acid such that the blocking and/or stimulating agent is produced in a human cell transformed with the expression vector.

Expression control regions are regulatory polynucleotides (sometimes referred to herein as elements), such as promoters and enhancers, that influence expression of an operably linked nucleic acid. An expression control region of an expression vector of the invention is capable of expressing operably linked encoding nucleic acid in a human cell. In an embodiment, the cell is a tumor cell. In another embodiment, the cell is a non-tumor cell. In an embodiment, the expression control region confers regulatable expression to an operably linked nucleic acid. A signal (sometimes referred to as a stimulus) can increase or decrease expression of a nucleic acid operably linked to such an expression control region. Such expression control regions that increase expression in response to a signal are often referred to as inducible. Such expression control regions that decrease expression in response to a signal are often referred to as repressible. Typically, the amount of increase or decrease conferred by such elements is proportional to the amount of signal present; the greater the amount of signal, the greater the increase or decrease in expression.

In an embodiment, the present technology contemplates the use of inducible promoters capable of effecting high level of expression transiently in response to a cue. For example, when in the proximity of a tumor cell, a cell transformed with an expression vector for the heterodimeric protein (and/or additional agents) comprising such an expression control sequence is induced to transiently produce a high level of the agent by exposing the transformed cell to an appropriate cue. Illustrative inducible expression control regions include those comprising an inducible promoter that is stimulated with a cue such as a small molecule chemical compound. Particular examples can be found, for example, in U.S. Pat. Nos. 5,989,910, 5,935,934, 6,015,709, and 6,004,941, each of which is incorporated herein by reference in its entirety.

Expression control regions and locus control regions include full-length promoter sequences, such as native promoter and enhancer elements, as well as subsequences or polynucleotide variants which retain all or part of full-length or non-variant function. As used herein, the term "functional" and grammatical variants thereof, when used in reference to a nucleic acid sequence, subsequence or fragment, means that the sequence has one or more functions of native nucleic acid sequence (*e.g.*, non-variant or unmodified sequence).

As used herein, “operable linkage” refers to a physical juxtaposition of the components so described as to permit them to function in their intended manner. In the example of an expression control element in operable linkage with a nucleic acid, the relationship is such that the control element modulates expression of the nucleic acid. Typically, an expression control region that modulates transcription is juxtaposed near the 5' end of the transcribed nucleic acid (*i.e.*, “upstream”). Expression control regions can also be located at the 3’ end of the transcribed sequence (*i.e.*, “downstream”) or within the transcript (*e.g.*, in an intron). Expression control elements can be located at a distance away from the transcribed sequence (*e.g.*, 100 to 500, 500

to 1000, 2000 to 5000, or more nucleotides from the nucleic acid). A specific example of an expression control element is a promoter, which is usually located 5' of the transcribed sequence. Another example of an expression control element is an enhancer, which can be located 5' or 3' of the transcribed sequence, or within the transcribed sequence.

Expression systems functional in human cells are well known in the art, and include viral systems. Generally, a promoter functional in a human cell is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and typically a TATA box located 25-30 base pairs upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A promoter will also typically contain an upstream promoter element (enhancer element), typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, and the CMV promoter.

Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenylation signals include those derived from SV40. Introns may also be included in expression constructs.

There are a variety of techniques available for introducing nucleic acids into viable cells. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, polymer-based systems, DEAE-dextran, viral transduction, the calcium phosphate precipitation method, *etc.* For *in vivo* gene transfer, a number of techniques and reagents may also be used, including liposomes; natural polymer-based delivery vehicles, such as chitosan and gelatin; viral vectors are also suitable for *in vivo* transduction. In some situations, it is desirable to provide a targeting agent, such as an antibody or ligand specific for a tumor cell surface membrane protein. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.*, capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique

of receptor-mediated endocytosis is described, for example, by Wu *et al.*, J. Biol. Chem. 262, 4429-4432 (1987); and Wagner *et al.*, Proc. Natl. Acad. Sci. USA 87, 3410-3414 (1990).

Where appropriate, gene delivery agents such as, *e.g.*, integration sequences can also be employed. Numerous integration sequences are known in the art (*see*, *e.g.*, Nunes-Duby *et al.*, Nucleic Acids Res. 26:391-406, 1998; Sadwoski, J. Bacteriol., 165:341-357, 1986; Bestor, Cell, 122(3):322-325, 2005; Plasterk *et al.*, TIG 15:326-332, 1999; Kootstra *et al.*, Ann. Rev. Pharm. Toxicol., 43:413-439, 2003). These include recombinases and transposases. Examples include Cre (Sternberg and Hamilton, J. Mol. Biol., 150:467-486, 1981), lambda (Nash, Nature, 247, 543-545, 1974), FIp (Broach, *et al.*, Cell, 29:227-234, 1982), R (Matsuzaki, *et al.*, J. Bacteriology, 172:610-618, 1990), cpC31 (*see*, *e.g.*, Groth *et al.*, J. Mol. Biol. 335:667-678, 2004), sleeping beauty, transposases of the mariner family (Plasterk *et al.*, supra), and components for integrating viruses such as AAV, retroviruses, and antiviruses having components that provide for virus integration such as the LTR sequences of retroviruses or lentivirus and the ITR sequences of AAV (Kootstra *et al.*, Ann. Rev. Pharm. Toxicol., 43:413-439, 2003). In addition, direct and targeted genetic integration strategies may be used to insert nucleic acid sequences encoding the chimeric fusion proteins including CRISPR/CAS9, zinc finger, TALEN, and meganuclease gene-editing technologies.

In one aspect, the invention provides expression vectors for the expression of the heterodimeric proteins (and/or additional agents) that are viral vectors. Many viral vectors useful for gene therapy are known (*see*, *e.g.*, Lundstrom, Trends Biotechnol., 21: 1 17, 122, 2003. Illustrative viral vectors include those selected from Antiviruses (LV), retroviruses (RV), adenoviruses (AV), adeno-associated viruses (AAV), and α viruses, though other viral vectors may also be used. For *in vivo* uses, viral vectors that do not integrate into the host genome are suitable for use, such as α viruses and adenoviruses. Illustrative types of α viruses include Sindbis virus, Venezuelan equine encephalitis (VEE) virus, and Semliki Forest virus (SFV). For *in vitro* uses, viral vectors that integrate into the host genome are suitable, such as retroviruses, AAV, and Antiviruses. In one embodiment, the invention provides methods of transducing a human cell *in vivo*, comprising contacting a solid tumor *in vivo* with a viral vector of the invention.

In various embodiments, the present technology provides a host cell, comprising the expression vector comprising the heterodimeric protein described herein.

Expression vectors can be introduced into host cells for producing the present heterodimeric proteins. Cells may be cultured *in vitro* or genetically engineered, for example. Useful mammalian host cells include, without limitation, cells derived from humans, monkeys, and rodents (*see*, for example, Kriegler in "Gene Transfer and Expression: A Laboratory Manual," 1990, New York, Freeman & Co.). These include monkey kidney cell lines transformed by

SV40 (*e.g.*, COS-7, ATCC CRL 1651); human embryonic kidney lines (*e.g.*, 293, 293-EBNA, or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J Gen Virol* 1977, 36:59); baby hamster kidney cells (*e.g.*, BHK, ATCC CCL 10); Chinese hamster ovary-cells-DHFR (*e.g.*, CHO, Urlaub and Chasin, *Proc Natl Acad Sci USA* 1980, 77:4216); DG44 CHO cells, CHO-K1 cells, mouse sertoli cells (Mather, *Biol Reprod* 1980, 23:243-251); mouse fibroblast cells (*e.g.*, NIH-3T3), monkey kidney cells (*e.g.*, CV1 ATCC CCL 70); African green monkey kidney cells. (*e.g.*, VERO-76, ATCC CRL-1587); human cervical carcinoma cells (*e.g.*, HELA, ATCC CCL 2); canine kidney cells (*e.g.*, MDCK, ATCC CCL 34); buffalo rat liver cells (*e.g.*, BRL 3A, ATCC CRL 1442); human lung cells (*e.g.*, W138, ATCC CCL 75); human liver cells (*e.g.*, Hep G2, HB 8065); and mouse mammary tumor cells (*e.g.*, MMT 060562, ATCC CCL51). Illustrative cancer cell types for expressing the fusion proteins described herein include mouse fibroblast cell line, NIH3T3, mouse Lewis lung carcinoma cell line, LLC, mouse mastocytoma cell line, P815, mouse lymphoma cell line, EL4 and its ovalbumin transfectant, *E.G7*, mouse melanoma cell line, B16F10, mouse fibrosarcoma cell line, MC57, and human small cell lung carcinoma cell lines, SCLC#2 and SCLC#7.

Host cells can be obtained from normal or affected subjects, including healthy humans, cancer patients, and patients with an infectious disease, private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers.

Cells that can be used for production of the present heterodimeric proteins *in vitro*, *ex vivo*, and/or *in vivo* include, without limitation, epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells (*e.g.*, as obtained from bone marrow), umbilical cord blood, peripheral blood, fetal liver, *etc.* The choice of cell type depends on the type of tumor or infectious disease being treated or prevented, and can be determined by one of skill in the art.

Production and purification of Fc-containing macromolecules (such as Fc fusion proteins) has become a standardized process, with minor modifications between products. For example, many Fc containing macromolecules are produced by human embryonic kidney (HEK) cells (or variants thereof) or Chinese Hamster Ovary (CHO) cells (or variants thereof) or in some cases by bacterial or synthetic methods. Following production, the Fc containing macromolecules that are secreted by HEK or CHO cells are purified through binding to Protein A columns and subsequently 'polished' using various methods. Generally speaking, purified Fc containing macromolecules are stored in liquid form for some period of time, frozen for extended periods of time or in some cases lyophilized. In various embodiments, production of the heterodimeric proteins contemplated herein may have unique characteristics as compared

to traditional Fc containing macromolecules. In certain examples, the heterodimeric proteins may be purified using specific chromatography resins, or using chromatography methods that do not depend upon Protein A capture. In other embodiments, the heterodimeric proteins may be purified in an oligomeric state, or in multiple oligomeric states, and enriched for a specific oligomeric state using specific methods. Without being bound by theory, these methods could include treatment with specific buffers including specified salt concentrations, pH and additive compositions. In other examples, such methods could include treatments that favor one oligomeric state over another. The heterodimeric proteins obtained herein may be additionally 'polished' using methods that are specified in the art. In some embodiments, the heterodimeric proteins are highly stable and able to tolerate a wide range of pH exposure (between pH 3-12), are able to tolerate a large number of freeze/thaw stresses (greater than 3 freeze/thaw cycles) and are able to tolerate extended incubation at high temperatures (longer than 2 weeks at 40 degrees C). In other embodiments, the heterodimeric proteins are shown to remain intact, without evidence of degradation, deamidation, etc. under such stress conditions.

Subjects and/or Animals

In some embodiments, the subject and/or animal is a mammal, *e.g.*, a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, rabbit, sheep, or non-human primate, such as a monkey, chimpanzee, or baboon. In other embodiments, the subject and/or animal is a non-mammal, such, for example, a zebrafish. In some embodiments, the subject and/or animal may comprise fluorescently-tagged cells (with *e.g.,* GFP). In some embodiments, the subject and/or animal is a transgenic animal comprising a fluorescent cell.

In some embodiments, the subject and/or animal is a human. In some embodiments, the human is a pediatric human. In other embodiments, the human is an adult human. In other embodiments, the human is a geriatric human. In other embodiments, the human may be referred to as a patient.

In certain embodiments, the human has an age in a range of from about 0 months to about 6 months old, from about 6 to about 12 months old, from about 6 to about 18 months old, from about 18 to about 36 months old, from about 1 to about 5 years old, from about 5 to about 10 years old, from about 10 to about 15 years old, from about 15 to about 20 years old, from about 20 to about 25 years old, from about 25 to about 30 years old, from about 30 to about 35 years old, from about 35 to about 40 years old, from about 40 to about 45 years old, from about 45 to about 50 years old, from about 50 to about 55 years old, from about 55 to about 60 years old, from about 60 to about 65 years old, from about 65 to about 70 years old, from about 70 to about 75 years old, from about 75 to about 80 years old, from about 80 to about 85 years old,

from about 85 to about 90 years old, from about 90 to about 95 years old or from about 95 to about 100 years old.

In other embodiments, the subject is a non-human animal, and therefore the invention pertains to veterinary use. In a specific embodiment, the non-human animal is a household pet. In another specific embodiment, the non-human animal is a livestock animal.

Kits

The invention provides kits that can simplify the administration of any agent described herein. An illustrative kit of the invention comprises any composition described herein in unit dosage form. In one embodiment, the unit dosage form is a container, such as a pre-filled syringe, which can be sterile, containing any agent described herein and a pharmaceutically acceptable carrier, diluent, excipient, or vehicle. The kit can further comprise a label or printed instructions instructing the use of any agent described herein. The kit may also include a lid speculum, topical anesthetic, and a cleaning agent for the administration location. The kit can also further comprise one or more additional agent described herein. In one embodiment, the kit comprises a container containing an effective amount of a composition of the invention and an effective amount of another composition, such those described herein.

Definitions

As used in this Specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive and covers both "or" and "and".

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About is understood to be within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from the context, all numerical values provided herein are modified by the term "about."

A stated range is understood to be any value between and at the limits of the stated range. As examples, a range between 1 and 5 includes 1, 2, 3, 4, and 5; a range between 1 and 10 includes 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10; and a range between 1 and 100 includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although other probes, compositions, methods, and kits similar, or equivalent, to those described herein can be used in the practice of the present technology, the preferred materials and methods are described herein. It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

Any aspect or embodiment described herein can be combined with any other aspect or embodiment as disclosed herein.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

The present invention is further illustrated by the following example, which should not be construed as limiting in any way.

Example 1: Construction and Characterization of Heterodimers Comprising Butyrophilin-like (BTNL) Family Proteins of the Present Technology

Various non-limiting protein engineering embodiments of the present technology are shown in **FIG. 1**. As shown in **FIG. 1** (top panel), the heterodimeric proteins of the present technology comprise a butyrophilin family member, a core domain, and an antigen-target. Accordingly, the exemplary heterodimeric proteins of the present technology include:

i) a human butyrophilin 3/8 heterodimer adjoined to either a human CD19-specific or PSMA-specific scFv;

ii) a human butyrophilin 3A1/3A2 heterodimer adjoined to either a human CD19-specific or PSMA-specific scFv;

iii) a human butyrophilin 3A1/3A3 heterodimer adjoined to either a human CD19-specific or PSMA-specific scFv; and

iv) a mouse butyrophilin 1/6 heterodimer adjoined to a mouse CD19-specific scFv.

The butyrophilin family members contemplated in a heterodimeric construct include but are not limited to: BTN1A1, BTN2A1, BTN2A2, BTN2A3, BTN3A3, BTNL2, BTNL9, BTNL10, SKINT, *etc* (**FIG. 1** (middle panel)). Other antigen-targets for a butyrophilin heterodimeric construct include but are not limited to: GD2, PSCA, BCMA, CD123, B7-H3, CD20, CD30, CD33, CD38, CEA, CLEC12A, DLL3, EGFRvIII, EpCAM, CD307, FLT3, GPC3, gpA33, HER2, MUC16, P-cadherin, SSTR2, mesothelin, *etc* (**FIG. 1** (middle panel)).

Without being bound by theory, the proposed mechanism of action for a butyrophilin heterodimer construct targeting either CD19 or PSMA as illustrated in the bottom panel. In this example, engagement of gamma delta T cells to CD19 or PSMA positive tumor cells is enhanced due to the butyrophilin heterodimer simultaneously engaging a tumor antigen and the gamma delta T cell receptor. The contemplated GAmma DELta T cell ENgager constructs are referred to herein as 'GADLEN' fusion proteins.

The sequences of exemplary embodiments of GADLEN fusion proteins are provided in the Table below (double underlined sequences are the leader sequence, single underlined is the linker):

SEQ ID NO Species Description	Sequence
66 Human BTNL3-Alpha-scFVh19	MEPGLSWVFLVAIIKGVQCQWQVTGPGKFVQALVGEDAVFSCSLFPETSAE AMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRV SLRLKNITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDG GIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQE NAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGKRGSK YGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYK CKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN VFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSGSDIQLTQSPASLAVSL GQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRF SGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK*
67 Human BTNL8-Beta-scFVlh19	MEPGLSWVFLVAIIKGVQCQWQVFGPDKPVQALVGEDAAFSCFLSPKTNA EAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRI SLRLENITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDR DIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQ ENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGEDG SKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKE YKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQE GNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSIQLTQSPAIMSA SPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFS GSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELK *
68 Human BTNL3-Alpha-scFVhPSMA	MEPGLSWVFLVAIIKGVQCQWQVTGPGKFVQALVGEDAVFSCSLFPETSAE AMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRV SLRLKNITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDG GIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQE NAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGKRGSK YGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYK CKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN VFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSGSGEVQLVESGGGLV QPGGSLTLSCAASRFMISEYHMHWVRQAPGKGLEWVSTINPAGTTDYAE SVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCDSYGYRGQGTQVTV*

69 Human BTNL8-Beta- scFVlPSMA	MEFGLSWVFLVAILKGVQCQWQVFGPDKPVQALVGEDAAFSCFLSPKTNA EAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRI SLRLENITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDR DIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQ ENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGEDG SKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKE YKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQE GNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSGQTVVTQEPSLT VSPGGTVTLTCASSTGAVTSGNYPNWVQQKPGQAPRGLIGGTKFLVPGTP ARFSGSLLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVFGGGTKLTVL*
70 Human BTN3A1- Alpha- scFvCD19	MEFGLSWVFLVAILKGVQCQFSVLGPSGPILAMVGEDADLPCHLFPTMSAE TMELKWVSSSLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAA LRIHNVTASDSGKYLCYFQDGDFYEKALVELKVAALGSDLHVDVKGYK DGGIHLECRSTGWYPQPQIQWSNNKGENIPTVEAPVVADGVGLYAVAAS VIMRGSSGEGVSCTIRSSLLGLEKTASISIADPFFRSAQRWIAALAGGSGSR KGGKRGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVV DVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQD WLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTV DKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSQVQLQQ SGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGD GDTNYNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTV GRYYYAMDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIQLTQSPASLAV SLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPP RFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK
71 Human BTN3A2- Beta- scFvCD19	MEFGLSWVFLVAILKGVQCQFSVLGPSGPILAMVGEDADLPCHLFPTMSAE TMELKWVSSSLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAA LRIHNVTASDSGKYLCYFQDGDFYEKALVELKVAALGSNLHVEVKGYED GGIHLECRSTGWYPQPQIQWSNAKGENIPAVEAPVVADGVGLYEVAASVI MRGGSGEGVSCIIRNSLLGLEKTASISIADPFFRSAQPWGSGSDEGGEDGS KYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDP EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEY KCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVK GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG NVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSQVQLQQSGAELV RPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNY NGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYY AMDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIQLTQSPASLAVSLGQR ATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSGS GSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK
72 Human BTNL3- Alpha- scFvCD19-2	MEFGLSWVFLVAILKGVQCQWQVTGPGKFVQALVGEDAVFSCSLFPETSAE AMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRV SLRLKNITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDG GIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQE NAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGKRGSK YGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYK CKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN VFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSQVQLQQSGAELVRPG SSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGK FKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMD

	YWGQGTTVTVSSGGGGSGGGGSGGGGSDIQLTQSPASLAVSLGQRATISC KASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSGSGSGTD FTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK
73 Human BTNL8-Beta-scFvCD19-2	MEFGLSWVFLVAILKGVQCQWQVFGPDKPVQALVGEDAAFSCFLSPKTNA EAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRI SLRLENITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDR DIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQ ENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGEDG SKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKE YKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQE GNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSQVQLQQSGAEL VRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTN YNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYY YAMDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIQLTQSPASLAVSLGQ RATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSG SGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK
74 Human BTNL3-KIHT22Y-scFvCD19-2	MEFGLSWVFLVAILKGVQCQWQVTGPGKFVQALVGEDAVFSCSLFPETSAE AMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRV SLRLKNITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDG GIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQE NAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASEPKSCDKTHTCPPC PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA LPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLYCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGKGGGSQVQLQQSGAELVRPGSSVKISCKAS GYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLTA DESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGTTV TVSSGGGGSGGGGSGGGGSDIQLTQSPASLAVSLGQRATISCKASQSVDY DGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSGSGSGTDFTLNIHPVE KVDAATYHCQQSTEDPWTFGGGTKLEIK
75 Human BTNL8-KIHY86T-scFvCD19-2	MEFGLSWVFLVAILKGVQCQWQVFGPDKPVQALVGEDAAFSCFLSPKTNA EAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRI SLRLENITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDR DIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQ ENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLYCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPGKGGGSQVQLQQSGAELVRPGSSVKISCKA SGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLT ADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGTT VTVSSGGGGSGGGGSGGGGSDIQLTQSPASLAVSLGQRATISCKASQSVD YDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSGSGSGTDFTLNIHP VEKVDAATYHCQQSTEDPWTFGGGTKLEIK
76 Mouse BTNL1-Alpha-scFvCD19V HVL	MEFGLSWVFLVAILKGVQCEVSWFSVKGPAEPITVLLGTEATLPCQLSPEQS AARMHIRWYRAQPTPAVLVFHNGQEQGEVQMPEYRGRTQMVRQAIDM GSVALQIQQVQASDDGLYHCQFTDGFTSQEVSMELRVIGLGSAPLVHMT GPENDGIRVLCSSSGWFPKPKVQWRDTSGNMLLSSSELQTQDREGLFQVE VSLLVTDRAIGNVICSIQNPMYDQEKSKAILLPEPFFPKTCPWKGSGSDEG GEDGVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPKVTCVVVDISK

	DDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGK EFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCM ITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNW EAGNTFTCSVLHEGLHNHHTEKSLSHSPGIRKGGKRGSGSEVQLQQSGAE LVRPGTSVKLSCKVSGDTITFYYMHFVKQRPGQGLEWIGRIDPEDESTKY SEKFKNKATLTADTSSNTAYLKLSSLTSEDTATYFCIYGGYYFDYWGQGV MVTVSSGGGGSGGGGSGGGGSDIQMTQSPASLSTSLGETVTIQCQASEDI YSGLAWYQQKPGKSPQLLIYGASDLQDGVPSRFSGSGSGTQYSLKITSMQ TEDEGVYFCQQGLTYPRTFGGGTKLELK
77 Mouse BTNL6-Beta- scFvCD19V HVL	*MEFGLSWVFLVAIIKGVQC*EQLPEYSQRTSLVKEQFHQGTAAVRILNVQAP DSGIYICHFKQGVFYEEAILELKVAAMGSVPEVYIKGPEDGGVCVVCITSG WYPEPQVHWKDSRGEKLTASLEIHSEDAQGLFRTETSLVVRDSSVRNVTC STFNPILGQEKAMAMFLPEPFFPKVSPWKPGSGSDEGGEDGVPRDCGCKP CICTVPEVSSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDD VEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAP IEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQ WNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLHE GLHNHHTEKSLSHSPGIRKGGKRGSGSEVQLQQSGAELVRPGTSVKLSCK VSGDTITFYYMHFVKQRPGQGLEWIGRIDPEDESTKYSEKFKNKATLTAD TSSNTAYLKLSSLTSEDTATYFCIYGGYYFDYWGQGVMVTVSSGGGGSG GGGSGGGGSDIQMTQSPASLSTSLGETVTIQCQASEDIYSGLAWYQQKPG KSPQLLIYGASDLQDGVPSRFSGSGSGTQYSLKITSMQTEDEGVYFCQQG LTYPRTFGGGTKLELK
78 Mouse BTNL1- Alpha- scFvCD19VL VH	*MEFGLSWVFLVAIIKGVQC*EVSWFSVKGPAEPITVLLGTEATLPCQLSPEQS AARMHIRWYRAQPTPAVLVFHNGQEQGEVQMPEYRGRTQMVRQAIDM GSVALQIQQVQASDDGLYHCQFTDGFTSQEVSMELRVIGLGSAPLVHMT GPENDGIRVLCSSSGWFPKPKVQWRDTSGNMLLSSSELQTQDREGLFQVE VSLLVTDRAIGNVICSIQNPMYDQEKSKAILLPEPFFPKTCPWKGSGSDEG GEDGVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPKVTCVVVDISK DDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGK EFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCM ITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNW EAGNTFTCSVLHEGLHNHHTEKSLSHSPGIRKGGKRGSGSDIQMTQSPAS LSTSLGETVTIQCQASEDIYSGLAWYQQKPGKSPQLLIYGASDLQDGVPSR FSGSGSGTQYSLKITSMQTEDEGVYFCQQGLTYPRTFGGGTKLELKGGGG SGGGGSGGGGSEVQLQQSGAELVRPGTSVKLSCKVSGDTITFYYMHFVK QRPGQGLEWIGRIDPEDESTKYSEKFKNKATLTADTSSNTAYLKLSSLTSE DTATYFCIYGGYYFDYWGQGVMVTVSS
79 Mouse BTNL6-Beta- scFvCD19VL VH	*MEFGLSWVFLVAIIKGVQC*EQLPEYSQRTSLVKEQFHQGTAAVRILNVQAP DSGIYICHFKQGVFYEEAILELKVAAMGSVPEVYIKGPEDGGVCVVCITSG WYPEPQVHWKDSRGEKLTASLEIHSEDAQGLFRTETSLVVRDSSVRNVTC STFNPILGQEKAMAMFLPEPFFPKVSPWKPGSGSDEGGEDGVPRDCGCKP CICTVPEVSSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDD VEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAP IEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQ WNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLHE GLHNHHTEKSLSHSPGIRKGGKRGSGSDIQMTQSPASLSTSLGETVTIQCQ ASEDIYSGLAWYQQKPGKSPQLLIYGASDLQDGVPSRFSGSGSGTQYSLKI TSMQTEDEGVYFCQQGLTYPRTFGGGTKLELKGGGGSGGGGSGGGGSEV QLQQSGAELVRPGTSVKLSCKVSGDTITFYYMHFVKQRPGQGLEWIGRID PEDESTKYSEKFKNKATLTADTSSNTAYLKLSSLTSEDTATYFCIYGGYYF DYWGQGVMVTVSS
80 Human	*MEFGLSWVFLVAIIKGVQC*QWQVTGPGKFVQALVGEDAVFSCSLFPETSAE AMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRV

BTNL3-A-19scFv3	SLRLKNITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDG GIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQE NAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGKRGSK YGPP**CPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQE DPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLS GKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLT VDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGK**DEGGEDGSDIQ MTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSR LHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKL EITGGGSGGGSGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVS WIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSL QTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSS*
81 Human BTNL8-A-19scFv3	*MEFGLSWVFLVAIIKGVQC*QWQVFGPDKPVQALVGEDAAFSCFLSPKTNA EAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRI SLRLENITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDR DIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQ ENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGEDG SKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKE YKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQE GNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSDIQMTQTTSSLS ASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFS GSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGGGSGGG SGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGL EWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYC AKHYYYGGSYAMDYWGQGTSVTVSS*
82 Human BTN3A1-A-19scFv3	*MEFGLSWVFLVAIIKGVQC*QFSVLGPSGPILAMVGEDADLPCHLFPTMSAE TMELKWVSSSLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAA LRIHNVTASDSGKYLCYFQDGDFYEKALVELKVAALGSDLHVDVKGYK DGGIHLECRSTGWYPQPQIQWSNNKGENIPTVEAPVVADGVGLYAVAAS VIMRGSSGEGVSCTIRSSLLGLEKTASISIADPFFRSAQRWIAALAGGSGSR KGGKRGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVV DVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQD WLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTV DKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSDIQMTQ TTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSG VPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGG GSGGGSGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQP PRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDT AIYYCAKHYYYGGSYAMDYWGQGTSVTVSS
83 Human BTN3A2-B-19scFv3	*MEFGLSWVFLVAIIKGVQC*QFSVLGPSGPILAMVGEDADLPCHLFPTMSAE TMELKWVSSSLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAA LRIHNVTASDSGKYLCYFQDGDFYEKALVELKVAALGSNLHVEVKGYED GGIHLECRSTGWYPQPQIQWSNAKGENIPAVEAPVVADGVGLYEVAASVI MRGGSGEGVSCIIRNSLLGLEKTASISIADPFFRSAQPWGSGSDEGGEDGS KYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDP EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEY KCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVK GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG NVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSDIQMTQTTSSLSA SLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSG

	SGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGGGSGGGS GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLE WLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCA KHYYYGGSYAMDYWGQGTSVTVSS
84 Human BTNL3-A- GD2scFv3	*MEFGLSWVFLVAIIKGVQC*QWQVTGPGKFVQALVGEDAVFSCSLFPETSAE AMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRV SLRLKNITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDG GIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQE NAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGKRGSK YGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYK CKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN VFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSDVVMTQTPLSLPVSLG DQASISCRSSQSLVHRNGNTYLHWYLQKPGQSPKLLIHKVSNRFSGVPDR FSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPPLTFGAGTKLELKGGGS GGGSGGGSEVQLLQSGPELEKPGASVMISCKASGSSFTGYNMNWVRQNI GKSLEWIGAIDPYYGGTSYNQKFKGRATLTVDKSSSTAYMHLKSLTSEDS AVYYCVSGMKYWGQGTSVTVSS
85 Human BTNL8-B- GD2scFv3	*MEFGLSWVFLVAIIKGVQC*QWQVFGPDKPVQALVGEDAAFSCFLSPKTNA EAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRI SLRLENITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDR DIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQ ENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGEDG SKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKE YKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQE GNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSDVVMTQTPLSL PVSLGDQASISCRSSQSLVHRNGNTYLHWYLQKPGQSPKLLIHKVSNRFS GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPPLTFGAGTKLEL KGGGSGGGSGGGSEVQLLQSGPELEKPGASVMISCKASGSSFTGYNMNW VRQNIGKSLEWIGAIDPYYGGTSYNQKFKGRATLTVDKSSSTAYMHLKSL TSEDSAVYYCVSGMKYWGQGTSVTVSS
86 Human BTN3A1-A- GD2scFv3	*MEFGLSWVFLVAIIKGVQC*QFSVLGPSGPILAMVGEDADLPCHLFPTMSAE TMELKWVSSSLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAA LRIHNVTASDSGKYLCYFQDGDFYEKALVELKVAALGSDLHVDVKGYK DGGIHLECRSTGWYPQPQIQWSNNKGENIPTVEAPVVADGVGLYAVAAS VIMRGSSGEGVSCTIRSSLLGLEKTASISIADPFFRSAQRWIAALAGGSGSR KGGKRGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVV DVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQD WLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTV DKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSDVVMT QTPLSLPVSLGDQASISCRSSQSLVHRNGNTYLHWYLQKPGQSPKLLIHK VSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPPLTFGA GTKLELKGGGSGGGSGGGSEVQLLQSGPELEKPGASVMISCKASGSSFTG YNMNWVRQNIGKSLEWIGAIDPYYGGTSYNQKFKGRATLTVDKSSSTAY MHLKSLTSEDSAVYYCVSGMKYWGQGTSVTVSS
87 Human BTN3A2-B- GD2scFv3	*MEFGLSWVFLVAIIKGVQC*QFSVLGPSGPILAMVGEDADLPCHLFPTMSAE TMELKWVSSSLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAA LRIHNVTASDSGKYLCYFQDGDFYEKALVELKVAALGSNLHVEVKGYED GGIHLECRSTGWYPQPQIQWSNAKGENIPAVEAPVVADGVGLYEVAASVI MRGGSGEGVSCIIRNSLLGLEKTASISIADPFFRSAQPWGSGSDEGGEDGS

	KYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDP EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEY KCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVK GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG NVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSDVVMTQTPLSLP VSLGDQASISCRSSQSLVHRNGNTYLHWYLQKPGQSPKLLIHKVSNRFSG VPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPPLTFGAGTKLELK GGGSGGGSGGGSEVQLLQSGPELEKPGASVMISCKASGSSFTGYNMNWV RQNIGKSLEWIGAIDPYYGGTSYNQKFKGRATLTVDKSSSTAYMHLKSLT SEDSAVYYCVSGMKYWGQGTSVTVSS
88-93	Intentionally omitted.
94 Human BTNL3- Alpha- vTIGIT	*MEFGLSWVFLVAILKGVQC*QWQVTGPGKFVQALVGEDAVFSCSLFPETSAE AMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRV SLRLKNITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDG GIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQE NAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGKRGSK YGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYK CKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN VFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSGSMMTGTIETTGNISA EKGGSIILQCHLSSTTAQVTQVNWEQQDQLLAICNADLGWHISPSFKDRV APGPGLGLTLQSLTVNDTGEYFCIYHTYPDGTYTGRIFL*
95 Human BTNL8-Beta- vTIGIT	*MEFGLSWVFLVAILKGVQC*QWQVFGPDKPVQALVGEDAAFSCFLSPKTNA EAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRI SLRLENITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDR DIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQ ENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGEDG SKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKE YKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQE GNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSMMTGTIETTGNI SAEKGGSIILQCHLSSTTAQVTQVNWEQQDQLLAICNADLGWHISPSFKD RVAPGPGLGLTLQSLTVNDTGEYFCIYHTYPDGTYTGRIFL*
96 Human BTNL3- Alpha- vLAG3	*MEFGLSWVFLVAILKGVQC*QWQVTGPGKFVQALVGEDAVFSCSLFPETSAE AMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRV SLRLKNITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDG GIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQE NAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGKRGSK YGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYK CKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGN VFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSGSGAPAQLPCSPTIPLQ DLSLLRRAGVTWQHQPDSGPPAAAPGHPLAPGPHPAAPSSWGPRPRRYT VLSVGPGGLRSGRLPLQPRVQLDERGRQRGDFSLWLRPARRADAGEYRA AVHLRDRALSCRLRLRLG*
97 Human BTNL8-Beta- vLAG3	*MEFGLSWVFLVAILKGVQC*QWQVFGPDKPVQALVGEDAAFSCFLSPKTNA EAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRI SLRLENITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDR DIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQ ENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGEDG SKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED

	PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKE YKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQE GNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSGAPAQLPCSPTI PLQDLSLLRRAGVTWQHQPDSGPPAAAPGHPLAPGPHPAAPSSWGPRPRR YTVLSVGPGGLRSGRLPLQPRVQLDERGRQRGDFSLWLRPARRADAGEY RAAVHLRDRALSCRLRLRLG*

The italicized portion of the sequences in the above table is a leader sequence. In some embodiments, the leader sequence is absent. In some embodiments, optionally with the leader sequence (as indicated with doubleunderlining elsewhere herein, or, in embodiments: MEFGLSWVFLVAIIKGVQC (SEQ ID NO: 47)) is omitted. In some embodiments, the nucleotide sequence encoding a GADLEN fusion protein of the present technology further comprise a Kozak sequence. In some embodiments, the nucleotide sequence of Kozak sequence is CCGCCACC (SEQ ID NO: 98).

Example 2: Characterization of GADLEN Proteins of the Present Techology

The BTNL1/6-CD19 scFv GADLEN protein was generated by dual-transfection of CHO cells with both a BTNL1-CD19 scFv ('alpha', predicted MW = 77.8 kDa) and a BTNL6-CD19 scFv ('beta', predicted MW = 71.2 kDa) construct, in which the so-called alpha and beta constructs contained linker domains which facilitated heterodimerization of the desired BTNL1/6-CD19 scFv GADLEN protein. The purified protein was analyzed by Western blot using non-reducing, reducing, and both reducing and deglycosylating conditions, following detection with an anti-mouse Fc antibody. As shown in **FIG. 2**, the purified mouse BTNL1/6-CD19 scFv GADLEN protein appeared as a high molecular weight band in non-reducing conditions (left lane), which resolved in two bands in reducing conditions (middle lane) and in and both reducing and deglycosylating conditions (right lane). These results indicate the presence of a disulfide-linked protein that reduces to two individual proteins (following disruption of the interchain disulfide bonds with β-mercaptoethanol) with molecular weights consistent with the predicted molecular weights for the alpha and beta chains. Based on the similarity between the reducing, and both reducing and deglycosylating conditions lanes, the GADLEN construct appears to have few glycosylations.

The purified mouse BTNL 1/6-CD19 scFv GADLEN was detected using an Fc-specific ELISA. As shown in **FIG. 3**, the purified mouse BTNL 1/6-CD19 scFv GADLEN could be quantitated using an Fc-specific ELISA method.

To understand whether the GADLEN proteins of the present technology can bind to cancer cells, flow cytometry-based experiments were performed. As shown in **FIG. 4**, two different mouse tumor cell lines (A20, mouse lymphoma) and WEHI-3 (mouse leukemia), which express CD19 at different densities (A20 at a higher density than WEHI-3), bound the BTNL 1/6-CD19 scFv GADLEN protein. A20 cells, which express at a higher density of CD19 compared to

WEHI-3 cells, bound also showed higher intensities of binding compared to WEHI-3 cells (**FIG. 4**).

In another assay, the purified mouse BTNL 1/6-CD19 scFv GADLEN construct was used to stain A20 cells, and followed by incubation with an APC-conjugated anti-mouse Fc antibody. Cells were detected by flow cytometry. As shown in **FIG. 5**, purified mouse BTNL 1/6-CD19 scFv GADLEN exhibited cell surface binding to A20 cells as detected by flow cytometry.

Another assay for detecting the binding of CD19 scFv GADLEN construct WEHI-3 cells was performed. In the assay, the purified mouse BTNL 1/6-CD19 scFv GADLEN construct was used to stain WEHI-3 cells, and was then detected with an APC-conjugated anti-mouse Fc antibody. As shown in **FIG. 6**, the purified mouse BTNL 1/6-CD19 scFv GADLEN showed cell surface binding to WEHI-3 cells as detected by flow cytometry.

These results demonstrate that GADLEN proteins of the present technology immunospecifically bind to cell surface. Accordingly, GADLEN proteins of the present technology are useful in the methods disclosed herein.

Example 3: In Vitro Activity of the GADLEN Proteins of the Present Techology

The *in vitro* activity was characterized using a cell lysis. In these studies, purified mouse gamma delta T cells (isolated from mouse intestinal epithelium) were co-cultured with mouse A20 lymphoma cells alone or together with an anti-mouse CD19 specific antibody (clone 1D3) at a gamma delta T cells:A20 tumor cell ratio of 1:1, for 18 hours with live imaging every hour in an Incucyte imager. A20 cells alone and gamma delta T cells alone were included as negative controls. The A20 tumor cells were labeled with green fluorescence, and the assay included a red-fluorescent dye specific for Annexin-5, as an indicator of cell death. **FIG. 7** provides representative results of a mouse gamma delta T cell/mouse tumor cell killing assay mediated by the CD19-specific antibody. The y-axis in the figure indicates the overlap between green and red fluorescence, which occurs when green-labeled A20 tumor cells undergo cell death and stain dual-positive for red-labeled Annexin-5. As shown in **FIG. 7**, the combination of A20 lymphoma cells, gamma delta T cells and anti-mouse CD19 specific antibody clone 1D3 (squares) showed higher level of cell lysis compared to untreated A20 cells and gamma delta T cells (triangles). A20 tumor cells alone (circles) or gamma delta T cells alone (diamonds), the negative controls, showed background lysis activity (**FIG. 7**).

Then, the *in vitro* activity of the purified mouse BTNL 1/6-CD19 scFv GADLEN was characterized using a cell lysis assay. Purified mouse gamma delta T cells (isolated from mouse intestinal epithelium) were co-cultured with mouse A20 lymphoma cells alone or together with BTNL1/6-CD19scFv GADLEN. A20 cells alone and gamma delta T cells alone were included as negative controls. The A20 tumor cells were labeled with green fluorescence, and the assay

included a red-fluorescent dye specific for Annexin-5, as an indicator of cell death. **FIG. 8** provides the representative results. The y-axis in the figure indicates the overlap between green and red fluorescence, which occurs when green-labeled A20 tumor cells undergo cell death and stain dual-positive for red-labeled Annexin-5. As shown in **FIG. 8**, BTNL1/6-CD19scFv GADLEN cultured with purified mouse gamma delta T cells (isolated from mouse intestinal epithelium) and mouse A20 lymphoma cells (circles) provided higher levels of lysis than untreated purified mouse gamma delta T cells and A20 lymphoma cells (triangles). A20 cells alone (inverted triangles) and gamma delta T cells alone (diamonds) showed only background levels of lysis. In **FIGs. 7** and **8**, the mean maximum A20 killing activity from **FIG. 7** is indicated with the dashed line, and the killing activity in the presence of the GADLEN construct was observed to be superior to that observed with the CD19 specific antibody. In both **FIGs. 7** and **8**, the elevated reading at time 0 hours suggests that rapid A20 cell killing occurs, since there is a ~30 minute delay between the addition of the cells and reagents and the initial image captured by the Incucyte reader and indicated as 0 hours.

These results demonstrate that GADLEN proteins of the present technology immunospecifically bind to and induce efficient cell lysis of target cells. Accordingly, GADLEN proteins of the present technology are useful in the methods disclosed herein.

To understand the mechanism of observed lysis, a different gamma delta T cells:tumor cell killing assay was performed and CD107a was studied in WEHI3 cells. CD107a is a marker of cell degranulation, which occurs when T cells release cytolytic granules containing perforin and granzymes. Mouse gamma delta T cells (isolated from intestinal epithelium) were co-cultured for 4-5 hours with WEHI-3 tumor cells alone, or in combination with an anti-CD19 antibody or an increasing concentration of the BTNL1/6-CD19scFv GADLEN construct. Gamma delta T cells alone was used as a negative control, and WEHI3 cells + gamma delta T cells were used to control the gamma delta T cells activity without an antibody. The proportion of gamma delta T cells staining positive for CD107a by flow cytometry was measured by flow cytometry following the indicated co-culture, and plotted as a bar graph of the proportion of gamma delta T cells staining positive for CD107a. As shown in **FIG. 9A**, compared to Gamma delta T cells alone, or WEHI3 cells + gamma delta T cells, addition of the GADLEN construct led to a dose-dependent CD107a expression in isolated gamma delta T cells. Notably, only a minor increase in CD107a expression was noted when gamma delta T cells were co-cultured with WEHI-3 tumor cells in the absence of the GADLEN construct. To further validate activation of gamma delta T cells, CD69, a cell surface marker of activated T cells, was studied by flow cytometry and plotted as a bar graph of the proportion of gamma delta T cells staining positive for CD69. As shown in **FIG. 9B**, addition of the GADLEN construct to WEHI3 cells + gamma delta T cells caused a dose-dependent upregulation of CD69. Notably, only minor

increase in CD69 expression was observed when gamma delta T cells were co-cultured with WEHI-3 tumor cells in the absence of the GADLEN construct.

The assay for upregulation of CD107a and CD69 was repeated in A20 cells. Mouse gamma delta T cells (isolated from intestinal epithelium) were co-cultured for 4-5 hours with A20 tumor cells alone, or in combination with an anti-CD19 antibody or an increasing concentration of the BTNL1/6-CD19scFv GADLEN construct. Gamma delta T cells alone was used as a negative control, and A20 cells + gamma delta T cells were used to control the gamma delta T cells activity without an antibody. The proportion of gamma delta T cells staining positive for CD107a by flow cytometry was measured by flow cytometry following the indicated co-culture, and plotted as a bar graph of the proportion of gamma delta T cells staining positive for CD107a. As shown in **FIG. 10A**, compared to Gamma delta T cells alone, or A20 cells + gamma delta T cells, addition of the GADLEN construct led to a dose-dependent CD107a expression in isolated gamma delta T cells. Notably, only a minor increase in CD107a expression was noted when gamma delta T cells were co-cultured with A20 tumor cells in the absence of the GADLEN construct. To further validate activation of gamma delta T cells, CD69, a cell surface marker of activated T cells, was studied by flow cytometry and plotted as a bar graph of the proportion of gamma delta T cells staining positive for CD69. As shown in **FIG. 10B**, addition of the GADLEN construct to A20 cells + gamma delta T cells caused a dose-dependent upregulation of CD69. Notably, only minor increase in CD69 expression was observed when gamma delta T cells were co-cultured with A20 tumor cells in the absence of the GADLEN construct.

These results demonstrate that GADLEN proteins of the present technology induce contemporaneous activation and targeting of gamma delta T cells to tumor cells. Accordingly, GADLEN proteins of the present technology are useful in the methods of modulating a patient's immune response disclosed herein.

Example 4: In Vivo Activity of the GADLEN Proteins of the Present Techology

The effect of GADLEN proteins of the present technology on B cells and T cells was evaluated next. Towards this goal, Balb.c mice were implanted in A20 tumor cells in the hind flank. When those tumors grew to approximately 40 mm^3, the mice were randomly assigned to four groups containing ≥ 5 mice. The four groups were dosed with (i) vehicle only, (ii) anti-PD1 antibody, (iii) anti-CD19 antibody, or (iv) the BTNL1/6-CD19scFv GADLEN protein (all dosed at 100 µg/mouse). 24 hours following the first treatment, peripheral blood was collected from each mouse and analyzed for the proportion of CD20+ B cells and CD3+ gamma delta TCR+ T cells by flow cytometry. As shown in **FIG. 11A**, the frequency of CD20+ B cells in the BTNL1/6-CD19scFv GADLEN protein treated group (1.29%) had reduced to by > 10 fold

compared to the vehicle only group (22.6%). In contrast, the frequency of CD20+ cells in the peripheral blood of anti-PD1 (18.5%) or anti-CD19 (19.9) antibody-treated groups had not significantly changed compared to the vehicle only group (22.6%). As shown in **FIG. 11B**, the frequency of gamma delta T cells in the peripheral blood of mice in the BTNL1/6-CD19scFv GADLEN protein treated group (8.12%) significantly increased compared to compared to the vehicle only group (0.46%). In contrast, the frequency of gamma delta T cells in the peripheral blood of mice in anti-PD1 (0.42%) or anti-CD19 (0.54) antibody-treated groups had not significantly changed compared to the vehicle only group (0.46%). These data indicated that whereas <1% of circulating CD3+ T cells expressed the gamma delta TCR in vehicle, anti-PD1 or anti-CD19 antibody treated mice, the proportion of gamma delta T cells has increased >10 fold within 24 hours of treatment with the BTNL1/6-CD19scFv GADLEN protein.

These results demonstrate that GADLEN proteins of the present technology stimulate the proliferation of gamma delta T cells *in vivo*. Accordingly, GADLEN proteins of the present technology are useful in the methods of treatment disclosed herein.

To extend these observation further, the effect of GADLEN proteins of the present technology on CD19+ and CD20+ B cells was evaluated. Balb.c mice were implanted in A20 tumor cells or WEHI-3 in the hind flank. When those tumors grew to approximately 40 mm^3, the mice were randomly assigned to four groups each containing ≥ 5 mice. The four groups were dosed with (i) vehicle only, (ii) anti-PD1 antibody, (iii) anti-CD19 antibody, or (iv) the BTNL1/6-CD19scFv GADLEN protein (all dosed at 100 μg/mouse). 24 hours following the first treatment, peripheral blood was collected from each mouse and analyzed for the proportion of CD19+ B cells and CD20+ B cells by flow cytometry. Shown in **FIGs. 12A-12D** are bar graphs showing CD19+ (**FIGs. 12A** and **12C**) or CD20+ B cells (**FIGs. 12B** and **12D**) plotted as mean ± SEM with ≥ 5 mice/group bearing A20 tumors (**FIGs. 12A-12B**) or WEHI-3 tumors (**FIGs. 12C-12D**).

As shown in **FIG. 12A**, the treatment with either anti-CD19 antibody, or the BTNL1/6-CD19scFv GADLEN protein led to a significant reduction in detection of CD19+ B cells compared to vehicle only control-treated mice in mice bearing A20 tumors. Similarly, as shown in **FIG. 12C**, the treatment with either anti-CD19 antibody, or the BTNL1/6-CD19scFv GADLEN protein led to a significant reduction in detection of CD19+ B cells compared to vehicle only control-treated mice in mice bearing WEHI-3 tumors. To understand whether the anti-CD19 antibody had an effect on detection of CD19 antigen, CD20 expression was evaluated. As shown in **FIG. 12B**, the frequency of CD20+ B cells significantly reduced in the BTNL1/6-CD19scFv GADLEN protein-treated mice compared to vehicle only control-treated mice in mice bearing A20 tumors. In contrast, the frequency of CD20+ B cells in the anti-CD19-treated mice did not show significant reduction. Similarly, as shown in **FIG. 12D**, the

frequency of CD20+ B cells significantly reduced in the BTNL1/6-CD19scFv GADLEN protein-treated mice compared to vehicle only control-treated mice in Balb.c mice with established WEHI-3 tumors. In contrast, the frequency of CD20+ B cells in the anti-CD19-treated mice did not show significant reduction.

These data illustrate that while the anti-CD19 treatment antibody competed with the CD19 detection antibody, the antibody treatment had not truly depleted B cells in the peripheral blood because the frequenc of CD20+ B cells (where there is no competition between the CD19 treatment antibody and the CD20 detection antibody) had not significantly changed. In contrast, all mice treated with the BTNL1/6-CD19scFv GADLEN protein showed near complete depletion of peripheral blood B cells within 24 hours of treatment.

The effect of BTNL1/6-CD19scFv GADLEN protein on gamma delta T cells was evaluated. Balb.c mice were implanted in A20 tumor cells or WEHI-3 in the hind flank. When those tumors grew to approximately 40 mm^3, the mice were randomly assigned to four groups each containing ≥ 5 mice. The four groups were dosed with (i) vehicle only, (ii) anti-PD1 antibody, (iii) anti-CD19 antibody, or (iv) the BTNL1/6-CD19scFv GADLEN protein (all dosed at 100 µg/mouse). 24 hours following the first treatment, peripheral blood was collected from each mouse and analyzed for the proportion of gamma delta cells by flow cytometry.

As shown in **FIG. 13A**, the frequency of gamma delta T cells in mice bearing established A20 tumors significantly increased in in the BTNL1/6-CD19scFv GADLEN protein-treated mice compared to vehicle only control-treated mice in mice bearing A20 tumors. In contrast, the treatment with anti-PD1 antibody or anti-CD19 antibody did not have a significant effect on the frequency of gamma delta T cells in mice bearing established A20 tumors compared to vehicle only control-treated mice. Likewise, as shown in **FIG. 13B** , the frequency of gamma delta T cells in mice bearing established WEHI-3 tumors significantly increased in in the BTNL1/6-CD19scFv GADLEN protein-treated mice compared to vehicle only control-treated mice in mice bearing A20 tumors. In contrast, the treatment with anti-PD1 antibody or anti-CD19 antibody did not have a significant effect on the frequency of gamma delta T cells in mice bearing established WEHI-3 tumors compared to vehicle only control-treated mice. These data illustrated that the BTNL1/6-CD19scFv GADLEN protein had stimulated rapid proliferation of mouse gamma delta T cells within 24 hours of treatment.

These results demonstrate that GADLEN proteins of the present technology induce a near complete depletion of peripheral blood B cells and stimulate rapid proliferation of gamma delta T cells within 24 hours of treatment and thereby modulate the immune response in a subject in need thereof. Accordingly, GADLEN proteins of the present technology are useful in the methods of treatment disclosed herein.

The anti-tumor activity of BTNL1/6-Fc-CD19scFv GADLEN protein was evaluated using the WEHI3 tumor model. WEHI-3 cells were inoculated subcutaneously on the rear flank. When the average starting tumor volume (STV) reached 90.92 mm^3 (day 0), treatments were begun. The treatment groups were anti-PD1 (clone RMP1-14), anti-CD19 (clone 1D3), and BTNL1/6-Fc-CD19scFv were each given in 3 doses IP; 100µg per dose on days 0, 3, and 6. Vehicle alone was used as a negative control. Tumor growth was assessed over a 17-day time course. Tumor volumes were measured and plotted as a function of time. As shown in **FIG. 32A**, the average tumor growth of a WEHI-3 (leukemia) tumors educed in BTNL1/6-Fc-CD19scFv-treated mice compared to the vehicle alone-treated mice. Tumor growth was also accessed on day 8 and day 10. As shown in **FIG. 32B**, the BTNL1/6-Fc-CD19scFv GADLEN-treated mice showed significantly smaller tumors compared to the vehicle alone-treated mice on day 8. The activity of BTNL1/6-Fc-CD19scFv GADLEN was more significant than that of anti-CD19 (clone 1D3) on day 8 (**FIG. 32B**). Similarly, as shown in **FIG. 32C**, the BTNL1/6-Fc-CD19scFv GADLEN-treated mice showed significantly smaller tumors compared to the vehicle alone-treated mice on day 10. The activity of BTNL1/6-Fc-CD19scFv GADLEN was more significant than that of anti-CD19 (clone 1D3) on day 10 (**FIG. 32C**).

The effect of BTNL1/6-Fc-CD19scFv GADLEN treatment on survival was evaluated using Kaplan-Meier curve of mice from **FIGs. 32A-32C**. Significance between survival curves was determined using Mantel-Cox. As shown in **FIG. 33**, anti-PD1 (clone RMP1-14), anti-CD19 (clone 1D3), and BTNL1/6-Fc-CD19scFv, showed an improved Mantel-Cox survival p=0.1283, 0.0145, and 0.0015, respectively. Therefore, while anti-PD1 (clone RMP1-14), anti-CD19 (clone 1D3), and BTNL1/6-Fc-CD19scFv showed improved survival, the activity of BTNL1/6-Fc-CD19scFv GADLEN was more significant than that of anti-PD1 (clone RMP1-14) and anti-CD19 (clone 1D3) (**FIG. 33**).

These results demonstrate that GADLEN proteins of the present technology have a significant anti-tumor activity, which provides a significantly improved survival in subjects in need thereof. Accordingly, GADLEN proteins of the present technology are useful in the methods of treatment of cancer disclosed herein.

Example 5: Characterization of BTNL3/8-CD19 scFv GADLEN Protein and BTN3A1/3A2-CD19 scFv GADLEN protein of the Present Techology

The BTNL3/8-CD19 scFv GADLEN protein was generated by dual-transfection of CHO cells with both a BTNL3-CD19 scFv ('alpha', predicted MW = 78.4 kDa) and a BTNL8-CD19 scFv ('beta', predicted MW = 78.9 kDa) construct (see the cartoon on the top of **FIG. 14**), in which the so-called alpha and beta constructs contained linker domains which facilitated heterodimerization of the desired BTNL 3/8-CD19 scFv GADLEN protein. The BTNL3/8-

CD19 scFv GADLEN protein was purified and analyzed by western blot using non-reducing, reducing, and both reducing and deglycosylating conditions, following detection with an anti-human BTNL8 antibody or anti-human Fc antibody.

As shown in **FIG. 16**, BTNL3/8-CD19 scFv GADLEN protein was recognized by both an anti-human BTNL8 antibody (left blot) or anti-human Fc antibody (right blot). The purified non-reduced human BTNL3/8-CD19 scFv GADLEN protein showed mobility consistent with a dimer of a BTNL3-CD19 scFv ('alpha', predicted MW = 78.4 kDa) and a BTNL8-CD19 scFv ('beta', predicted MW = 78.9 kDa) (left lanes in **FIG. 14**). Reducing (middle lanes in **FIG. 14**) and both reducing and deglycosylating (right lanes in **FIG. 14**) produced bands expected for BTNL3-CD19 scFv ('alpha', predicted MW = 78.4 kDa) and BTNL8-CD19 scFv ('beta', predicted MW = 78.9 kDa). These results indicated the presence of a disulfide-linked protein that reduces to a single band of roughly half the molecular weight of the non-reduced band (following disruption of the interchain disulfide bonds with β-mercaptoethanol). Because the molecular weights of the alpha and beta chains are so similar, a doublet band could not be detected under reducing conditions in this study. A small reduction in molecular weight was observed in both reduced and deglycosylated protein (third lanes) compared to reduced protein (second lanes), indicating that the GADLEN construct is likely to be a glycosylated protein. As shown in **FIG. 15**, depicts the purified human BTNL 3/8-CD19 scFv GADLEN protein could be quantitated using an Fc-specific ELISA method.

BTN3A1/3A2-CD19 scFv GADLEN protein was generated by dual-transfection of CHO cells with both a BTN3A1-CD19 scFv ('alpha', predicted MW = 77.4 kDa) and a BTN3A2-CD19 scFv ('beta', predicted MW = 77.7 kDa) construct ((see the cartoon on the top of **FIG. 16**), in which the so-called alpha and beta constructs contained linker domains which facilitated heterodimerization of the desired BTN 3A1/3A2-CD19 scFv GADLEN protein. The BTN3A1/3A2-CD19 scFv GADLEN protein was purified and analyzed by western blot using non-reducing, reducing, and both reducing and deglycosylating conditions, following detection with an anti-human BTN3A1/2 antibody or anti-human Fc antibody.

As shown in **FIG. 16**, BTN3A1/3A2-CD19 scFv GADLEN protein was recognized by both an anti-human BTN3A1/2 antibody (left blot in **FIG. 16**) or anti-human Fc antibody (right blot in **FIG. 16**). The non-reduced BTN3A1/3A2-CD19 scFv GADLEN protein (left lanes in **FIG. 16**) showed a mobility consistent with a dimer of the BTN3A1-CD19 scFv ('alpha', predicted MW = 77.4 kDa) and the BTN3A2-CD19 scFv ('beta', predicted MW = 77.7 kDa). In contrast, reduced (middle lanes in **FIG. 16**) as well as both reduced and deglycosylated (right lanes in **FIG. 16**) revealed the monomers: BTN3A1-CD19 scFv ('alpha', predicted MW = 77.4 kDa) and the BTN3A2-CD19 scFv ('beta', predicted MW = 77.7 kDa). These results indicated the presence of a disulfide-linked protein that reduces to a single band of roughly half the molecular

weight of the non-reduced band (following disruption of the interchain disulfide bonds with β-mercaptoethanol). Because the molecular weights of the alpha and beta chains are similar, a doublet band could not be detected under reducing conditions in this study. There was a small reduction in molecular weight between the reduced and both reduced and deglycosylated lanes, indicating that the GADLEN construct appears to be a glycosylated protein. A series of background bands were also present in the gel, which are likely due to either non-specific binding for each antibody and/or cleaved or incompletely translated proteins which are common in transient transfection productions with multiple plasmids.

As shown in **FIG. 17**, the purified human BTNL 3A1/3A2-CD19 scFv GADLEN could be quantitated using an Fc-specific ELISA method. A positive control (recombinant BTN3A1-Fc; squares in **FIG. 17**) was compared to the BTN3A1/3A2-CD19scFv construct (diamonds in **FIG. 17**), as well as to a control BTNL3/8-KIH-CD19scFv protein that includes the 'knob-in-hole' mutations in the linker domain to facilitate heterodimerization (circles in **FIG. 17**).

Example 6: In Vitro Activity of BTNL3/8-CD19 scFv GADLEN Protein and BTN3A1/3A2-CD19 scFv GADLEN protein of the Present Techology

The assay for upregulation of CD107a was performed using the BTNL3/8-CD19 scFv GADLEN protein and BTN3A1/3A2-CD19 scFv GADLEN protein in human Raji tumor cells (human Burkitt lymphoma cell line). Human gamma delta T cells isolated from human peripheral blood (total of >11 replicates from >3 human donors) were co-cultured for 4-5 hours with Raji tumor cells alone, or together with the anti-human CD20 antibody (Rituximab), BTNL3/8-CD19scFv, BTN3A1/3A2-CD19scFv, HMBPP (positive control) or BTN3A1/3A2-CD19scFv in addition to HMBPP. . Gamma delta T cells alone was used as a negative control, and Raji cells + gamma delta T cells were used to control the gamma delta T cells activity without an antibody. The proportion of gamma delta T cells staining positive for CD107a by flow cytometry was measured by flow cytometry following the indicated co-culture, and plotted as a bar graph of the proportion of gamma delta T cells staining positive for CD107a.

As shown in **FIG. 18A**, the frequency of CD107a+ human gamma delta T cells significantly increased following the co-culture with CD20 antibody (Rituximab), BTNL3/8-CD19scFv, BTN3A1/3A2-CD19scFv, HMBPP (positive control) or BTN3A1/3A2-CD19scFv in addition to HMBPP, compared to a combination human gamma delta T cells and Raji cells.

The assay for upregulation of CD69 was performed using the BTNL3/8-CD19 scFv GADLEN Protein and BTN3A1/3A2-CD19 scFv GADLEN protein in human Raji tumor cells (human Burkitt lymphoma cell line) from the same co-cultures as for the assay for upregulation of CD107a.

As shown in **FIG. 18B**, the proportion of human gamma delta T cells expressing the activation marker, significantly increased following the co-culture with CD20 antibody (Rituximab), BTNL3/8-CD19scFv, BTN3A1/3A2-CD19scFv, HMBPP (positive control) or BTN3A1/3A2-CD19scFv in addition to HMBPP, compared to a combination human gamma delta T cells and Raji cells. The data indicate that the BTNL3/8-CD19scFv and BTN3A1/3A2-CD19scFv GADLEN constructs both cause CD107a degranulation and CD69 expression in human gamma delta T cells, similar to what was observed in mouse gamma delta T cells using the species-specific construct. The increased activity of the BTN3A1/3A2 construct in this assay is likely a result of the higher proportion of Vγ9+ gamma delta T cells in peripheral blood, which are the gamma delta T cell population that selectively respond to a BTN3A1/3A2 heterodimer. Gamma delta T cells isolated from human intestinal epithelium would be comparatively enriched for Vγ4+ gamma delta T cells, and therefore be selectively responsive to a BTNL3/8 GADLEN construct.

Whether BTNL3/8-CD19 scFv GADLEN protein and BTN3A1/3A2-CD19 scFv GADLEN protein can mediate killing of cancer cells was studied. In these studies, purified human gamma delta T cells (isolated from human peripheral blood mononuclear cells) were co-cultured with human CD19+ Raji cells together with increasing concentrations (1-25μg/ml) of BTN3A1/A2-CD19scFv or BTNL3/8-Fc-CD19scFv heterodimer proteins at a gamma delta T cells:Raji tumor cell ratio of 1:1, for 8 hours with live imaging every hour in an Incucyte imager. The Raji tumor cells were labeled with green fluorescence, and the assay included a red-fluorescent dye specific for Annexin-5, as an indicator of cell death. Raji cells alone and gamma delta T cells alone were included as negative controls. The integrated intensity of red fluorescence, which occurs when Raji tumor cells undergo cell death and stain positive for red-labeled Annexin-5 was plotted as a function of time. As shown in **FIG. 34A**, BTN3A1/3A2-CD19 scFv GADLEN, in the repsence of gamma delta T cells, mediated increased killing of Raji cells (circles, squares and triangles) compared to Raji cells alone (diamonds) or gamma delta T cells alone (inverted triangle). 12.5 (squares) or 25 μg/ml BTN3A1/3A2-CD19 scFv GADLEN (circles) doses caused more efficient killing compared to 1 μg/ml (triangles) dose. Similarly, ss shown in **FIG. 34A**, BTNL3/8-CD19 scFv GADLEN protein, in the repsence of gamma delta T cells, mediated increased killing of Raji cells (circles, squares and triangles) compared to Raji cells alone (diamonds) or gamma delta T cells alone (inverted triangle). 12.5 (squares) or 25 μg/ml BTNL3/8-CD19 scFv GADLEN protein (circles) doses caused more efficient killing compared to 1 μg/ml (triangles) dose.

These results demonstrate that GADLEN proteins of the present technology induce contemporaneous activation and targeting of gamma delta T cells to tumor cells, causing

efficient cancer cell killing. Accordingly, GADLEN proteins of the present technology are useful in the methods of modulating a patient's immune response disclosed herein.

Example 7: Various GADLEN proteins of the Present Techology

Prepared in this example are heterodimeric proteins in which each polypeptide chain comprises a butyrophilin-like (BTNL) family protein linked by a charge polarized core domain to a portion of the extracellular domain of a Type I transmembrane protein. Similar to the heterodimeric proteins shown in **FIG. 1**, these heterodimeric proteins each comprise two polypeptide chains; however, here, a first polypeptide chain (*i.e.,* an alpha strand) comprises a portion of BTNL3 and a second polypeptide chain (*i.e.,* a beta strand) comprises a portion of BTNL8.

Without wishing to be bound by theory, typically the immune system is roughly divided into 'innate' immunity and 'adaptive' immunity. Innate immune responses are akin to a front-line response system which can provide rapid, albeit incomplete, protection to a variety of threats through recognition of pathogen- or cell damage-associated molecular patterns. Adaptive immunity can be triggered by innate immunity, takes longer to become fully activated, tends to be significantly more potent, is generally antigen-specific and, importantly, includes long-lasting memory responses. Without wishing to be bound by theory, there is not a clean division of labor between innate and adaptive immune responses, and there are a growing number of phenotypically-described immune cell types and effector molecules which straddle the typical definition between innate and adaptive immunity.

Without wishing to be bound by theory, one of the cell types that straddles the interface between innate and adaptive immunity are a sub-population of T cells wherein the T cell receptor is derived through somatic gene rearrangement of V gamma and V delta genes as opposed to V alpha and V beta genes. This cell population, known as gamma delta T cells, is found at lower frequencies than alpha beta T cells, but is particularly abundant at epithelial surfaces including, without limitation, the gastrointestinal tract, the skin, and the uterus. Some gamma delta T cells are also found in the blood, and may be important for recognizing specific antigens including phosphor-antigens in the blood. Interestingly, across a range of thirty-nine human tumors and a dataset derived from ~39,000 human cancer patients, one of the most favorable prognostic factors uncovered was the proportion of gamma delta T cells found within biopsied tumors. This information suggests, *inter alia*, that manipulation of gamma delta T cells may have important consequences in the treatment of human cancers.

Recently, the molecular mechanisms by which the gamma delta T cell receptor identify cognate antigen have been better defined. Interestingly, without wishing to be bound by theory, one of the primary activation mechanisms for gamma delta T cells appears to be mediated through an evolutionarily conserved family of receptors known as butyrophilin-like (BTNL) proteins.

There are at least two btnl proteins in mice (btnl 1 and btnl 6) and at least 6 BTNL proteins in humans (including BTNL1, BTNL3, BTNL6, BTNL8, BTN3A1, BTN3A2, and BTN3A3). In humans, activation of gamma delta T cells by BTNL family proteins is dependent upon heterodimerization of different individual BTNL proteins. In gut epithelium, a heterodimer comprising BTNL1, BTNL3, BTNL6 and BTNL8 is required for efficient gamma delta T cell activation. In the blood, a heterodimer comprising BTN3A1 and BTN3A2 appears to be most important for recognition of phospho-antigens. Thus, heterodimerization of BTNL proteins appears to be an important mechanism for activation of gamma delta T cells in specific tissues, and distinct heterodimers of BTNL proteins may help guide immunity to specific antigens across a range of tissues. The specific domain of BTNL family proteins that interact with the gamma delta T cell receptor are derived from the V-domain of BTNL proteins.

Due to the requirement for BTNL protein heterodimerization, and the potentially important role for gamma delta T cells in cancer and other human diseases, therapeutic proteins which could provide functional BTNL heterodimers are useful for the treatment of human diseases, including cancer. Such heterodimeric proteins include those derived from fusion proteins (including Fc fusion proteins), or from therapeutic proteins derived from monoclonal antibody binding domains (including antibodies, bi-specific antibodies, tri-specific antibodies, quadra-specific antibodies, etc. and also including fusion partners between native protein binding domains (further including BTNL proteins) and antibodies or antibody fragments). In some instances, the therapeutic protein is derived from an Fc-containing fusion protein wherein the therapeutic protein was specifically designed to facilitate heterodimerization between two individual polypeptide chains, wherein one polypeptide chain includes one BTNL protein (BTNL1/BTNL3 for example) and the other polypeptide chain includes another BTNL protein (BTNL6/BTNL8 for example). In further instances, the BTNL1/6 containing heterodimer is further comprised of a second set of therapeutic proteins which may provide a dual-mechanism of action to the BTNL1/6 containing fusion protein. As an example, the second mechanism of action provides targeting of the BTNL1/6 heterodimer to a specific tissue or tumor antigen, and in another example the second mechanism of action provides a second co-stimulatory signal to immune cells, and in yet another example the second mechanism of action may provide a mechanism to block immune inhibitory signals including so-called "checkpoint" molecules in human cancers.

In further instances, the BTNL3/8 containing heterodimer is further comprised of a second set of therapeutic proteins which may provide a dual-mechanism of action to the BTNL3/8 containing fusion protein. As an example, the second mechanism of action provides targeting of the BTNL3/8 heterodimer to a specific tissue or tumor antigen, and in another example the second mechanism of action provides a second co-stimulatory signal to immune cells, and in

yet another example the second mechanism of action may provide a mechanism to block immune inhibitory signals including so-called "checkpoint" molecules in human cancers.

Heterodimeric proteins relevant to this non- limiting example include the following specific combinations of alpha strands and beta strands:

(1) an alpha strand comprising a V-set domain of BTNL3 linked by a charge polarized core domain to a portion of the extracellular domain of T cell immunoreceptor with Ig and ITIM domains (TIGIT) and a beta strand comprising a V-set domain of BTNL8 linked by a charge polarized core domain to a portion of the extracellular domain of TIGIT. Illustrative alpha and beta strands have the following amino acid sequences:

BTNL3-Alpha-vTIGIT

CCGCCACCMEFGLSWVFLVAIIKGVQCQWQVTGPGKFVQALVGEDAVFSCSLFPETS
AEAMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRVSLRLK
NITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDGGIQLLCLSSGWFP
QPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQENAGSILCSIHLAEQSHEVES
KVLIGETFFQPSPWRLASGSGSRKGGKRGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKD
QLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSV
LTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQV
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG
NVFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSGSMMTGTIETTGNISAEKGGSII
LQCHLSSTTAQVTQVNWEQQDQLLAICNADLGWHISPSFKDRVAPGPGLGLTLQSLT
VNDTGEYFCIYHTYPDGTYTGRIFL (SEQ ID NO: 24)

BTNL8-Beta-vTIGIT

QWQVFGPDKPVQALVGEDAAFSCFLSPKTNAEAMEVRFFRGQFSSVVHLYRDGKDQ
PFMQMPQYQGRTKLVKDSIAEGRISLRLENITVLDAGLYGCRISSQSYYQKAIWELQV
SALGSVPLISITGYVDRDIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGL
FDVEISLTVQENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGE
DGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQF
NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPS
SIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSL
GKRKGGKRGSGSMMTGTIETTGNISAEKGGSIILQCHLSSTTAQVTQVNWEQQDQLL
AICNADLGWHISPSFKDRVAPGPGLGLTLQSLTVNDTGEYFCIYHTYPDGTYTGRIFL
(SEQ ID NO: 25)

(2) an alpha strand comprising a V-set domain of BTNL3 linked by a charge polarized core domain to a portion of the extracellular domain of lymphocyte-activation gene 3 (LAG-3) and a beta strand comprising a V-set domain of BTNL8 linked by a charge polarized core domain to a portion of the extracellular domain of LAG-3. Illustrative alpha and beta strands have the following amino acid sequences:

BTNL3-Alpha-vLAG3

QWQVTGPGKFVQALVGEDAVFSCSLFPETSAEAMEVRFFRNQFHAVVHLYRDGED
WESKQMPQYRGRTEFVKDSIAGGRVSLRLKNITPSDIGLYGCWFSSQIYDEEATWEL
RVAALGSLPLISIVGYVDGGIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGY
SLYDVEISIIVQENAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGK
RGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQF
NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPS
SIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSL
GKDEGGEDGSGSGAPAQLPCSPTIPLQDLSLLRRAGVTWQHQPDSGPPAAAPGHPLA
PGPHPAAPSSWGPRPRRYTVLSVGPGGLRSGRLPLQPRVQLDERGRQRGDFSLWLRP
ARRADAGEYRAAVHLRDRALSCRLRLRLG (SEQ ID NO: 26)

BTNL8-Beta-vLAG3

QWQVFGPDKPVQALVGEDAAFSCFLSPKTNAEAMEVRFFRGQFSSVVHLYRDGKDQ
PFMQMPQYQGRTKLVKDSIAEGRISLRLENITVLDAGLYGCRISSQSYYQKAIWELQV
SALGSVPLISITGYVDRDIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGL
FDVEISLTVQENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGE
DGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQF
NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPS
SIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSL
GKRKGGKRGSGSGAPAQLPCSPTIPLQDLSLLRRAGVTWQHQPDSGPPAAAPGHPLA
PGPHPAAPSSWGPRPRRYTVLSVGPGGLRSGRLPLQPRVQLDERGRQRGDFSLWLRP
ARRADAGEYRAAVHLRDRALSCRLRLRLG (SEQ ID NO: 27)

(3) an alpha strand comprising a portion of the extracellular domain of TIGIT linked by a charge polarized core domain to a V-set domain of BTNL3 and a beta strand comprising a portion of the extracellular domain of TIGIT linked by a charge polarized core domain to a V-set domain of BTNL8. Illustrative alpha and beta strands have the following amino acid sequences:

TIGIT-Alpha-vBTNL3

MMTGTIETTGNISAEKGGSIILQCHLSSTTAQVTQVNWEQQDQLLAICNADLGWHISP
SFKDRVAPGPGLGLTLQSLTVNDTGEYFCIYHTYPDGTYTGRIFLEVLESSVAEHGAR
FQIPGSGSRKGGKRGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVV
VDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKE
YKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALH
NHYTQKSLSLSLGKDEGGEDGSGSPLISIVGYVDGGIQLLCLSSGWFPQPTAKWKGPQ
GQDLSSDSRANADGYSLYDVEISIIVQENAGSILCSIHLAEQSHEVE (SEQ ID NO: 28)

TIGIT-Beta-vBTNL8

MMTGTIETTGNISAEKGGSIILQCHLSSTTAQVTQVNWEQQDQLLAICNADLGWHISP
SFKDRVAPGPGLGLTLQSLTVNDTGEYFCIYHTYPDGTYTGRIFLEVLESSVAEHGAR
FQIPGSGSDEGGEDGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVV
VDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKE
YKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALH
NHYTQKSLSLSLGKRKGGKRGSGSALGSVPLISITGYVDRDIQLLCQSSGWFPRPTAK
WKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQENAGSISCSMRHAHLSREVESRVQ
(SEQ ID NO: 29)

(4) an alpha strand comprising a portion of the extracellular domain of LAG-3 linked by a charge polarized core domain to a V-set domain of BTNL3 and a beta strand comprising a portion of the extracellular domain of LAG-3 linked by a charge polarized core domain to a V-set domain of BTNL8. Illustrative alpha and beta strands have the following amino acid sequences:

LAG3-Alpha-vBTNL3

VPVVWAQEGAPAQLPCSPTIPLQDLSLLRRAGVTWQHQPDSGPPAAAPGHPLAPGPH
PAAPSSWGPRPRRYTVLSVGPGGLRSGRLPLQPRVQLDERGRQRGDFSLWLRPARRA
DAGEYRAAVHLRDRALSCRLRLRLGQASMTASPPGSLRASDWVILNCSFSRPDRPAS
VHWFRNRGQGRVPVRESPHHHLAESFLFLPQVSPMDSGPWGCILTYRDGFNVSIMYN
LTVLGLEPPTPLTVYAGAGSRVGLPCRLPAGVGTRSFLTAKWTPPGGGPDLLVTGDN
GDFTLRLEDVSQAQAGTYTCHIHLQEQQLNATVTLAIITVTPKSFGSPGSLGKLLCEV
TPVSGQERFVWSSLDTPSQRSFSGPWLEAQEAQLLSQPWQCQLYQGERLLGAAVYFT
ELSSPGAQRSGRAPGALPAGHLGSGSRKGGKRGSKYGPPCPPCPAPEFLGGPSVFLFP
PKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTY
RVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEM
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKS

RWQEGNVFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSGSPLISIVGYVDGGIQLL
CLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQENAGSILCSIHLA
EQSHEVE (SEQ ID NO: 30)

LAG3-Beta-vBTNL8

VPVVWAQEGAPAQLPCSPTIPLQDLSLLRRAGVTWQHQPDSGPPAAAPGHPLAPGPH
PAAPSSWGPRPRRYTVLSVGPGGLRSGRLPLQPRVQLDERGRQRGDFSLWLRPARRA
DAGEYRAAVHLRDRALSCRLRLRLGQASMTASPPGSLRASDWVILNCSFSRPDRPAS
VHWFRNRGQGRVPVRESPHHHLAESFLFLPQVSPMDSGPWGCILTYRDGFNVSIMYN
LTVLGLEPPTPLTVYAGAGSRVGLPCRLPAGVGTRSFLTAKWTPPGGGPDLLVTGDN
GDFTLRLEDVSQAQAGTYTCHIHLQEQQLNATVTLAIITVTPKSFGSPGSLGKLLCEV
TPVSGQERFVWSSLDTPSQRSFSGPWLEAQEAQLLSQPWQCQLYQGERLLGAAVYFT
ELSSPGAQRSGRAPGALPAGHLGSGSDEGGEDGSKYGPPCPPCPAPEFLGGPSVFLFPP
KPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYR
VVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMT
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSR
WQEGNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSALGSVPLISITGYVDR
DIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQENAGSIS
CSMRHAHLSREVESRVQ (SEQ ID NO: 31)

(5) an alpha strand comprising a portion of the extracellular domain of programmed cell death protein 1 (PD-1) linked by a charge polarized core domain to a V-set domain of BTNL3 and a beta strand comprising a portion of the extracellular domain of PD-1 linked by a charge polarized core domain to a V-set domain of BTNL8. Illustrative alpha and beta strands have the following amino acid sequences:

PD1-Alpha-vBTNL3

PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTD
KLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQI
KESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLVGSGSRKGGKRGSKYGPPCPPCP
APEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNA
KTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPRE
PQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSGSPLI
SIVGYVDGGIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQ
ENAGSILCSIHLAEQSHEVE (SEQ ID NO: 32)

PD1-Beta-vBTNL8

PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTD
KLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQI
KESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLVGSGSDEGGEDGSKYGPPCPPCP
APEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNA
KTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPRE
PQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSAL
GSVPLISITGYVDRDIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGLFDV
EISLTVQENAGSISCSMRHAHLSREVESRVQ (SEQ ID NO: 33)

(6) an alpha strand comprising a V-set domain of BTNL3 linked by a charge polarized core domain to a portion of the extracellular domain of alpha CD19 (αCD19) and a beta strand comprising a V-set domain of BTNL8 linked by a charge polarized core domain to a portion of the antibody binding domain directed to CD19. Illustrative alpha and beta strands have the following amino acid sequences:

Alpha Strand

QWQVTGPGKFVQALVGEDAVFSCSLFPETSAEAMEVRFFRNQFHAVVHLYRDGED
WESKQMPQYRGRTEFVKDSIAGGRVSLRLKNITPSDIGLYGCWFSSQIYDEEATWEL
RVAALGSLPLISIVGYVDGGIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGY
SLYDVEISIIVQENAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGK
RGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQF
NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPS
SIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSL
GKDEGGEDGSGSDIQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPG
QPPKLLIYDASNLVSGIPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFG
GGTKLEIK*(SEQ ID NO: 35).

Beta Strand

QWQVFGPDKPVQALVGEDAAFSCFLSPKTNAEAMEVRFFRGQFSSVVHLYRDGKDQ
PFMQMPQYQGRTKLVKDSIAEGRISLRLENITVLDAGLYGCRISSQSYYQKAIWELQV
SALGSVPLISITGYVDRDIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGL
FDVEISLTVQENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGE
DGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQF
NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPS
SIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE

NNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSL
GKRKGGKRGSGSIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKR
WIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTK
LELK **(SEQ ID NO: 36).

(7) an alpha strand comprising a V-set domain of BTNL3 linked by a charge polarized core domain to a portion of an antibody derived binding domain directed to PSMA (αPSMA) and a beta strand comprising a V-set domain of BTNL8 linked by a charge polarized core domain to an antibody-derived binding domain directed to PSMA. Illustrative alpha and beta strands have the following amino acid sequences:

AlphaStrand

QWQVTGPGKFVQALVGEDAVFSCSLFPETSAEAMEVRFFRNQFHAVVHLYRDGED
WESKQMPQYRGRTEFVKDSIAGGRVSLRLKNITPSDIGLYGCWFSSQIYDEEATWEL
RVAALGSLPLISIVGYVDGGIQLLCLSSGWFPQPTAKWKGPQGQDLSSDSRANADGY
SLYDVEISIIVQENAGSILCSIHLAEQSHEVESKVLIGETFFQPSPWRLASGSGSRKGGK
RGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQF
NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPS
SIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSL
GKDEGGEDGSGSGEVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHWVRQAPGK
GLEWVSTINPAGTTDYAESVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCDSYG
YRGQGTQVTV* (SEQ ID NO: 37).

Beta Strand

QWQVFGPDKPVQALVGEDAAFSCFLSPKTNAEAMEVRFFRGQFSSVVHLYRDGKDQ
PFMQMPQYQGRTKLVKDSIAEGRISLRLENITVLDAGLYGCRISSQSYYQKAIWELQV
SALGSVPLISITGYVDRDIQLLCQSSGWFPRPTAKWKGPQGQDLSTDSRTNRDMHGL
FDVEISLTVQENAGSISCSMRHAHLSREVESRVQIGDTFFEPISWHLATKGSGSDEGGE
DGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQF
NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPS
SIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSL
GKRKGGKRGSGSGQTVVTQEPSLTVSPGGTVTLTCASSTGAVTSGNYPNWVQQKPG
QAPRGLIGGTKFLVPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVF
GGGTKLTVL* (SEQ ID NO: 38).

(8) an alpha strand comprising a V-set domain of BTNL3 linked by a charge polarized core domain to a portion of the extracellular domain of alpha CD19 (αCD19) and a beta strand comprising a V-set domain of BTNL8 linked by a charge polarized core domain to a portion of the antibody binding domain directed to CD19. Illustrative alpha and beta strands have the following amino acid sequences:

Alpha Strand

CCGCCACCMEFGLSWVFLVAIIKGVQCQWQVTGPGKFVQALVGEDAVFSCSLFPETS
AEAMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRVSLRLK
NITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDGGIQLLCLSSGWFP
QPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQENAGSILCSIHLAEQSHEVES
KVLIGETFFQPSPWRLASGSGSRKGGKRGSKYGPPCPPCPAPEFLGGPSVFLFPPKPKD
QLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSV
LTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQV
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG
NVFSCSVLHEALHNHYTQKSLSLSLGKDEGGEDGSQVQLQQSGAELVRPGSSVKISC
KASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLTADESSS
TAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGTTVTVSSGGGGSGGG
GSGGGGSDIQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKL
LIYDASNLVSGIPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKL
EIK (SEQ ID NO: 39).

Beta Strand

CCGCCACCMEFGLSWVFLVAIIKGVQCQWQVFGPDKPVQALVGEDAAFSCFLSPKT
NAEAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRISLRLE
NITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDRDIQLLCQSSGWFP
RPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQENAGSISCSMRHAHLSREVE
SRVQIGDTFFEPISWHLATKGSGSDEGGEDGSKYGPPCPPCPAPEFLGGPSVFLFPPKP
KDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVV
SVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKN
QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQ
EGNVFSCSVLHEALHNHYTQKSLSLSLGKRKGGKRGSGSQVQLQQSGAELVRPGSSV
KISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLTAD
ESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGTTVTVSSGGGGS
GGGGSGGGGSDIQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQP
PKLLIYDASNLVSGIPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGG
TKLEIK (SEQ ID NO: 40).

(9) an alpha strand comprising a V-set domain of BTNL3 linked by a charge polarized core domain to a portion of the extracellular domain of alpha CD19 (αCD19) and a beta strand comprising a V-set domain of BTNL8 linked by a charge polarized core domain to a portion of the antibody binding domain directed to CD19. Illustrative alpha and beta strands have the following amino acid sequences:

Alpha Strand

CCGCCACCMEFGLSWVFLVAIIKGVQCQWQVTGPGKFVQALVGEDAVFSCSLFPETS
AEAMEVRFFRNQFHAVVHLYRDGEDWESKQMPQYRGRTEFVKDSIAGGRVSLRLK
NITPSDIGLYGCWFSSQIYDEEATWELRVAALGSLPLISIVGYVDGGIQLLCLSSGWFP
QPTAKWKGPQGQDLSSDSRANADGYSLYDVEISIIVQENAGSILCSIHLAEQSHEVES
KVLIGETFFQPSPWRLASEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP
EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD
WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLYCLV
KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGKGGGSQVQLQQSGAELVRPGSSVKISCKASGYAFSSY
WMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLTADESSSTAYMQLSSLA
SEDSAVYFCARRETTTVGRYYYAMDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIQ
LTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSG
IPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK (SEQ ID NO: 41).

Beta Strand

CCGCCACCMEFGLSWVFLVAIIKGVQCQWQVFGPDKPVQALVGEDAAFSCFLSPKT
NAEAMEVRFFRGQFSSVVHLYRDGKDQPFMQMPQYQGRTKLVKDSIAEGRISLRLE
NITVLDAGLYGCRISSQSYYQKAIWELQVSALGSVPLISITGYVDRDIQLLCQSSGWFP
RPTAKWKGPQGQDLSTDSRTNRDMHGLFDVEISLTVQENAGSISCSMRHAHLSREVE
SRVQIGDTFFEPISWHLATKEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC
LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLTSKLTVDKSRWQQGNVFS
CSVMHEALHNHYTQKSLSLSPGKGGGSQVQLQQSGAELVRPGSSVKISCKASGYAFS
SYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLTADESSSTAYMQLSS
LASEDSAVYFCARRETTTVGRYYYAMDYWGQGTTVTVSSGGGGSGGGGSGGGGSD
IQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLV
SGIPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIK (SEQ ID NO: 42).

(10) an alpha strand comprising a V-set domain of mBTNL1 linked by a charge polarized core domain to a portion of the extracellular domain of alpha CD19 (αCD19) and a beta strand comprising a V-set domain of mBTNL6 linked by a charge polarized core domain to a portion of the antibody binding domain directed to CD19. Illustrative alpha and beta strands have the following amino acid sequences:

Alpha Strand

CCGCCACCMEFGLSWVFLVAIIKGVQCEVSWFSVKGPAEPITVLLGTEATLPCQLSPE
QSAARMHIRWYRAQPTPAVLVFHNGQEQGEVQMPEYRGRTQMVRQAIDMGSVALQ
IQQVQASDDGLYHCQFTDGFTSQEVSMELRVIGLGSAPLVHMTGPENDGIRVLCSSS
GWFPKPKVQWRDTSGNMLLSSSELQTQDREGLFQVEVSLLVTDRAIGNVICSIQNPM
YDQEKSKAILLPEPFFPKTCPWKGSGSDEGGEDGVPRDCGCKPCICTVPEVSSVFIFPP
KPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSV
SELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKD
KVSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNW
EAGNTFTCSVLHEGLHNHHTEKSLSHSPGIRKGGKRGSGSEVQLQQSGAELVRPGTS
VKLSCKVSGDTITFYYMHFVKQRPGQGLEWIGRIDPEDESTKYSEKFKNKATLTADT
SSNTAYLKLSSLTSEDTATYFCIYGGYYFDYWGQGVMVTVSSGGGGSGGGGSGGGG
SDIQMTQSPASLSTSLGETVTIQCQASEDIYSGLAWYQQKPGKSPQLLIYGASDLQDG
VPSRFSGSGSGTQYSLKITSMQTEDEGVYFCQQGLTYPRTFGGGTKLELK (SEQ ID
NO: 43).

Beta Strand

CCGCCACCMEFGLSWVFLVAIIKGVQCEQLPEYSQRTSLVKEQFHQGTAAVRILNVQ
APDSGIYICHFKQGVFYEEAILELKVAAMGSVPEVYIKGPEDGGVCVVCITSGWYPEP
QVHWKDSRGEKLTASLEIHSEDAQGLFRTETSLVVRDSSVRNVTCSTFNPILGQEKA
MAMFLPEPFFPKVSPWKPGSGSDEGGEDGVPRDCGCKPCICTVPEVSSVFIFPPKPKD
VLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPI
MHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSL
TCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGN
TFTCSVLHEGLHNHHTEKSLSHSPGIRKGGKRGSGSEVQLQQSGAELVRPGTSVKLSC
KVSGDTITFYYMHFVKQRPGQGLEWIGRIDPEDESTKYSEKFKNKATLTADTSSNTA
YLKLSSLTSEDTATYFCIYGGYYFDYWGQGVMVTVSSGGGGSGGGGSGGGGSDIQM
TQSPASLSTSLGETVTIQCQASEDIYSGLAWYQQKPGKSPQLLIYGASDLQDGVPSRFS
GSGSGTQYSLKITSMQTEDEGVYFCQQGLTYPRTFGGGTKLELK (SEQ ID NO: 44).

(11) an alpha strand comprising a V-set domain of mBTNL1 linked by a charge polarized core domain to a portion of the extracellular domain of alpha CD19 (αCD19) and a beta strand

comprising a V-set domain of mBTNL6 linked by a charge polarized core domain to a portion of the antibody binding domain directed to CD19. Illustrative alpha and beta strands have the following amino acid sequences:

Alpha Strand

CCGCCACCMEFGLSWVFLVAIIKGVQCEVSWFSVKGPAEPITVLLGTEATLPCQLSPE
QSAARMHIRWYRAQPTPAVLVFHNGQEQGEVQMPEYRGRTQMVRQAIDMGSVALQ
IQQVQASDDGLYHCQFTDGFTSQEVSMELRVIGLGSAPLVHMTGPENDGIRVLCSSS
GWFPKPKVQWRDTSGNMLLSSSELQTQDREGLFQVEVSLLVTDRAIGNVICSIQNPM
YDQEKSKAILLPEPFFPKTCPWKGSGSDEGGEDGVPRDCGCKPCICTVPEVSSVFIFPP
KPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSV
SELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKD
KVSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNW
EAGNTFTCSVLHEGLHNHHTEKSLSHSPGIRKGGKRGSGSDIQMTQSPASLSTSLGET
VTIQCQASEDIYSGLAWYQQKPGKSPQLLIYGASDLQDGVPSRFSGSGSGTQYSLKIT
SMQTEDEGVYFCQQGLTYPRTFGGGTKLELKGGGGSGGGGSGGGGSEVQLQQSGAE
LVRPGTSVKLSCKVSGDTITFYYMHFVKQRPGQGLEWIGRIDPEDESTKYSEKFKNK
ATLTADTSSNTAYLKLSSLTSEDTATYFCIYGGYYFDYWGQGVMVTVSS (SEQ ID NO: 45).

Beta Strand

CCGCCACCMEFGLSWVFLVAIIKGVQCEQLPEYSQRTSLVKEQFHQGTAAVRILNVQ
APDSGIYICHFKQGVFYEEAILELKVAAMGSVPEVYIKGPEDGGVCVVCITSGWYPEP
QVHWKDSRGEKLTASLEIHSEDAQGLFRTETSLVVRDSSVRNVTCSTFNPILGQEKA
MAMFLPEPFFPKVSPWKPGSGSDEGGEDGVPRDCGCKPCICTVPEVSSVFIFPPKPKD
VLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPI
MHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSL
TCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGN
TFTCSVLHEGLHNHHTEKSLSHSPGIRKGGKRGSGSDIQMTQSPASLSTSLGETVTIQC
QASEDIYSGLAWYQQKPGKSPQLLIYGASDLQDGVPSRFSGSGSGTQYSLKITSMQTE
DEGVYFCQQGLTYPRTFGGGTKLELKGGGGSGGGGSGGGGSEVQLQQSGAELVRPG
TSVKLSCKVSGDTITFYYMHFVKQRPGQGLEWIGRIDPEDESTKYSEKFKNKATLTA
DTSSNTAYLKLSSLTSEDTATYFCIYGGYYFDYWGQGVMVTVSS (SEQ ID NO: 46).

In embodiments, the charge polarized core domain of the above-mentioned illustrative strands (*i.e.,* BTNL1- αCD19, BTNL3-αCD19, BTNL6-αCD19, BTNL8-αCD19, BTNL3-αPSMA, and BTNL8-αPSMA) comprises a polypeptide linker, optionally selected from a flexible amino acid sequence, IgG hinge region, or antibody sequence. In embodiments, the linker is a

synthetic linker, optionally PEG. In embodiments, the linker comprises the hinge-CH2-CH3 Fc domain derived from IgG1, optionally human IgG1. In embodiments, the linker comprises the hinge-CH2-CH3 Fc domain derived from IgG4, optionally human IgG4.

In embodiments, each of the above-mentioned illustrative strands (i.e., BTNL3-Alpha-vTIGIT, BTNL8-Beta-vTIGIT, BTNL3-Alpha-vLAG3, BTNL8-Beta-vLAG3, TIGIT-Alpha-vBTNL3, TIGIT-Beta-vBTNL8, LAG3-Alpha-vBTNL3, LAG3-Beta-vBTNL8, PD1-Alpha-vBTNL3, PD1-Beta-vBTNL8, BTNL3-αCD19, BTNL8-αCD19, BTNL3-αPSMA, and BTNL8-αPSMA) comprises an illustrative core domain having the following amino acid sequence:

SKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNW
YVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIE
KTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN
YKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGK
(SEQ ID NO: 34)

Since one end of each strand comprises a targeting sequence (*e.g.,* the portion of the extracellular domain of TIGIT, LAG-3, and PD-1 which is capable of binding to its ligand/receptor on the surface of a cancer cell), the heterochimeric proteins of this Example are particularly useful in the treatment of cancers. Here, the targeting sequence localizes the BTNL3/8 heterodimer to the surface of a cancer cell.

In this example, several heterodimeric proteins were generated based on the methods disclosed herein. For example, the following heterodimeric proteins were constructed: (1) a heterodimeric protein comprising BTNL3/8 linked by a charge polarized core domain to αCD19; (2) a heterodimeric protein comprising BTNL3/8 linked by a charge polarized core domain to αPSMA; (3) a heterodimeric protein comprising BTNL3/8 linked by a charge polarized core domain to vTIGIT; (4) a heterodimeric protein comprising BTNL3/8 linked by a charge polarized core domain to vLAG3; (5) a heterodimeric protein comprising LAG3 linked by a charge polarized core domain to vBTNL3/8, (6) a heterodimeric protein comprising PD1 linked by a charge polarized core domain to vBTNL3/8, (7) a heterodimeric protein comprising BTNL1/6 linked by a charge polarized core domain to αCD19; and (8) a heterodimeric protein comprising mBTNL1/6 linked by a charge polarized core domain to αCD19.

Several assays were performed to characterize the generated heterodimers and demonstrate their utility in the treatment of cancers.

FIG. 16 shows an ELISA assay that was developed to demonstrate specific detection of the BTNL3/8-Fc-αCD19 heterodimer protein. Microtiter plates were coated overnight with decreasing concentrations of the BTNL3/8-Fc-αCD19 heterodimeric protein (heterodimeric protein; HdA), and then blocked and incubated with CD19-His recombinant protein. An HRP-

conjugated anti-His monoclonal antibody was used for detection of the HdA-protein complex. As shown in **FIG. 16**, the presence of the BTNL3/8-Fc-αCD19 heterodimer was observed through capture with CD19-His recombinant protein, and detection with an HRP-conjugated anti-His monoclonal antibody. **FIG. 17** is a graph showing the percentage of binding of the BTNL3/8-Fc-αCD19 heterodimer protein to isolated human γδTCR T cells. Increasing amounts of the BTNL3/8-Fc-αCD19 heterodimer protein were co-incubated with 10^6 human γδTCR T cells freshly isolated from a healthy donor, followed by counter staining with a cocktail of antibodies, including anti- BTNL3/8. Graphed data is pre-gated on γδTCR+ T cells. The results of **FIG. 17** show that the BTNL3/8-Fc-αCD19 heterodimer protein binds to human γδTCR T cells. **FIG. 18** are graphs showing binding of the BTNL3/8-Fc-αCD19 heterodimer protein to B-cell lines. B-cell lines with and without CD19 expression were co-incubated with increasing amounts of the BTNL3/8-Fc-αCD19 heterodimer protein, then stained with a cocktail of antibodies for Flow Cytometry analysis. As shown in **FIG. 18**, the BTNL3/8-Fc-αCD19 heterodimer protein binds to Toledo cells (*e.g.,* at about 10μg/ml). Minimal binding was observed to Raji and SR cells. The results of this experiment demonstrate the BTNL3/8-Fc-αCD19 heterodimer protein binds to B-cells in a site specific manner. **FIG. 19** is a graph showing the ability of the BTNL3/8-Fc-αCD19 heterodimer protein to tether γδ T-cells to Raji cells. In these experiments, γδ T-cells were labeled with green cell tracker dye, and Raji tumor cells were labeled with deep red cell tracker dye, and then the labeled γδ T-cells and Raji tumor cells were co-cultured in the presence of the BTNL3/8-Fc-CD19. Flow cytometry was used to assess the ability of the BTNL3/8-Fc-αCD19 heterodimer protein to tether γδ T-cells to Raji cells. As shown in **FIG. 19**, the BTNL3/8-Fc-αCD19 heterodimer protein tethered the γδ T-cells to Raji cells. **FIG. 20** is a graph showing the ability of the BTNL3/8-Fc-αCD19 heterodimer protein to tether γδ T-cells to Toledo cells. In these experiments, γδ T-cells were labeled with αCD3(PE-Cy7) antibody, and Toledo tumor cells were labeled with αCD20 (BV650) antibody. The cells were then co-cultured in the presence of the BTNL3/8-Fc-αCD19 heterodimer protein or control proteins (*i.e.,* Igg4, BTNL3-Fc), and then analyzed using flow cytometry. As shown in **FIG. 20**, the BTNL3/8-Fc-αCD19 heterodimer protein tethered the γδ T-cells to Toledo cells.

FIG. 21 shows an ELISA assay that was developed to demonstrate specific detection of the BTNL3/8-Fc-αPSMA heterodimer protein. In these experimetns, microtiter plates were coated overnight with decreasing concentrations of the BTNL3/8-Fc-αPSMA heterodimer protein (heterodimeric protein HdA), and then blocked, and incubated with a PSMA-His recombinant protein. An HRP-conjugated anti-His monoclonal antibody was used for detection of the HdA-protein complex. As shown in **FIG. 21**, the presence of the BTNL3/8-Fc-αPSMA heterodimer was observed through capture with the PSMA-His recombinant protein, and detection with an

HRP-conjugated anti-His monoclonal antibody. **FIG. 22** is a graph showing the percentage of binding of the BTNL3/8-Fc-αPSMA heterodimer protein to isolated human γδTCR T cells. Increasing amounts of the BTNL3/8-Fc-αPSMA heterodimer protein were co-incubated with 10^6 human γδTCR T cells freshly isolated from a healthy donor, followed by counter staining with a cocktail of antibodies, including anti- BTNL3/8. Graphed data is pre-gated on γδTCR+ T cells. The results of **FIG. 22** show that the BTNL3/8-Fc-αPSMA heterodimer protein binds to human γδTCR T cells. **FIG. 23** are graphs showing binding of the BTNL3/8-Fc-αPSMA heterodimer protein to prostate cancer cell lines. Prostate cancer cell lines with (*i.e.,* LNCap) and without (PC3) PSMA expression were co-incubated with increasing amounts of the BTNL3/8-Fc-αPSMA heterodimer protein, then stained with a cocktail of antibodies for flow cytometry analysis. As shown in **FIG. 23**, the BTNL3/8-Fc-αPSMA heterodimer protein binds to LNCap cells that express PSMA in a dose dependent manner but not to PC3 cells, demonstrating the utility of the heterodimeric proteins disclosed herein in the treatment of cancers.

FIG. 24 are graphs showing binding of the BTNL3/8-Fc-vTIGIT heterodimer protein to isolated human γδTCR T cells. The BTNL3/8-Fc-vTIGIT heterodimer protein was co-incubated with 10^6 human γδTCR T cells freshly isolated from a healthy donor, and counter stained with a cocktail of antibodies, including an anti-BTNL3/8 antibody. The results show that the BTNL3/8-Fc-vTIGIT heterodimer protein binds to γδTCR T cells.

FIG. 25 are graphs showing binding of the BTNL3/8-Fc-vLAG3 heterodimer protein to isolated human γδTCR T cells. The BTNL3/8-Fc-vLAG3 heterodimer protein was co-incubated with 10^6 human γδTCR T cells freshly isolated from a healthy donor, and counter stained with a cocktail of antibodies, including an anti-BTNL3/8 antibody. The results show that the BTNL3/8-Fc-vLAG3 heterodimer protein binds to γδTCR T cells.

FIG. 26 is a Western blot showing detection of the TIGIT-Fc-vBTNL3/8 heterodimer protein under different conditions using anti-human TIGIT antibody (left), anti-human Fc antibody (middle), or anti-human BTNL8 antibody (right). **FIG. 27** are graphs showing binding of the TIGIT-Fc-vBTNL3/8 heterodimer protein to isolated human γδTCR T cells. The TIGIT-Fc-vBTNL3/8 heterodimer protein was co-incubated with 10^6 human γδTCR T cells freshly isolated from a healthy donor, and counter stained with a cocktail of antibodies, including an anti-TIGIT antibody. The results show that the TIGIT-Fc-vBTNL3/8 heterodimer protein binds to γδTCR T cells.

FIG. 28 are graphs showing binding of the LAG3-Fc-vBTNL3/8 heterodimer protein to isolated human γδTCR T cells. The LAG3-Fc-vBTNL3/8 heterodimer protein was co-incubated with 10^6 human γδTCR T cells freshly isolated from a healthy donor, and counter

stained with a cocktail of antibodies, including an anti- LAG3 antibody. The results show that the LAG3-Fc-vBTNL3/8 heterodimer protein binds to γδTCR T cells.

The experiments in this example demonstrate, inter alia, the utility of the heterodimeric proteins disclosed herein to treat cancer in a subject.

Sequences of other heterodimer proteins of the current technology are as follows:

The sequence of an illustrative core domain is provided below:

SKYGPP**CPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQF NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKG LPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYT QKSLSLSLGK**IEGRMD (SEQ ID NO: 15)

The sequence of an illustrative charge polarized core domain (positive – negative) is provided below:

GSGSRKGGKRGSKYGPP***CPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKC KVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHY TQKSLSLSLGK***DEGGEDGSGS (SEQ ID NO: 16)

The sequence of an illustrative charge polarized core domain (negative - positive) is provided below:

GSGSDEGGEDGSKYGPP***CPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKC KVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHY TQKSLSLSLGK***RKGGKRGSGS (SEQ ID NO: 17)

EQUIVALENTS

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

INCORPORATION BY REFERENCE

All patents and publications referenced herein are hereby incorporated by reference in their entireties.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present technology is not entitled to antedate such publication by virtue of prior invention.

As used herein, all headings are simply for organization and are not intended to limit the disclosure in any manner. The content of any individual section may be equally applicable to all sections.

CLAIMS

What is claimed is:

1. A heterodimeric protein comprising:

(a) a first domain comprising one or more butyrophilin family proteins, or a fragment thereof;

(b) a second domain comprising a targeting domain, the targeting domain being selected from an (i) antibody, antibody-like molecule, or antigen binding fragment thereof, and (ii) a extracellular domain; and

(c) a linker that adjoins the first and second domain.

2. The heterodimeric protein of claim 1, wherein the first domain comprises two of the same butyrophilin family proteins.

3. The heterodimeric protein of claim 1, wherein the first domain comprises two different butyrophilin family proteins.

4. The heterodimeric protein of any one of claims 1-3, wherein the butyrophilin family proteins, or a fragment thereof comprise a V-type domain.

5. The heterodimeric protein of any one of claims 1-4, wherein the butyrophilin family proteins, or a fragment thereof are derived from native full length proteins wherein the native full length proteins comprise a B30.2 domain.

6. The heterodimeric protein of any one of claims 1-5, wherein the butyrophilin family proteins are selected from BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT

7. The heterodimeric protein of any one of claims 1-6, wherein the first domain comprises:

(a) any one of BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT; and

(b) any one of BTNL1, BTN1A1, BTNL2, BTN2A1, BTN2A2, BTN2A3, BTNL3, BTN3A1, BTN3A2, BTN3A3, BTNL6, BTNL8, BTNL9, BTNL10, and SKINT.

8. The heterodimeric protein of any one of claims 1-7, wherein the first domain comprises:

(a) any one of human BTNL1, human BTN1A1, human BTNL2, human BTN2A1, human BTN2A2, human BTN2A3, human BTNL3, human BTN3A1, human BTN3A2, human BTN3A3, human BTNL6, human BTNL8, human BTNL9, and human BTNL10, and

(b) any one of human BTNL1, human BTN1A1, human BTNL2, human BTN2A1, human BTN2A2, human BTN2A3, human BTNL3, human BTN3A1, human BTN3A2, human BTN3A3, human BTNL6, human BTNL8, human BTNL9, and human BTNL10,.

9. The heterodimeric protein of any one of claims 1-8, wherein the first domain comprises one or more fragments of the butyrophilin family proteins, wherein the fragment is capable of binding a gamma delta T cell receptor and is optionally an extracellular domain.

10. The heterodimeric protein of any one of claims 1-9, wherein the first domain comprises a polypeptide having an amino acid sequence of:

(a) any one of SEQ ID NOs: 49-52; and

(b) any one of SEQ ID NOs: 49-52.

11. The heterodimeric protein of any one of claims 1-9, wherein the first domain comprises:

(a) BTNL3 and BTNL8;

(b) BTN3A1 and BTN3A2;

(c) BTN3A1 and BTN3A3; or

(d) BTNL1 and BTNL6.

12. The heterodimeric protein of any one of claims 1-11, wherein the first domain comprises:

(a) human BTNL3 and human BTNL8;

(b) human BTN3A1 and human BTN3A2; or

(c) human BTN3A1 and human BTN3A3.

13. The heterodimeric protein of any one of claims 1-12, wherein the first domain comprises a polypeptide having

(a) an amino acid sequence having at least 90%, or 95%, or 97%, or 98%, or 99% identity with SEQ ID NO: 49, and an amino acid sequence having at least 90%, or 95%, or 97%, or 98%, or 99% identity with SEQ ID NO: 52; or

(b) an amino acid sequence having at least 90%, or 95%, or 97%, or 98%, or 99% identity with SEQ ID NO: 50, and an amino acid sequence having at least 90%, or 95%, or 97%, or 98%, or 99% identity with SEQ ID NO: 51.

14. The heterodimeric protein of claim 12 or claim 13, wherein the first domain comprises a polypeptide having an amino acid sequence of

(a) SEQ ID NO: 49 and SEQ ID NO: 52; or

(b) SEQ ID NO: 50 and SEQ ID NO: 51.

15. The heterodimeric protein of any one of claims 1-14, wherein the targeting domain is an antibody, or antigen binding fragment thereof.

16. The heterodimeric protein of any one of claims 1-15, wherein the targeting domain is an antibody-like molecule, or antigen binding fragment thereof.

17. The heterodimeric protein of claim 16, wherein the antibody-like molecule is selected from a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), a DARPin; a Tetranectin; an Affibody; a Transbody; an Anticalin; an AdNectin; an Affilin; an Affimer, a Microbody; an aptamer; an alterase; a plastic antibody; a phylomer; a stradobody; a maxibody; an evibody; a fynomer, an armadillo repeat protein, a Kunitz domain, an avimer, an atrimer, a probody, an immunobody, a triomab, a troybody; a pepbody; a vaccibody, a UniBody; a DuoBody, a Fv, a Fab, a Fab′, and a $F(ab')_2$.

18. The heterodimeric protein of claim 16, wherein the antibody-like molecule is an scFv.

19. The heterodimeric protein of any one of claims 1-18, wherein the targeting domain is an extracellular domain.

20. The heterodimeric protein of any one of claims 1-19, wherein the targeting domain is capable of binding an antigen on the surface of a cancer cell.

21. The heterodimeric protein of claim 19 or claim 20, wherein the targeting domain specifically binds one of CD19, PSMA, GD2, PSCA, BCMA, CD123, B7-H3, CD20, CD30, CD33, CD38, CEA, CLEC12A, DLL3, EGFRvIII, EpCAM, CD307, FLT3, GPC3, gpA33, HER2, MUC16, P-cadherin, SSTR2, and mesothelin.

22. The heterodimeric protein of any one of claims 19-21, wherein the targeting domain comprises a portion of the extracellular domain of LAG-3, PD-1, TIGIT, CD19, or PSMA.

23. The heterodimeric protein of any one of claims 19-22, wherein the targeting domain specifically binds CD19.

24. The heterodimeric protein of any one of claims 19-22,, wherein the targeting domain specifically binds PSMA.

25. The heterodimeric protein of any one of claims 1-23, wherein the targeting domain is a polypeptide having an amino acid sequence with at least 90%, or 95%, or 97%, or 98%, or 99% identity with a polypeptide selected from SEQ ID NOs: 53-60.

26. The heterodimeric protein of claim 25, wherein the targeting domain is a polypeptide having an amino acid sequence of selected from ome or more of SEQ ID NOs: 53-60.

27. The heterodimeric protein of any one of claims 1-26, wherein the linker comprises (a) a first charge polarized core domain adjoined to a butyrophilin family protein, optionally at the carboxy terminus, and (b) a second charge polarized core domain adjoined to a butyrophilin family protein, optionally at the carboxy terminus.

28. The linker of claim 27, wherein the linker forms a heterodimer through electrostatic interactions between positively charged amino acid residues and negatively charged amino acid residues on the first and second charge polarized core domains.

29. The heterodimeric protein of claim 27 or claim 28, wherein the first and/or second charge polarized core domain comprises a polypeptide linker, optionally selected from a flexible amino acid sequence, IgG hinge region, or antibody sequence.

30. The heterodimeric protein of any one of claims 27-29, wherein the linker is a synthetic linker, optionally PEG.

31. The heterodimeric protein of any one of claims 27-30, wherein the linker comprises the hinge-CH2-CH3 Fc domain derived from IgG1, optionally human IgG1.

32. The heterodimeric protein of any one of claims 27-30, wherein the linker comprises the hinge-CH2-CH3 Fc domain derived from IgG4, optionally human IgG4.

33. The heterodimeric protein of any one of claims 27-32, wherein the first and/or second charge polarized core domain further comprise peptides having positively and/or negatively charged amino acid residues at the amino and/or carboxy terminus of the charge polarized core domain.

34. The heterodimeric protein of claim 28, wherein the positively charged amino acid residues include one or more of amino acids selected from His, Lys, and Arg.

35. The heterodimeric protein of claim 27 or claim 28, wherein the positively charged amino acid residues are present in a peptide comprising positively charged amino acid residues in the first and/or the second charge polarized core domains.

36. The heterodimeric protein of claim 35, wherein the peptide comprising positively charged amino acid residues comprises a sequence selected from $Y_nX_nY_nX_nY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 1), $YY_nXX_nYY_nXX_nYY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 3), and $Y_nX_nCY_nX_nY_n$ (where X is a positively charged amino acid such as arginine, histidine or lysine and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 5).

37. The heterodimeric protein of claim 35 or claim 36, wherein the peptide comprising positively charged amino acid residues comprises the sequence RKGGKR (SEQ ID NO: 11) or GSGSRKGGKRGS (SEQ ID NO: 12).

38. The heterodimeric protein of any one of claims 27-37, wherein the negatively charged amino acid residues may include one or more amino acids selected from Asp and Glu.

39. The heterodimeric protein of negatively 27-38, wherein the negatively charged amino acid residues are present in a peptide comprising negatively charged amino acid residues in the first and/or the second charge polarized core domains.

40. The heterodimeric protein of claim 39, wherein the peptide comprising negatively charged amino acid residues comprises a sequence selected from $Y_nZ_nY_nZ_nY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 2), $YY_nZZ_nYY_nZZ_nYY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 4), and $Y_nZ_nCY_nZ_nY_n$ (where Z is a negatively charged amino acid such as aspartic acid or glutamic acid and Y is a spacer amino acid such as serine or glycine) (SEQ ID NO: 6).

41. The heterodimeric protein of claim 39 or claim 40, wherein the peptide comprising negatively charged amino acid residues comprises the sequence DEGGED (SEQ ID NO: 13) or GSGSDEGGEDGS (SEQ ID NO: 14).

42. The heterodimeric protein of any one of claims 1-41, wherein the first domain and/or the heterodimeric protein modulates or is capable of modulating a γδ (gamma delta) T cell.

43. The heterodimeric protein of claim 42, wherein the gamma delta T cell is selected from a cell expressing Vγ4, Vγ9δ2, or Vγ7δ4.

44. The heterodimeric protein of claim 42 or claim 43, wherein the first domain comprises BTNL3 and BTNL8 and it modulates a Vγ4-expressing T cell.

45. The heterodimeric protein of claim 42 or claim 43, wherein the first domain comprises:

(a) BTNL3A1 and BTNL3A2 and it modulates a Vγ9δ2-expressing T cell,

(b) BTNL3A1 and BTNLA3 and it modulates a Vγ9δ2-expressing T cell, or

(c) BTNL1 and BTNL6 and it modulates a Vγ7δ4-expressing T cell.

46. The heterodimeric protein of any one of claims 42-45, wherein the modulation of a gamma delta T cell is activation of a gamma delta T cell.

47. The heterodimeric protein of any one of claims 1-46, wherein the heterodimeric protein is capable of forming a synapse between a gamma delta T cell and a tumor cell and/or the heterodimeric protein is capable of contemporaneous activation and targeting of gamma delta T cells to tumor cells.

48. The heterodimeric protein of any one of the above claims, wherein the heterodimeric protein is a complex of two polypeptide chains.

49. The heterodimeric protein of any one of the above claims, wherein the heterodimeric protein comprises an alpha chain and a beta chain wherein the alpha chain and the beta chain each independently comprise

(a) a first domain comprising a butyrophilin family protein, or fragment thereof;

(b) a second domain comprising a targeting domain, the targeting domain being selected from an (i) antibody, antibody-like molecule, or antigen binding fragment thereof, and (ii) a extracellular domain; and

(c) a linker that adjoins the first and second domain.

50. The heterodimeric protein of claim 49, wherein the alpha chain and the beta chain self-associate to form the heterodimer.

51. The heterodimeric protein of any one of claims 1-50, wherein the heterodimeric protein comprises one or more amino acid sequences having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 98%, or at least 99% sequence identity to:

(a) any extracellular domain ("ECD") sequence of Table 1, and/or

(b) one or more of SEQ ID NOs: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, each optionally with a leader sequence omitted.

52. A pharmaceutical composition, comprising the heterodimeric protein of any of claims 1-51.

53. An expression vector, comprising a nucleic acid encoding the first and/or second polypeptide chains of the heterodimeric protein of any of claims 1-51.

54. The expression vector of claim 53, wherein the expression vector is a mammalian expression vector.

55. The expression vector of claim 53 or claim 54, wherein the expression vector comprises DNA or RNA.

56. A host cell, comprising the expression vector of any one of claims 53-55.

57. A method of contemporaneous activation and targeting of gamma delta T cells to tumor cells comprising administering to a subject in need thereof an effective amount of a pharmaceutical composition of claim 52 to a subject in need thereof.

58. A method of modulating a patient's immune response, comprising administering an effective amount of a pharmaceutical composition of claim 52 to a subject in need thereof.

59. A method of stimulating proliferation of gamma delta T cells, comprising:

administering an effective amount of a pharmaceutical composition of claim 52 to a subject in need thereof thereby causing an in vivo proliferation of gamma delta T cells and/or

contacting an effective amount of a pharmaceutical composition of claim 52 with a cell derived from a subject in need thereof thereby causing an ex vivo proliferation of gamma delta T cells.

60. The method of any one of claims 57-59, wherein the subject's T cells are activated by the first domain.

61. The method of any one of claims 57-59 wherein the subject has a tumor and the gamma delta T cells modulate cells of the tumor.

62. A method of treating cancer, comprising administering to a subject in need thereof an effective amount of a pharmaceutical composition of claim 52 to a subject in need thereof.

63. The method of claim 62, wherein the cancer is a lymphoma.

64. The method of claim 62, wherein the cancer is a leukemia.

65. The method of any one of claims 62-64, wherein the cancer is a Hodgkin's and non-Hodgkin's lymphoma, B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; or chronic myeloblastic leukemia.

66. The method of claim 62, wherein the cancer is basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate

grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (*e.g.* that associated with brain tumors), and Meigs' syndrome.

67. The method of claim 62 or claim 66, wherein the cancer is prostate cancer.

68. The method of any one of claims 62, 66, or 67, wherein the cancer is an epithelial-derived carcinoma.

69. The method of any one of claims claim 62-68, wherein the cancer is known to express the antigenic target of the second domain of the heterodimeric protein.

70. The method of any one of claims claim 62-69, wherein the cancer has mutations which limit recognition by alpha beta T cells, optionally selected from mutations in MHC I, beta 2 microglobulin, and Transporter associated with antigen processing (TAP).

71. The method of any one of claims claim 62-70, wherein the subject is further administered autologous or allogeneic gamma delta T cells that were expanded *ex vivo*.

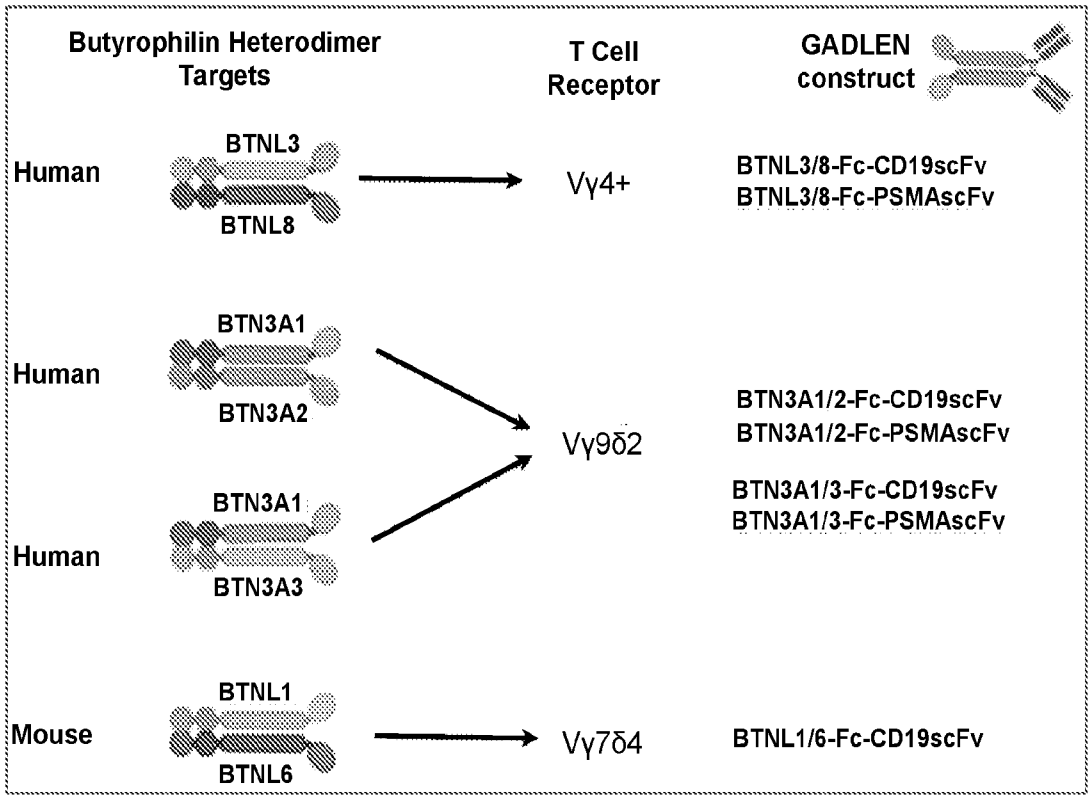
72. The method of claim 71, wherein the autologous or allogeneic gamma delta T cells express a Chimeric Antigen Receptor.

73. The method of any one of claims claim 62-70, wherein the subject is further administered autologous or allogeneic T cells that express a Chimeric Antigen Receptor.

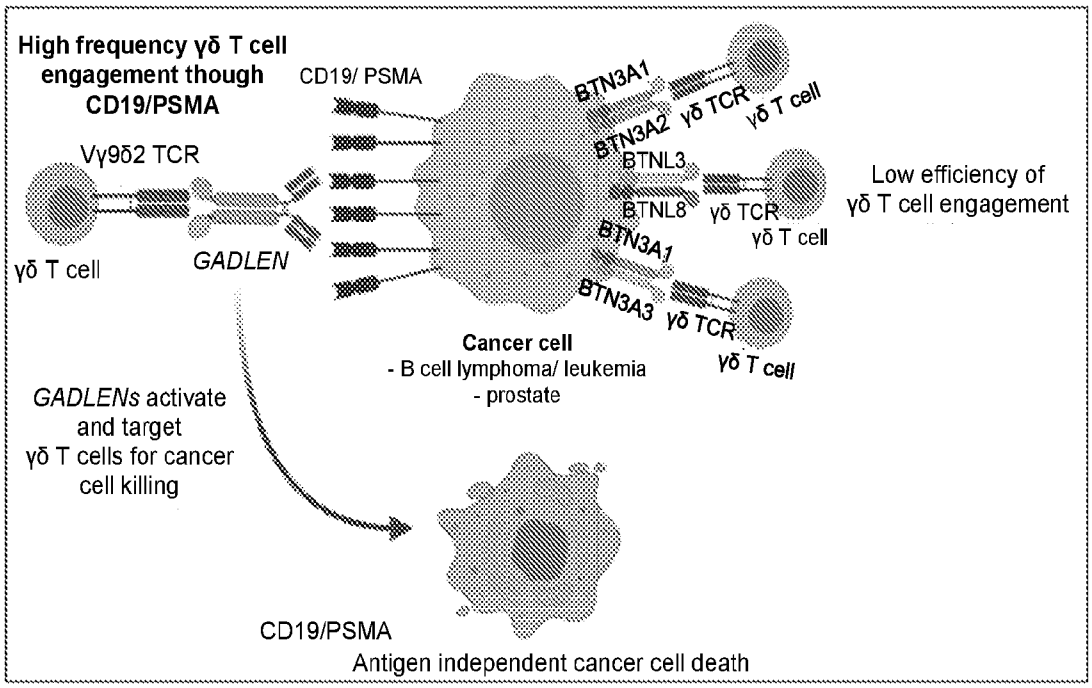
74. A method of treating an autoimmune disease or disorder, comprising administering an effective amount of a pharmaceutical composition of claim 52 to a subject in need thereof,

wherein the autoimmune disease or disorder is optionally selected from rheumatoid arthritis, systemic lupus erythematosus, diabetes mellitus, ankylosing spondylitis, Sjögren's syndrome, inflammatory bowel diseases (*e.g.*, colitis ulcerosa, Crohn's disease), multiple sclerosis, sarcoidosis, psoriasis, Grave's disease, Hashimoto's thyroiditis, , psoriasis, hypersensitivity reactions (*e.g.*, allergies, hay fever, asthma, and acute edema cause type I hypersensitivity reactions), and vasculitis.

FIG. 1



Other Human Butyrophilin Targets	Potential Cancer Antigens Targeted with scFv Scaffolds
BTN1A1, BTN2A1, BTN2A2, BTN2A3, BTN3A3, BTNL2, BTNL9, BTNL10, SKINT	GD2, PSCA, BCMA, CD123, B7-H3, CD20, CD30, CD33, CD38, CEA, CLEC12A, DLL3, EGFRvIII, EpCAM, CD307, FLT3, GPC3, gpA33, GPCR5D, HER2, MUC16, P-cadherin, SSTR2, mesothelin, etc.



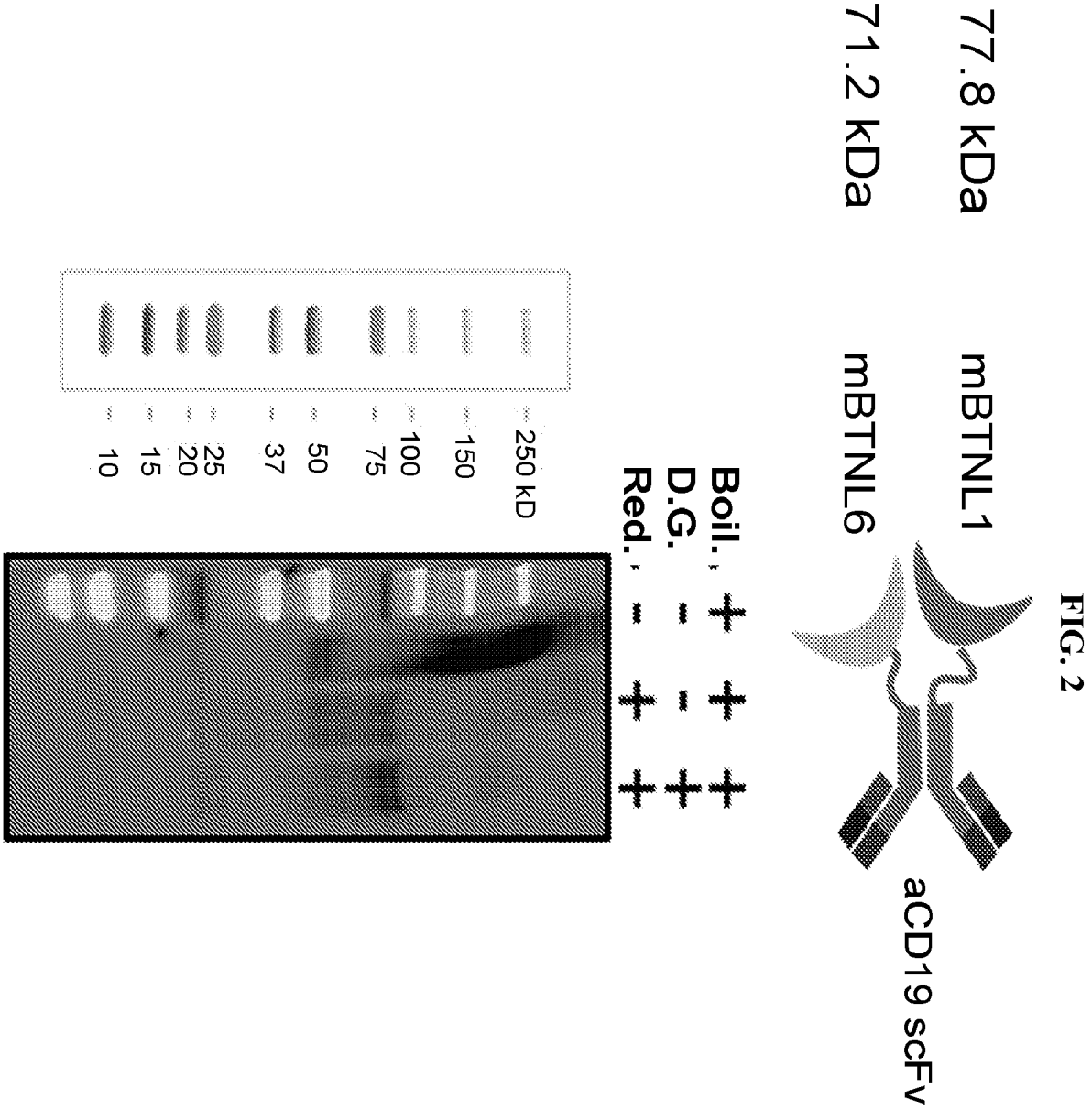

77.8 kDa
71.2 kDa
mBTNL1
mBTNL6
aCD19 scFv
Boil. + + +
D.G. - - +
Red. - + +
250 kD
150
100
75
50
37
25
20
15
10

FIG. 2

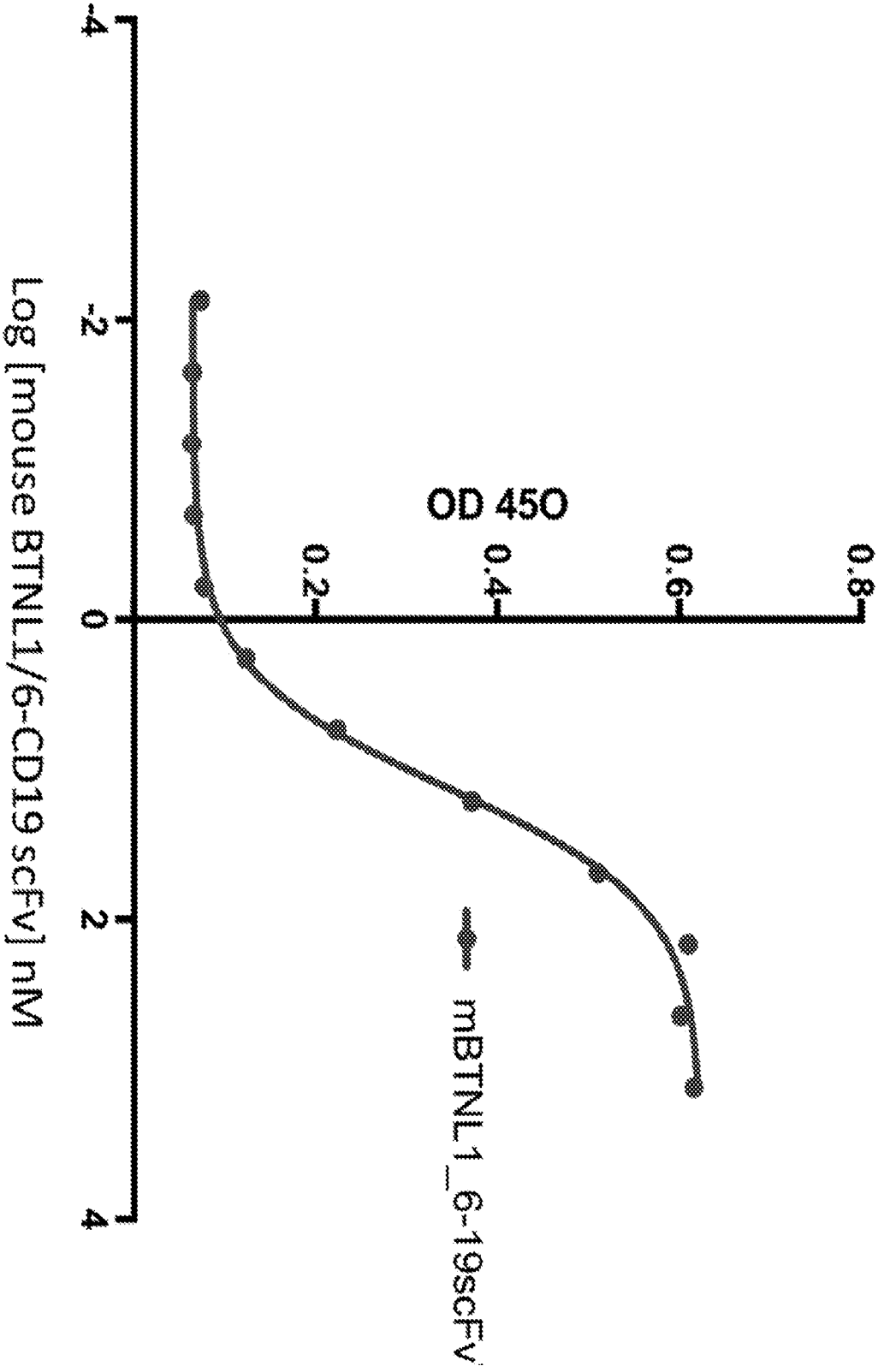

OD 450
0.2
0.4
0.6
0.8
0
-2
-4
2
4
Log [mouse BTNL1/6-CD19 scFv] nM
mBTNL1_6-19scFv

FIG. 3

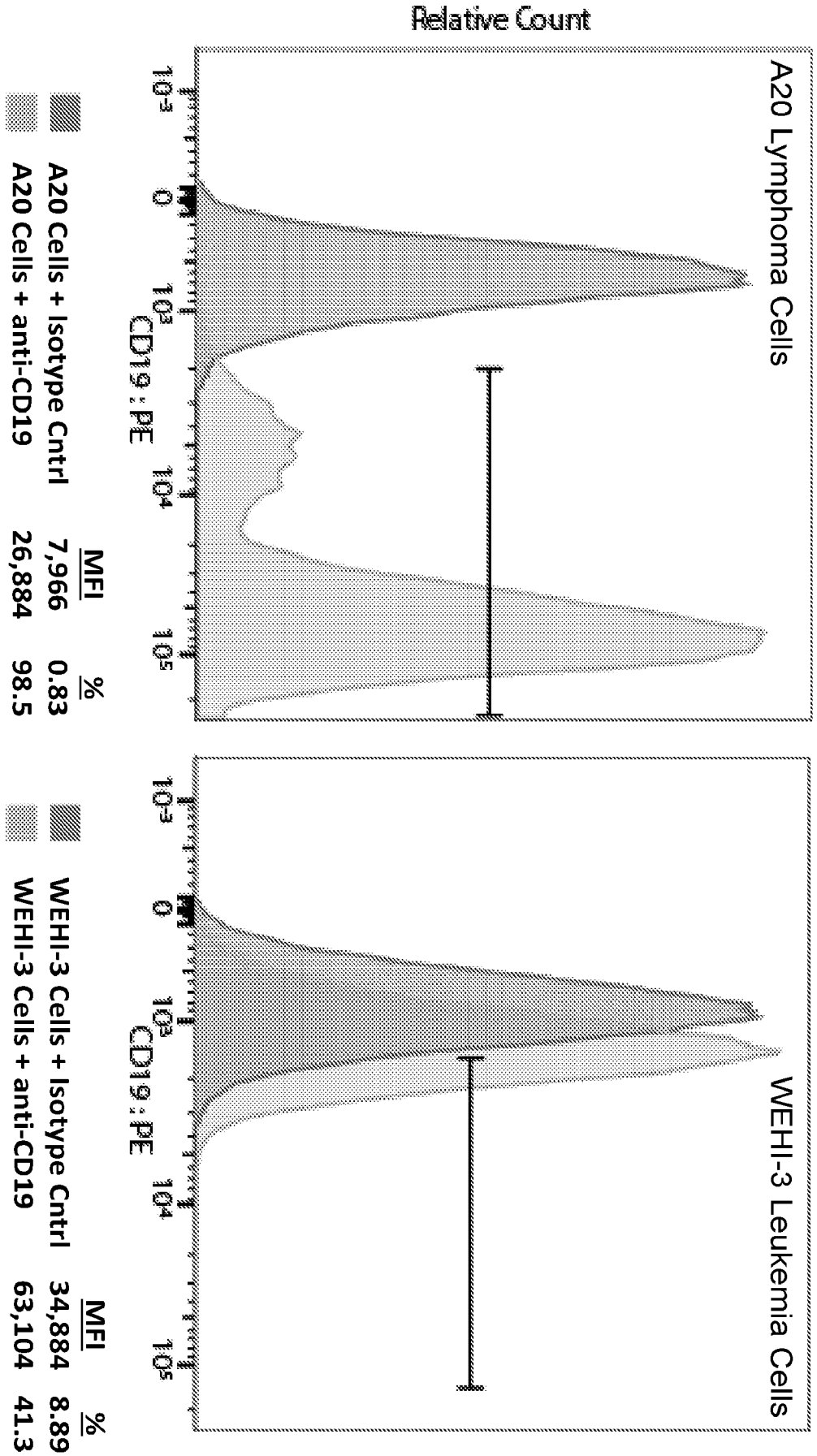

Relative Count
A20 Lymphoma Cells
CD19 : PE
A20 Cells + Isotype Cntrl
A20 Cells + anti-CD19
MFI
7,966
26,884
%
0.83
98.5
WEHI-3 Leukemia Cells
WEHI-3 Cells + Isotype Cntrl
WEHI-3 Cells + anti-CD19
MFI
34,884
63,104
%
8.89
41.3

FIG. 4

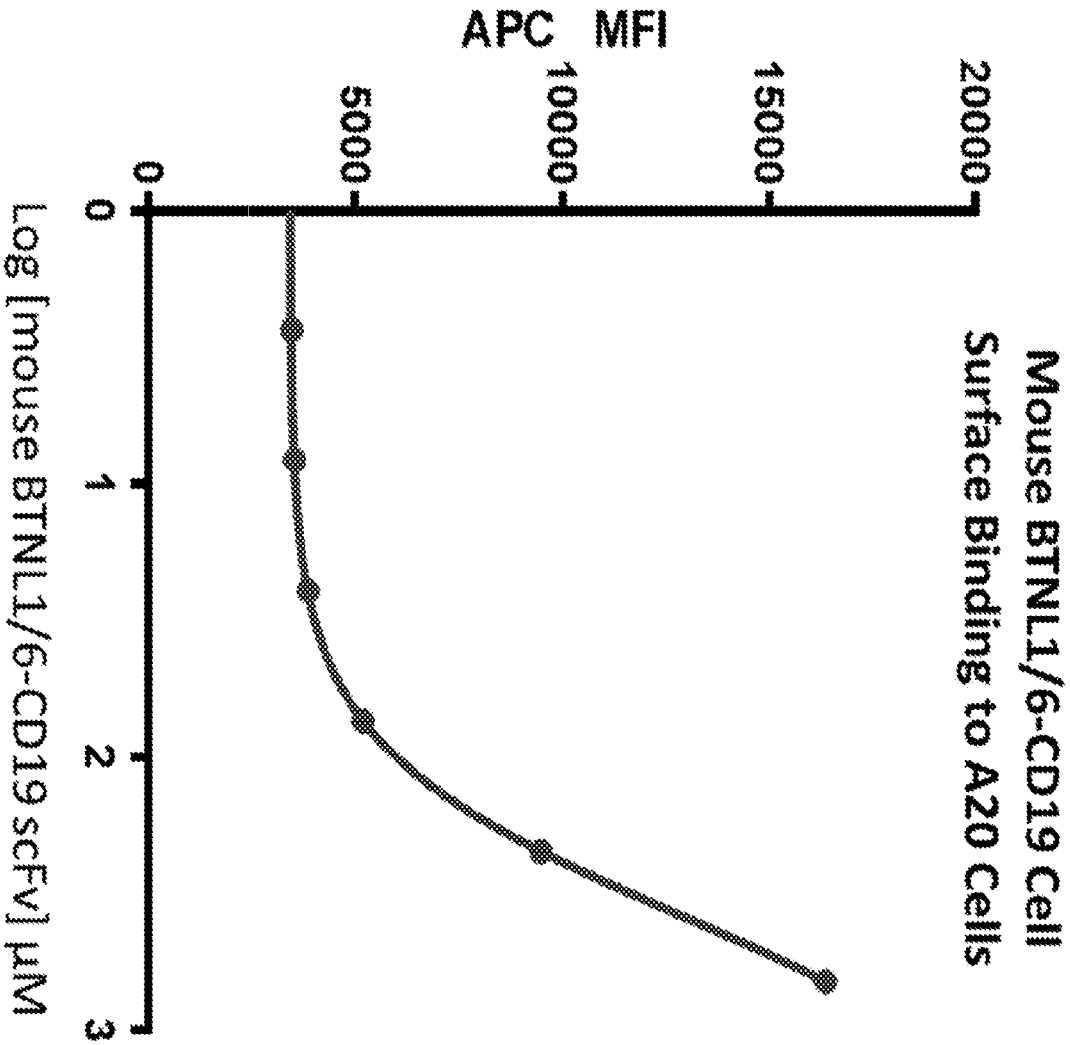

Mouse BTNL1/6-CD19 Cell
Surface Binding to A20 Cells
APC MFI
0
5000
10000
15000
20000
Log [mouse BTNL1/6-CD19 scFv] µM
0
1
2
3

FIG. 5

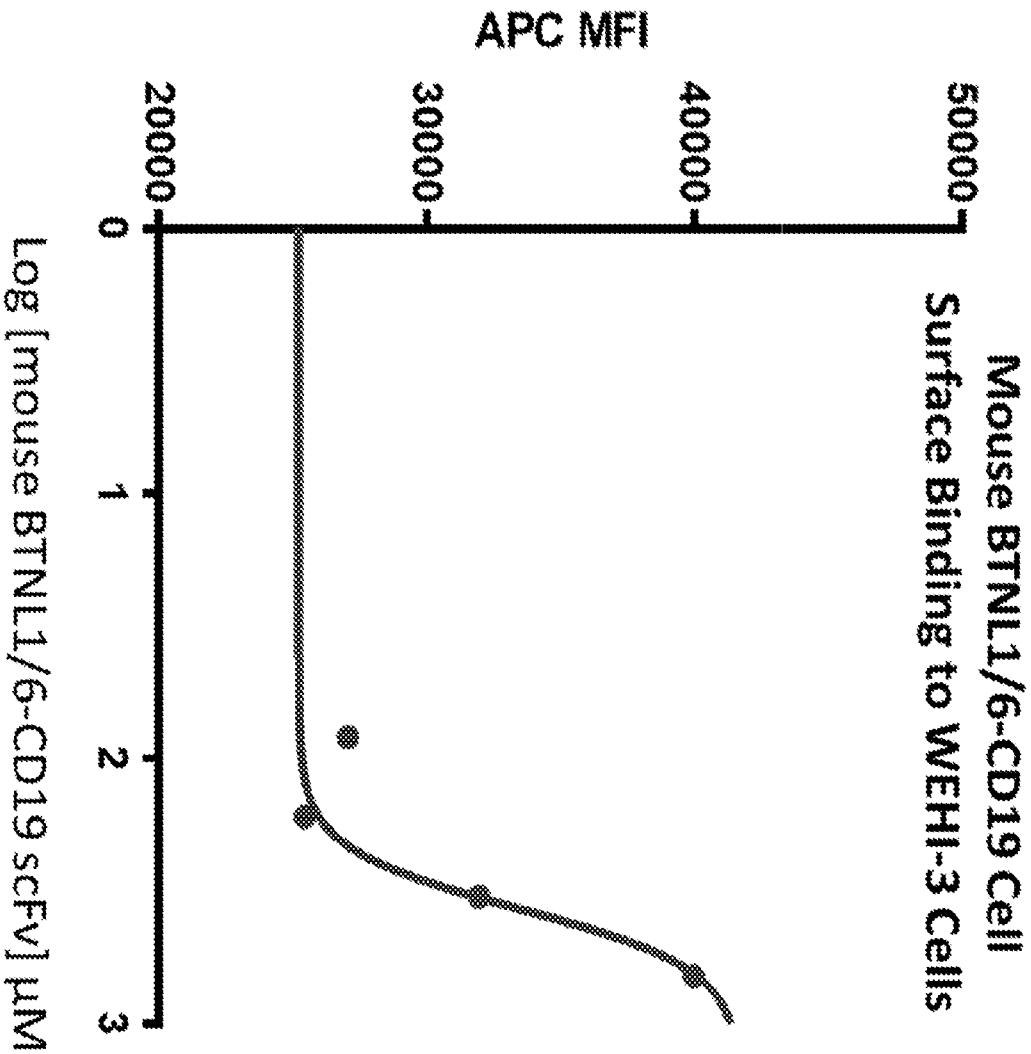

APC MFI
20000
30000
40000
50000
0
1
2
3
Log [mouse BTNL1/6-CD19 scFv] µM
Mouse BTNL1/6-CD19 Cell
Surface Binding to WEHI-3 Cells

FIG. 6

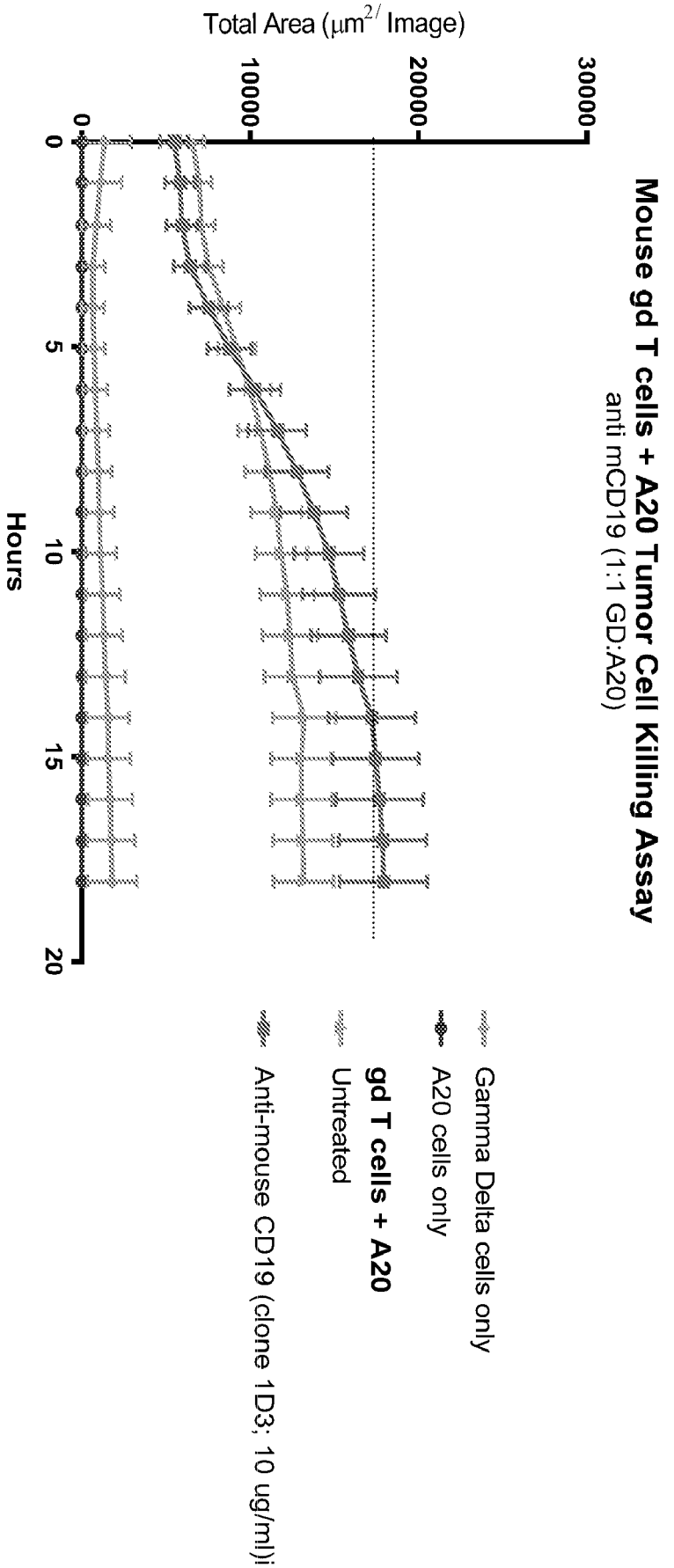

Mouse gd T cells + A20 Tumor Cell Killing Assay
anti mCD19 (1:1 GD:A20)
Total Area (μm2/ Image)
0
10000
20000
30000
Hours
0
5
10
15
20
Gamma Delta cells only
A20 cells only
gd T cells + A20
Untreated
Anti-mouse CD19 (clone 1D3; 10 ug/ml)i

FIG. 7

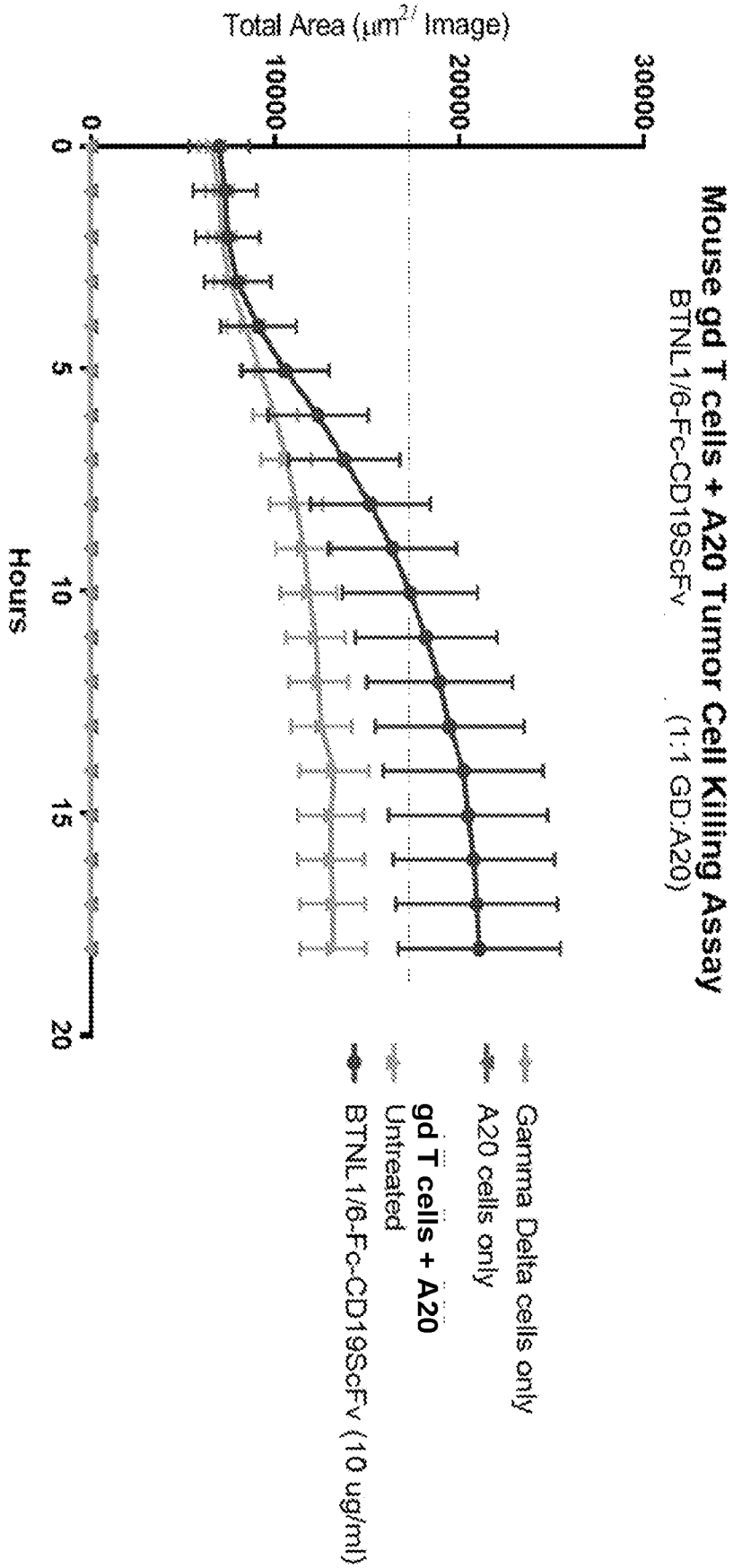

Mouse gd T cells + A20 Tumor Cell Killing Assay
BTNL1/6-Fc-CD19ScFv (1:1 GD:A20)
Total Area (µm²/ Image)
0
10000
20000
30000
Hours
0
5
10
15
20
Gamma Delta cells only
A20 cells only
gd T cells + A20
Untreated
BTNL1/6-Fc-CD19ScFv (10 ug/ml)

FIG. 8

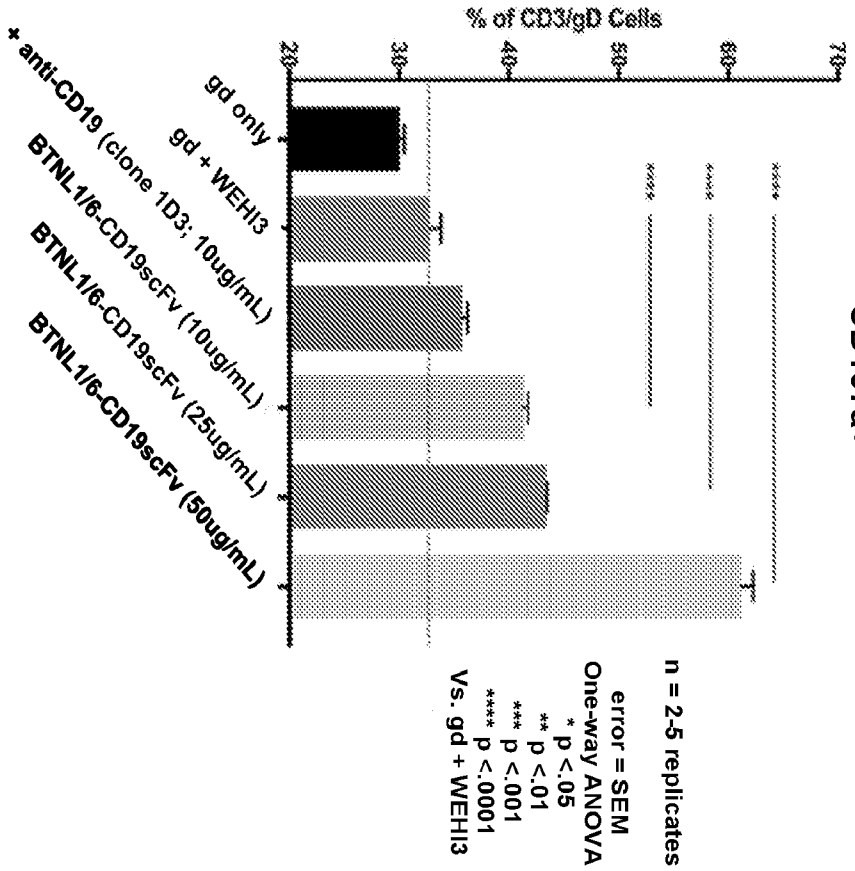

Mouse gd + WEHI3
CD107a+
% of CD3/gD Cells
20
30
40
50
60
70
gd only
gd + WEHI3
+ anti-CD19 (clone 1D3; 10ug/mL)
BTNL1/6-CD19scFv (10ug/mL)
BTNL1/6-CD19scFv (25ug/mL)
BTNL1/6-CD19scFv (50ug/mL)
****
****
****
n = 2-5 replicates
error = SEM
One-way ANOVA
* p <.05
** p <.01
*** p <.001
**** p <.0001
Vs. gd + WEHI3

FIG. 9A

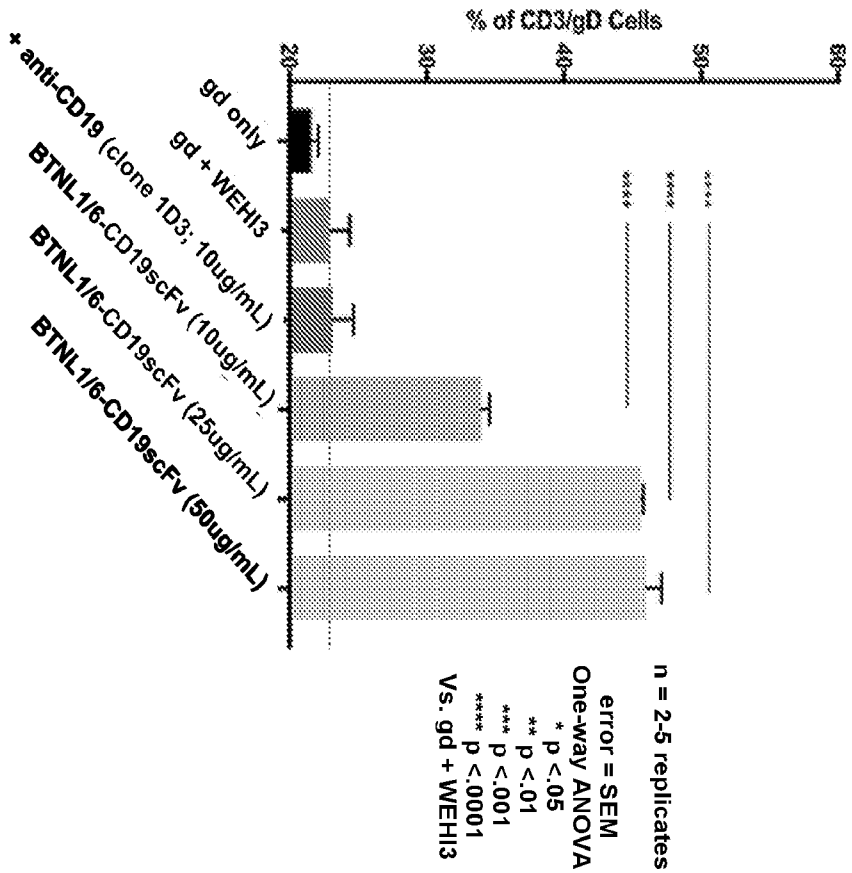

Mouse gd + WEHI3
CD69+
% of CD3/gD Cells
20
30
40
50
60
gd only
gd + WEHI3
+ anti-CD19 (clone 1D3; 10ug/mL)
BTNL1/6-CD19scFv (10ug/mL)
BTNL1/6-CD19scFv (25ug/mL)
BTNL1/6-CD19scFv (50ug/mL)
****
****
****
n = 2-5 replicates
error = SEM
One-way ANOVA
* p <.05
** p <.01
*** p <.001
**** p <.0001
Vs. gd + WEHI3

FIG. 9B

FIG. 10A

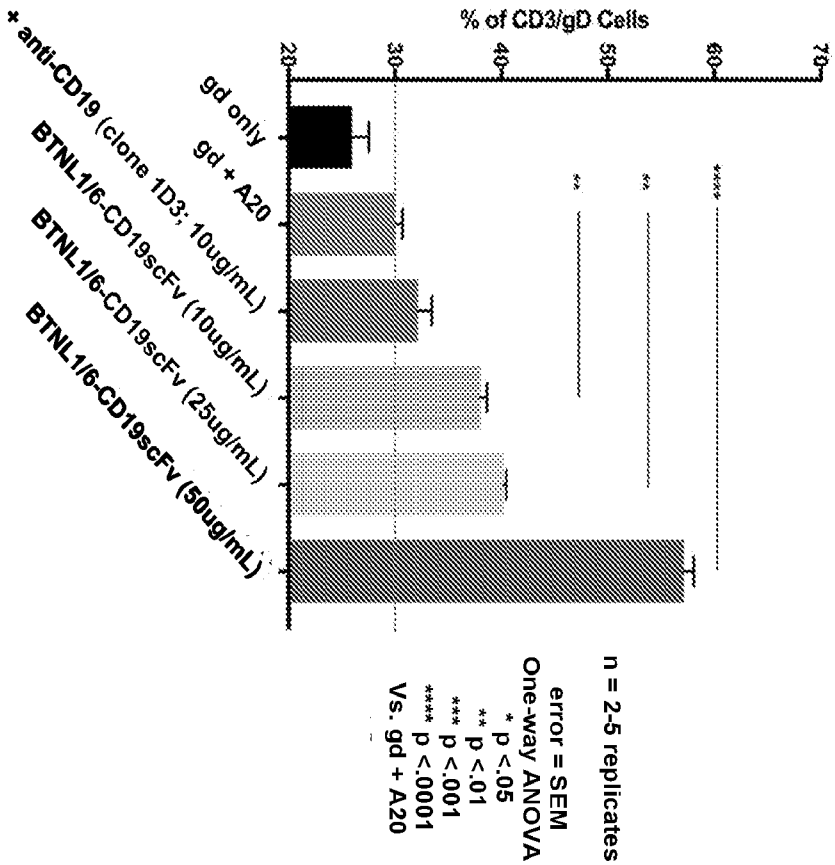


FIG. 10B

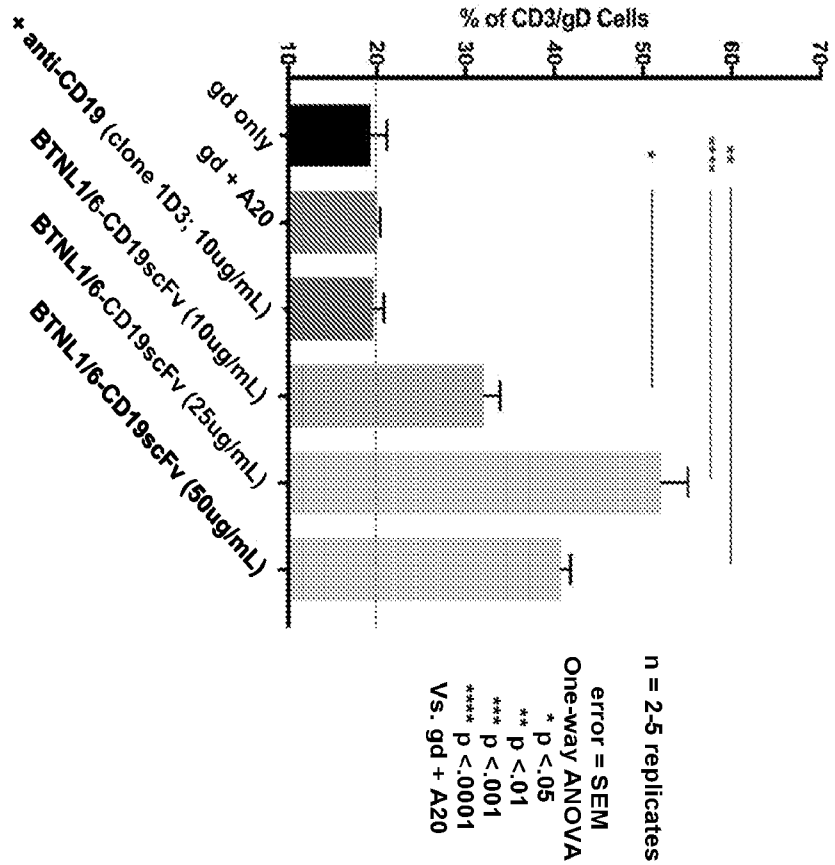


FIG. 11A

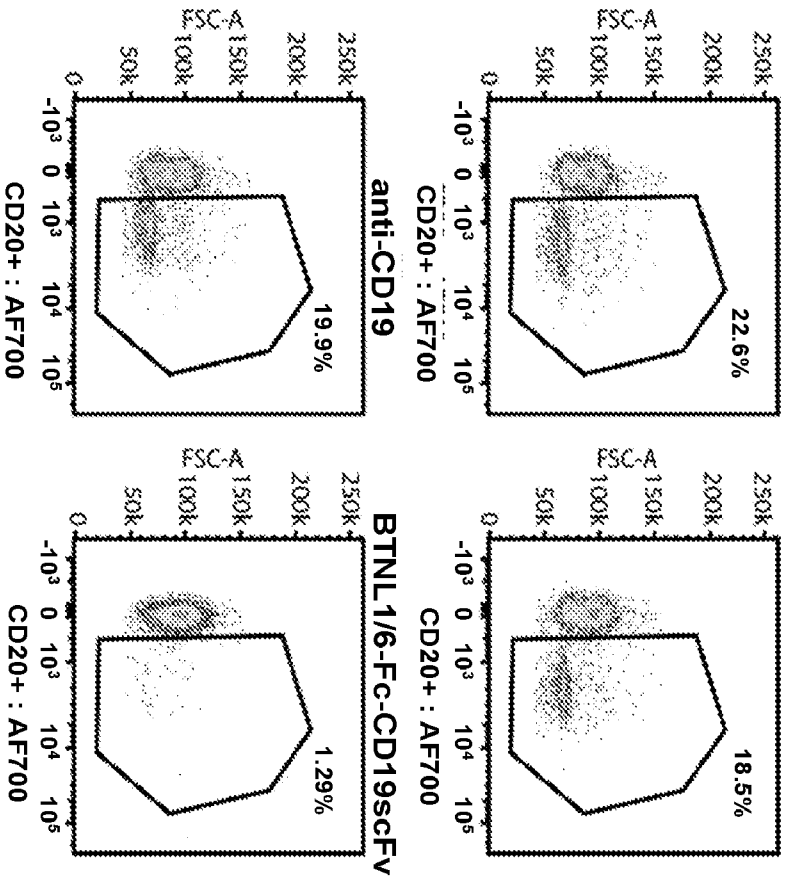


FIG. 11B

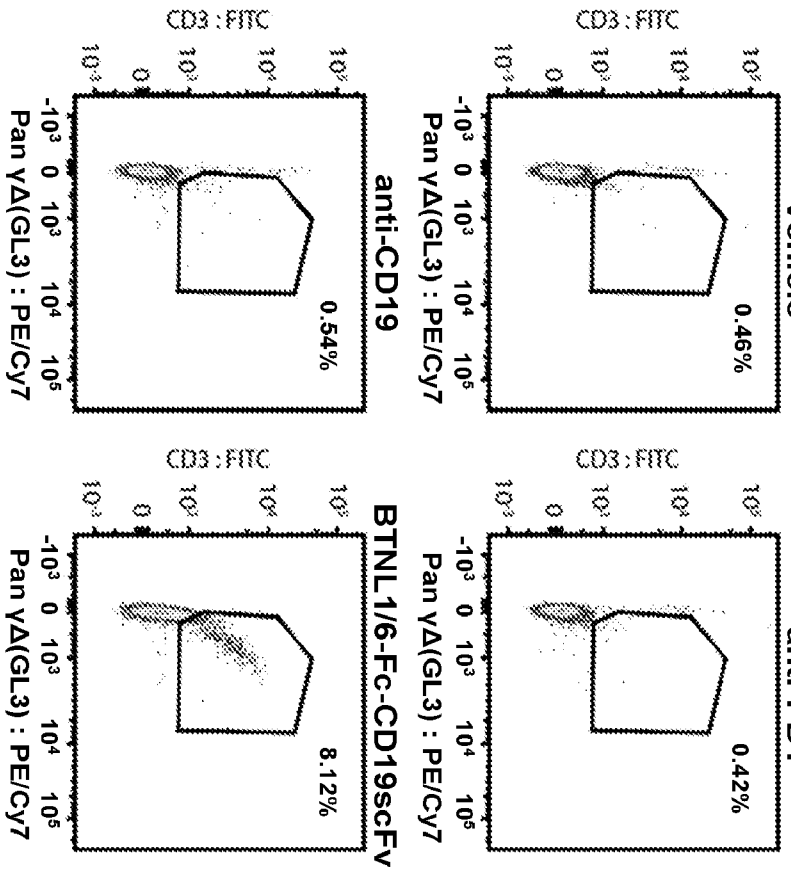


FIG. 12A

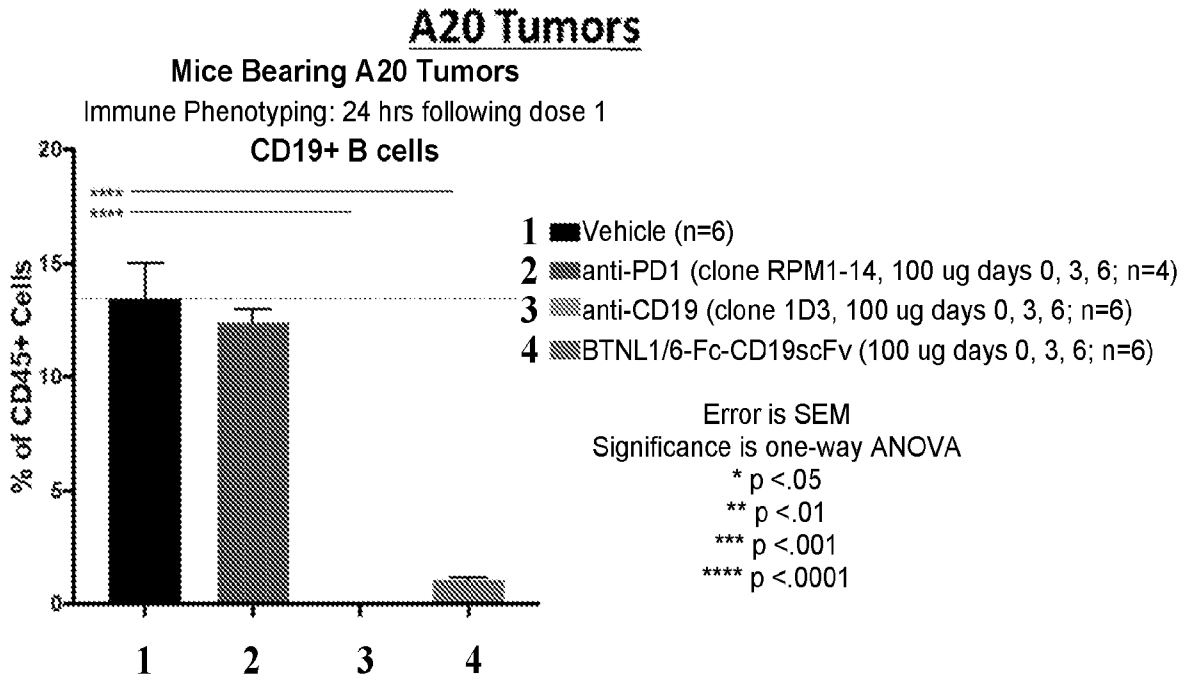


FIG. 12B

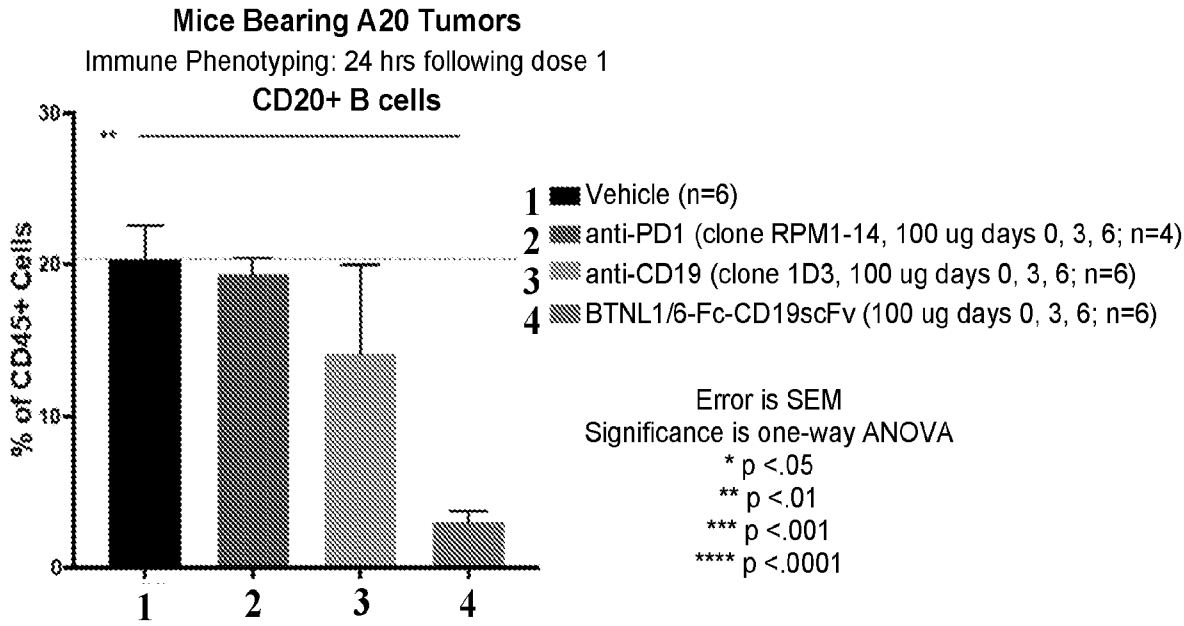


FIG. 12C

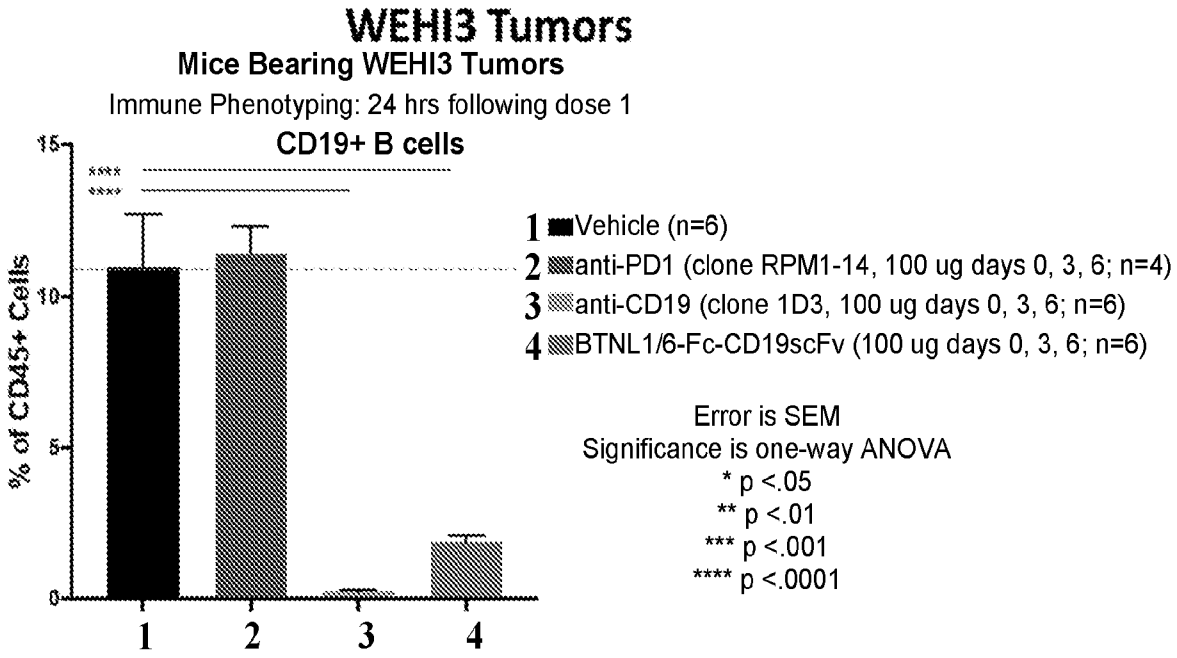


FIG. 12D

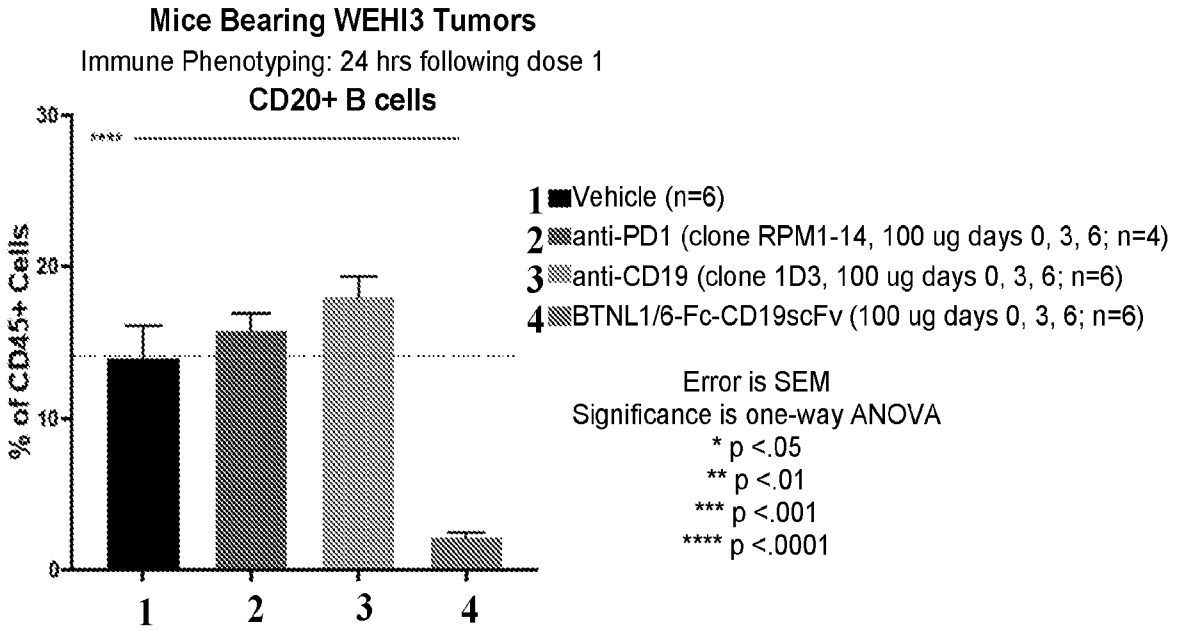


FIG. 13A

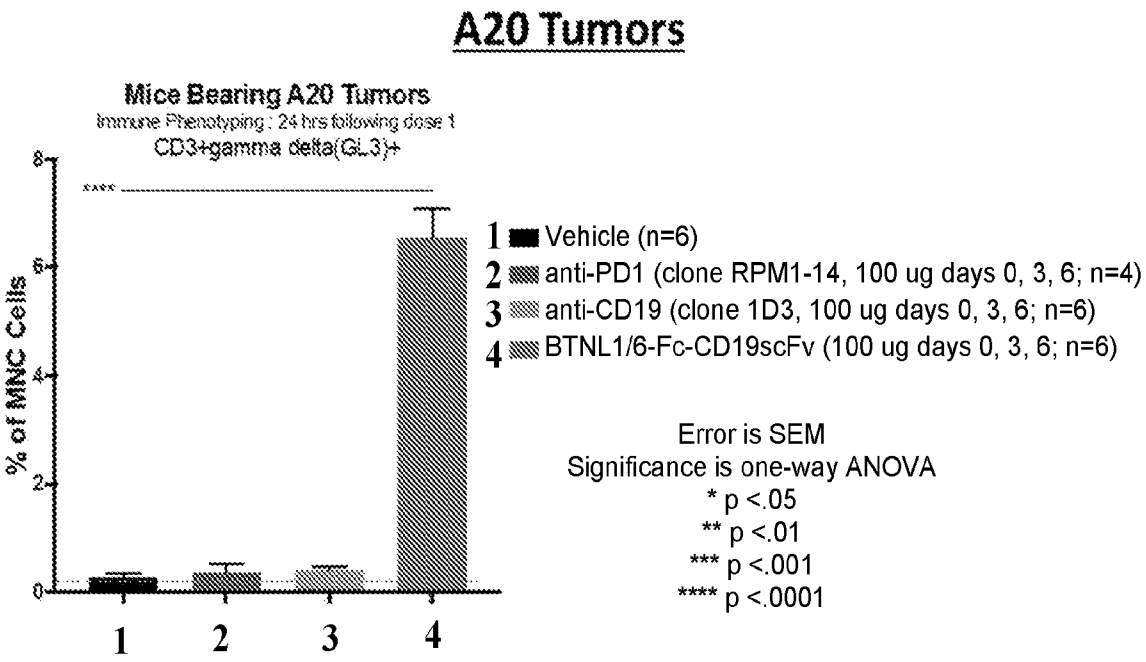
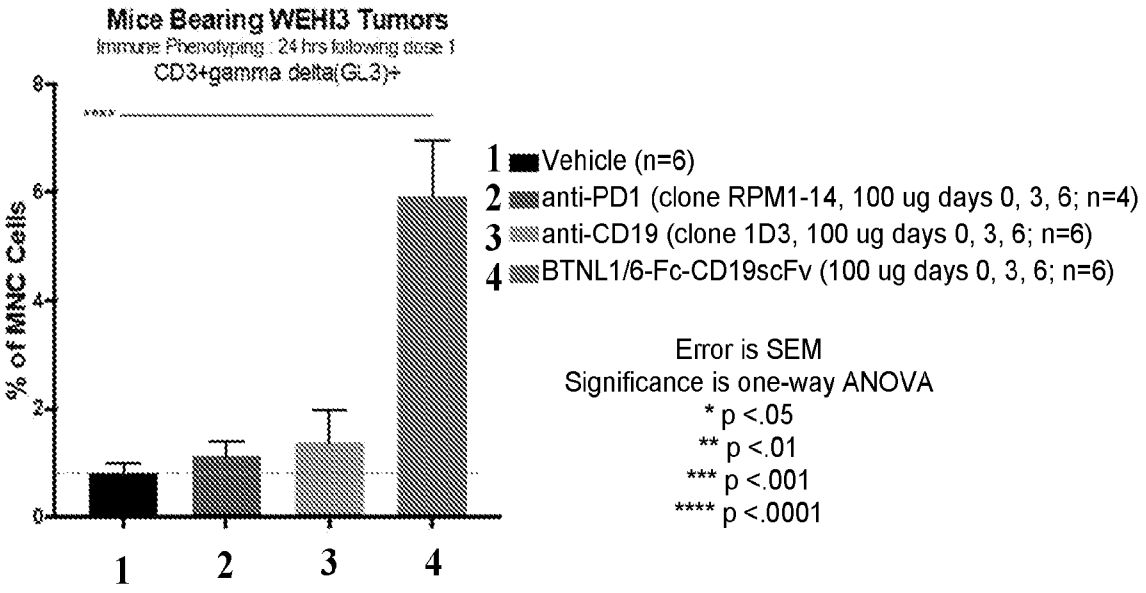


FIG. 13B



hBTNL3_8-19ScFv
78.4 kDa hBTNL3
78.9 kDa hBTNL8
aCD19 scFv
~250 kD
~150
~100
75
50
37
25
20
15
10
Boil. + + +
D.G. - - +
Red. - + +
BTNL8 (1:1000)
Fc (1:1000)

FIG. 14

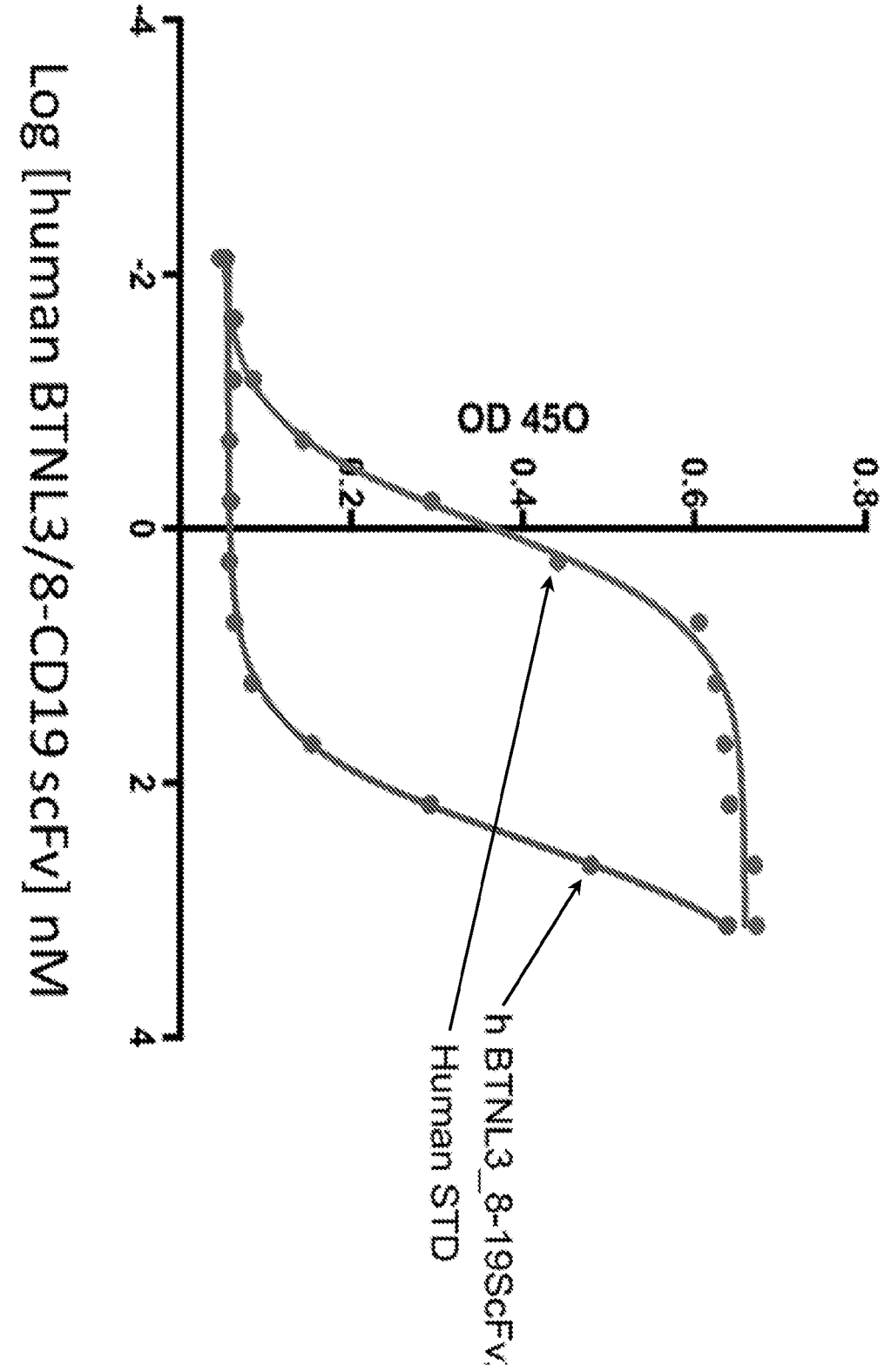

Log [human BTNL3/8-CD19 scFv] nM
-4
-2
0
2
4
OD 450
0.2
0.4
0.6
0.8
h BTNL3_8-19ScFv
Human STD

FIG. 15

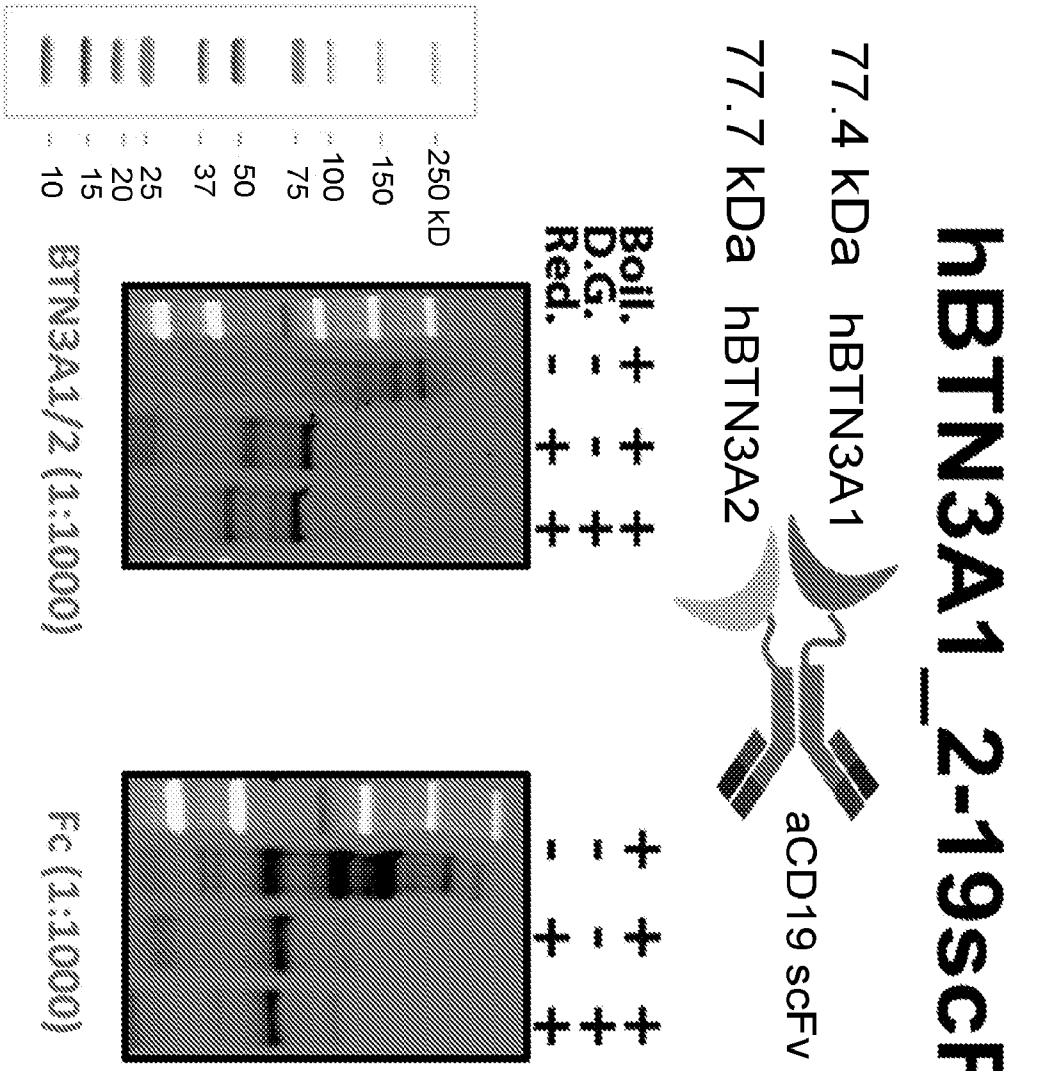

hBTN3A1_2-19scFv
77.4 kDa hBTN3A1
77.7 kDa hBTN3A2
aCD19 scFv
Boil. + + +
D.G. - - +
Red. - + +
250 kD
150
100
75
50
37
25
20
15
10
BTN3A1/2 (1:1000)
Fc (1:1000)

FIG. 16

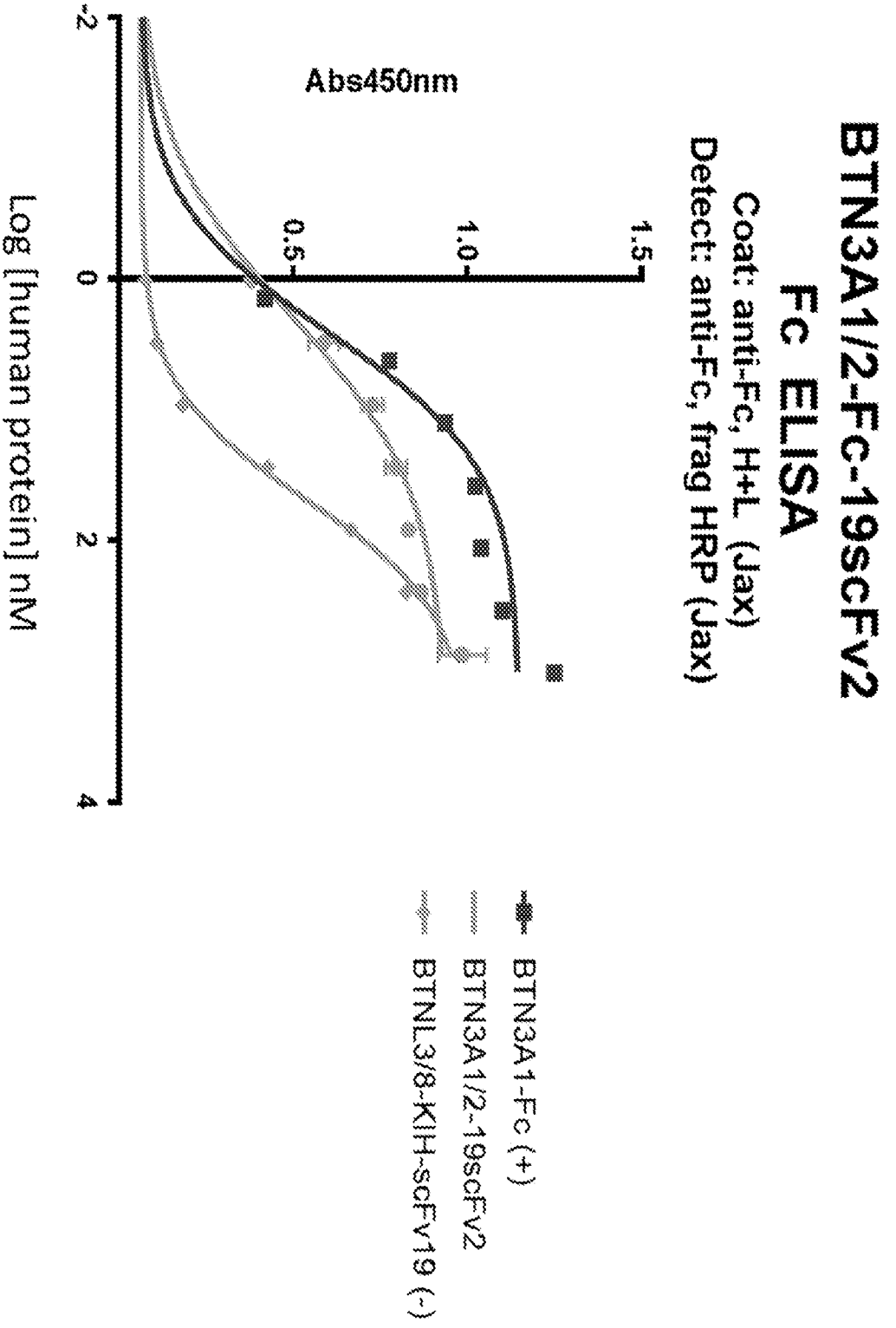

BTN3A1/2-Fc-19scFv2
Fc ELISA
Coat: anti-Fc, H+L (Jax)
Detect: anti-Fc, frag HRP (Jax)
Abs450nm
0.5
1.0
1.5
Log [human protein] nM
-2
0
2
4
BTN3A1-Fc (+)
BTN3A1/2-19scFv2
BTNL3/8-KIH-scFv19 (-)

FIG. 17

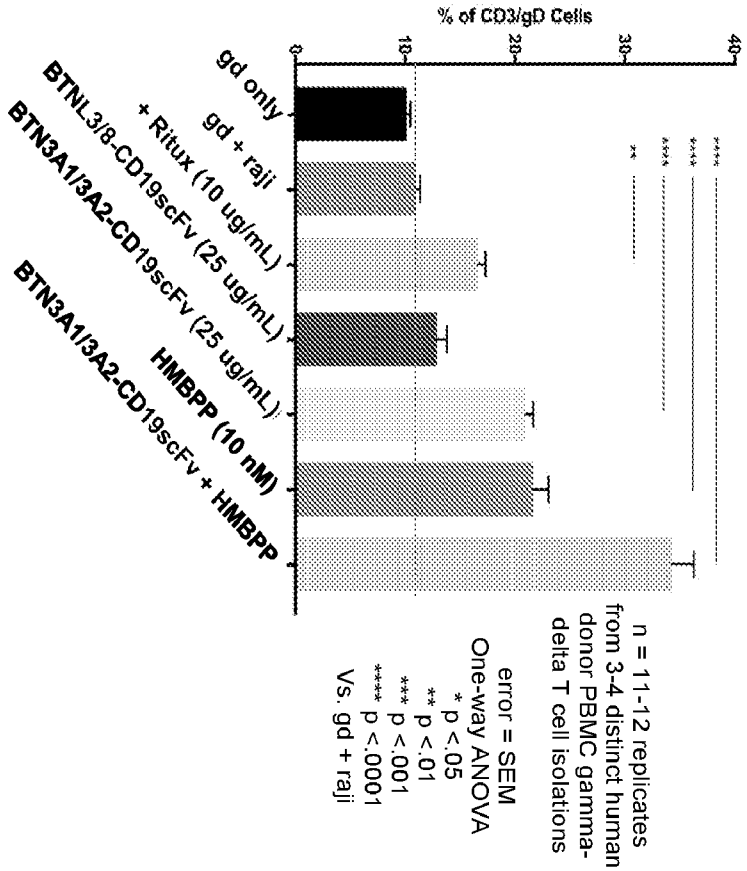


FIG. 18A

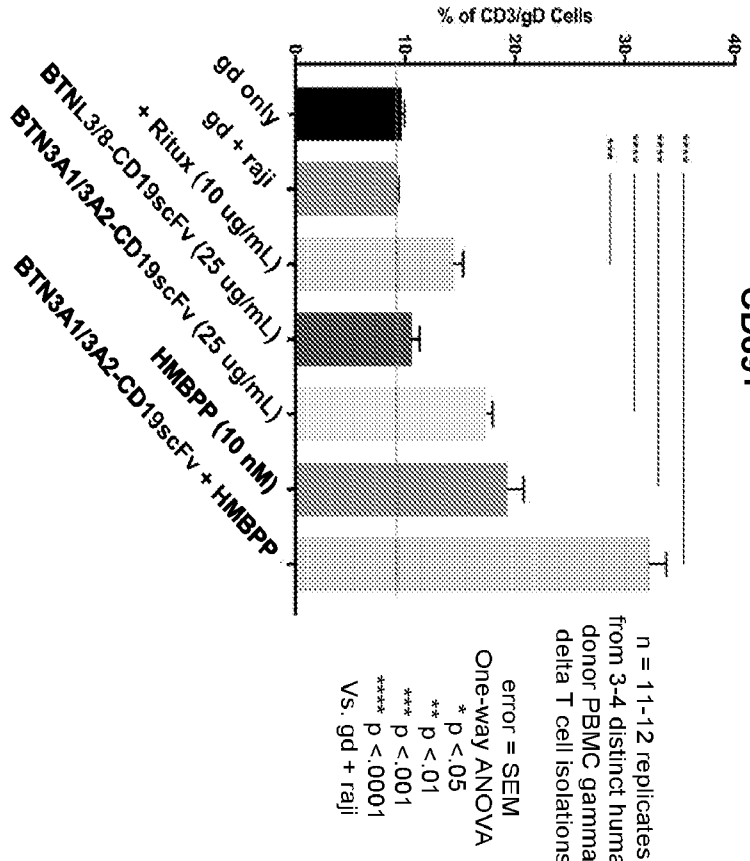


FIG. 18B

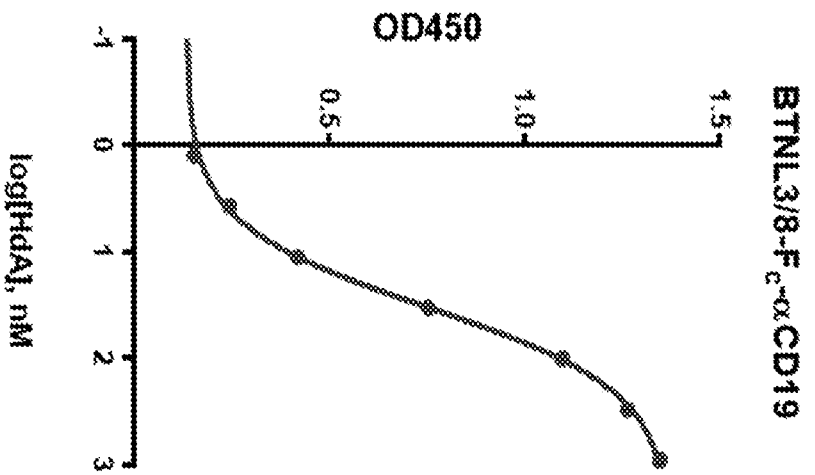


FIG. 19

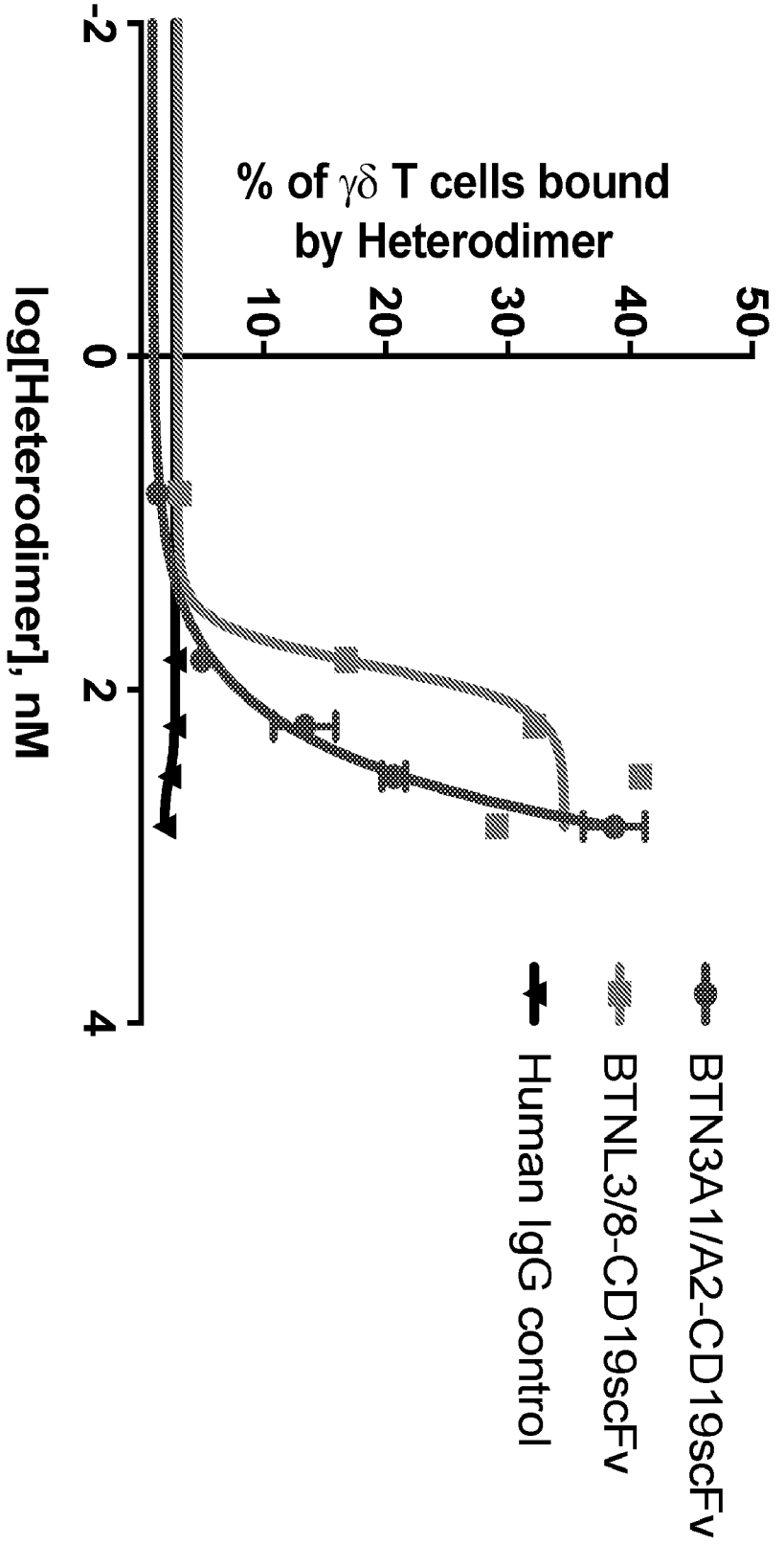

% of γδ T cells bound by Heterodimer
10
20
30
40
50
log[Heterodimer], nM
-2
0
2
4
BTN3A1/A2-CD19scFv
BTNL3/8-CD19scFv
Human IgG control

FIG. 20

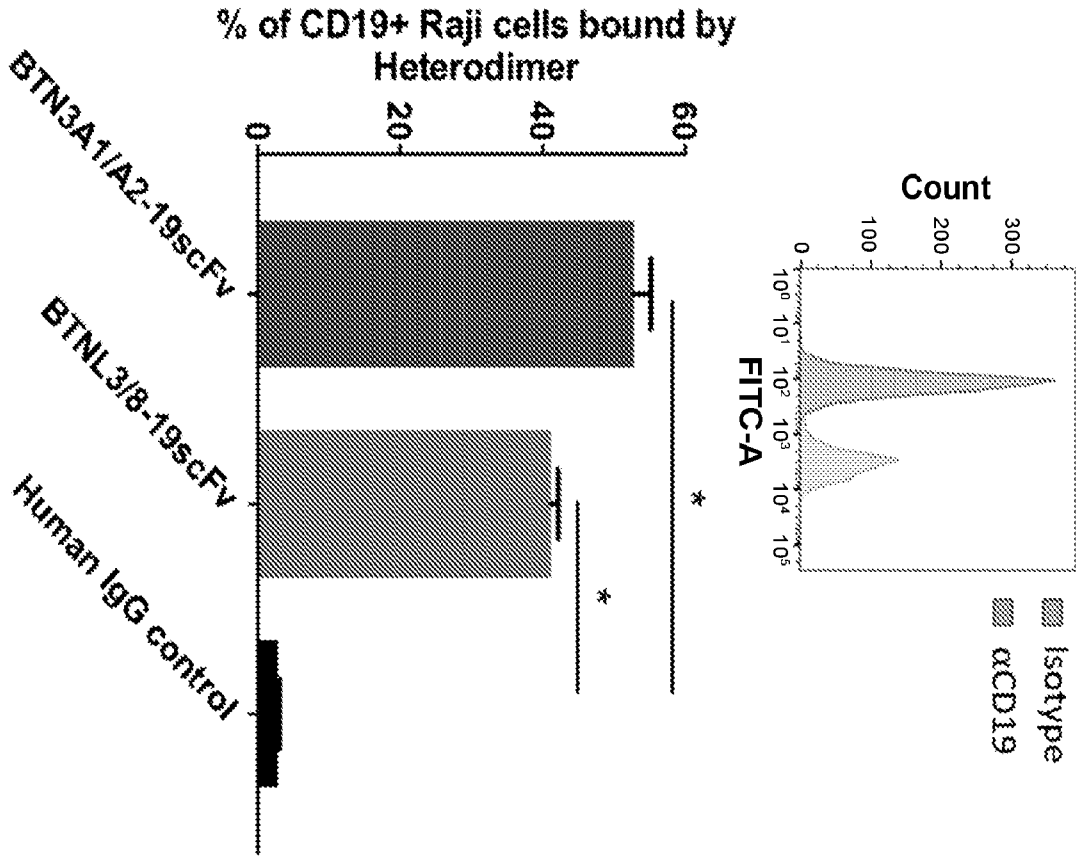

% of CD19+ Raji cells bound by Heterodimer
0
20
40
60
BTN3A1/A2-19scFv
BTNL3/8-19scFv
Human IgG control
*
*
Count
0
100
200
300
10^0 10^1 10^2 10^3 10^4 10^5
FITC-A
Isotype
αCD19

FIG. 21

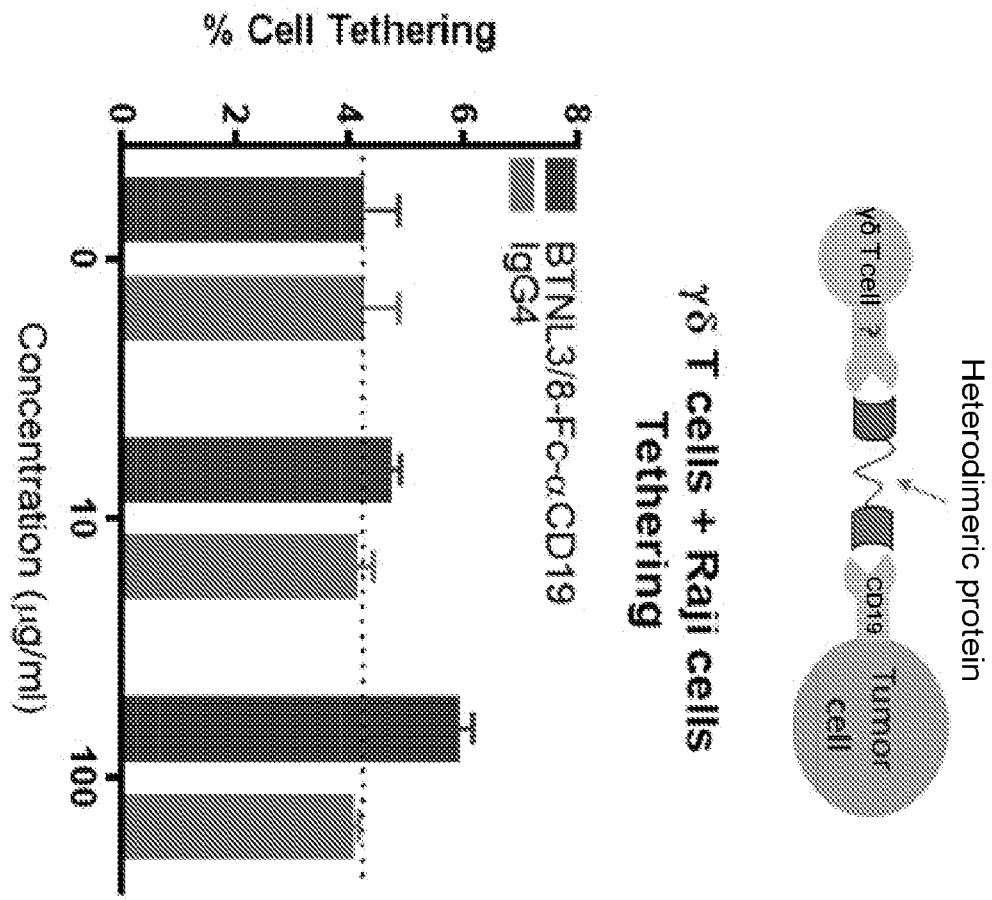

γδ T cell
Heterodimeric protein
CD19
Tumor cell
γδ T cells + Raji cells Tethering
% Cell Tethering
0
2
4
6
8
BTNL3/8-Fc-αCD19 IgG4
0
10
100
Concentration (μg/ml)

FIG. 22

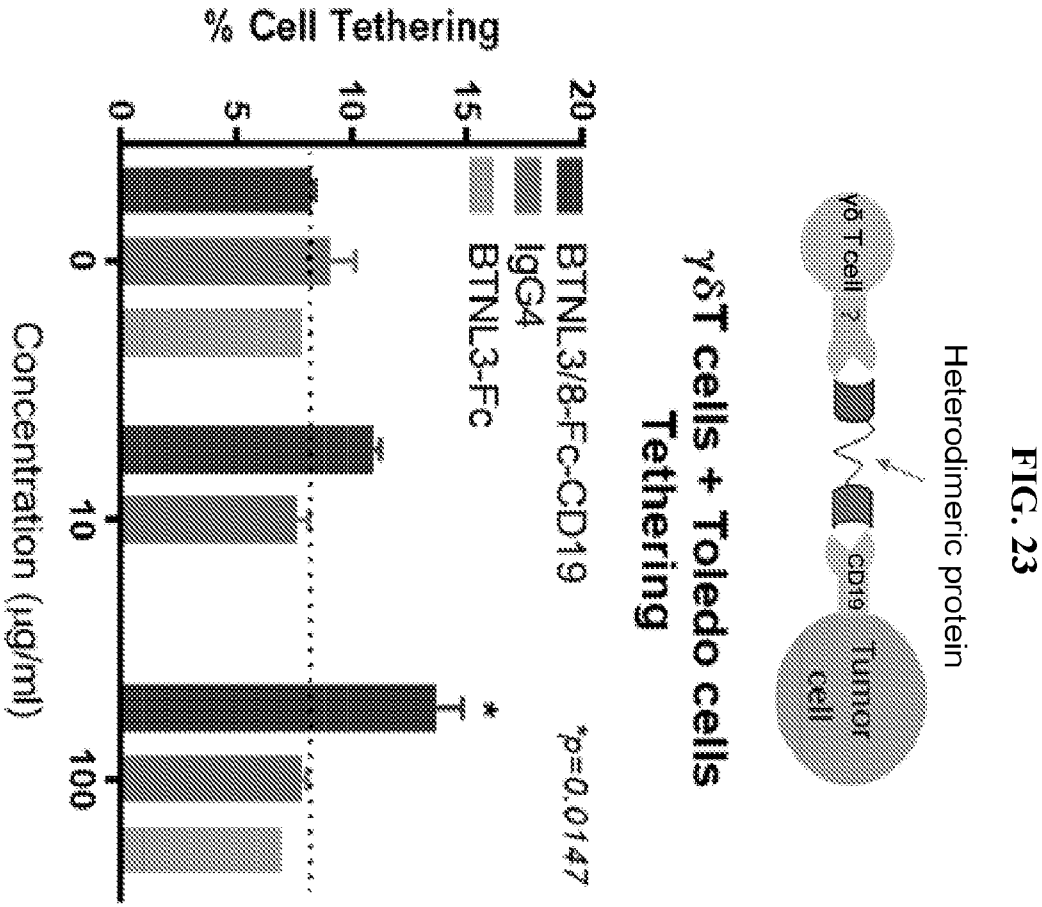

γδ T cell
Heterodimeric protein
CD19
Tumor cell
γδT cells + Toledo cells Tethering
% Cell Tethering
0
5
10
15
20
BTNL3/8-Fc-CD19
IgG4
BTNL3-Fc
*p=0.0147
0
10
100
Concentration (μg/ml)
*

FIG. 23

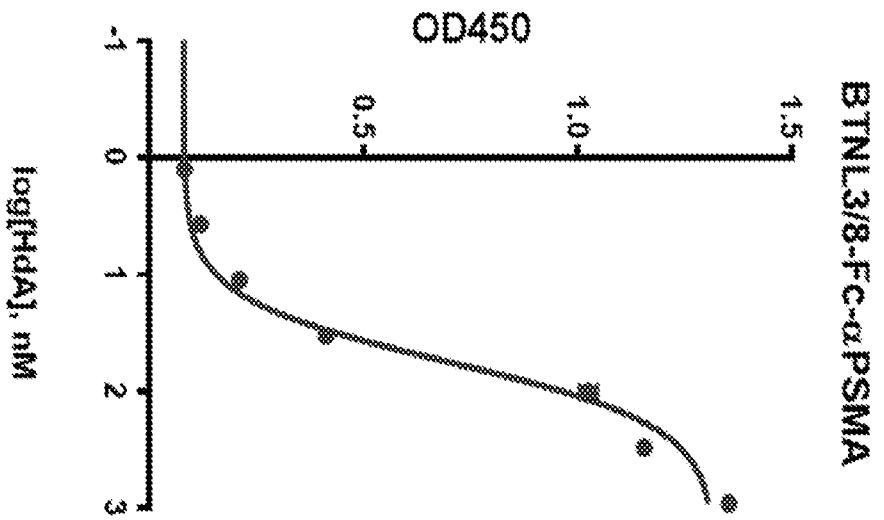


FIG. 24

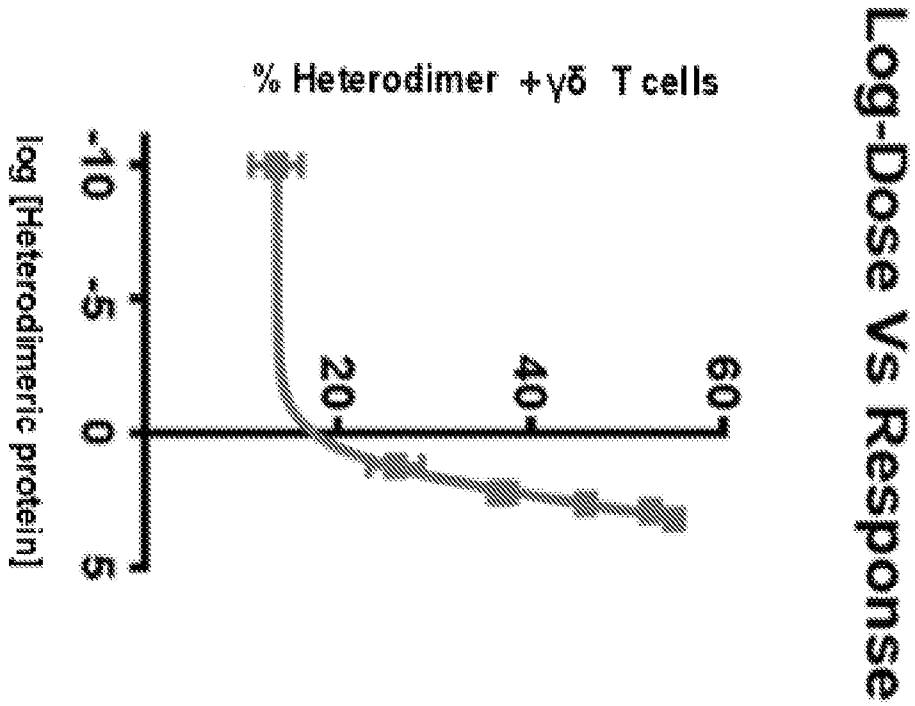

Log-Dose Vs Response
% Heterodimer +γδ T cells
log [Heterodimeric protein]
-10
-5
0
5
20
40
60

FIG. 25

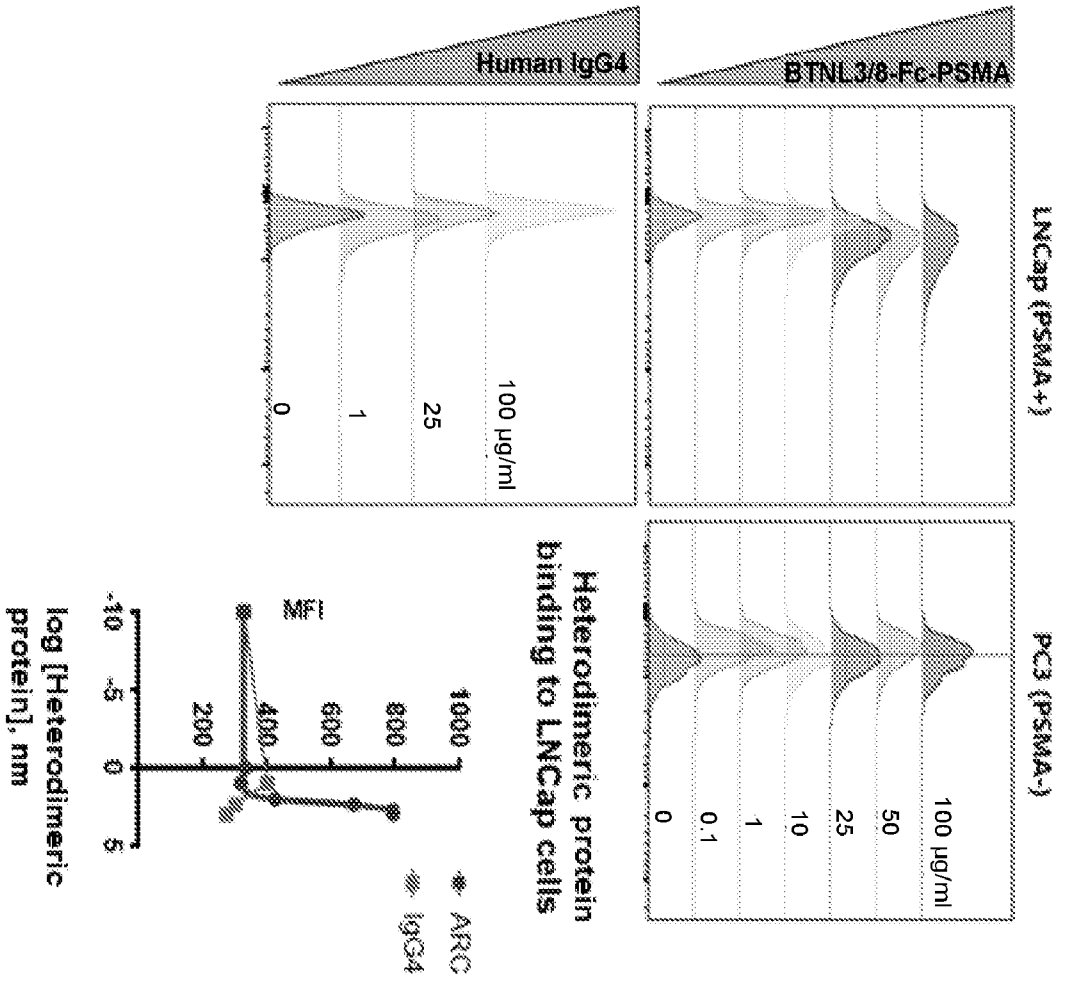

BTNL3/8-Fc-PSMA
Human IgG4
LNCap (PSMA+)
PC3 (PSMA-)
100 µg/ml
50
25
10
1
0.1
0
100 µg/ml
25
1
0
Heterodimeric protein binding to LNCap cells
MFI
1000
800
600
400
200
ARC
IgG4
-10
-5
0
5
log [Heterodimeric protein], nm

FIG. 26

FIG. 27

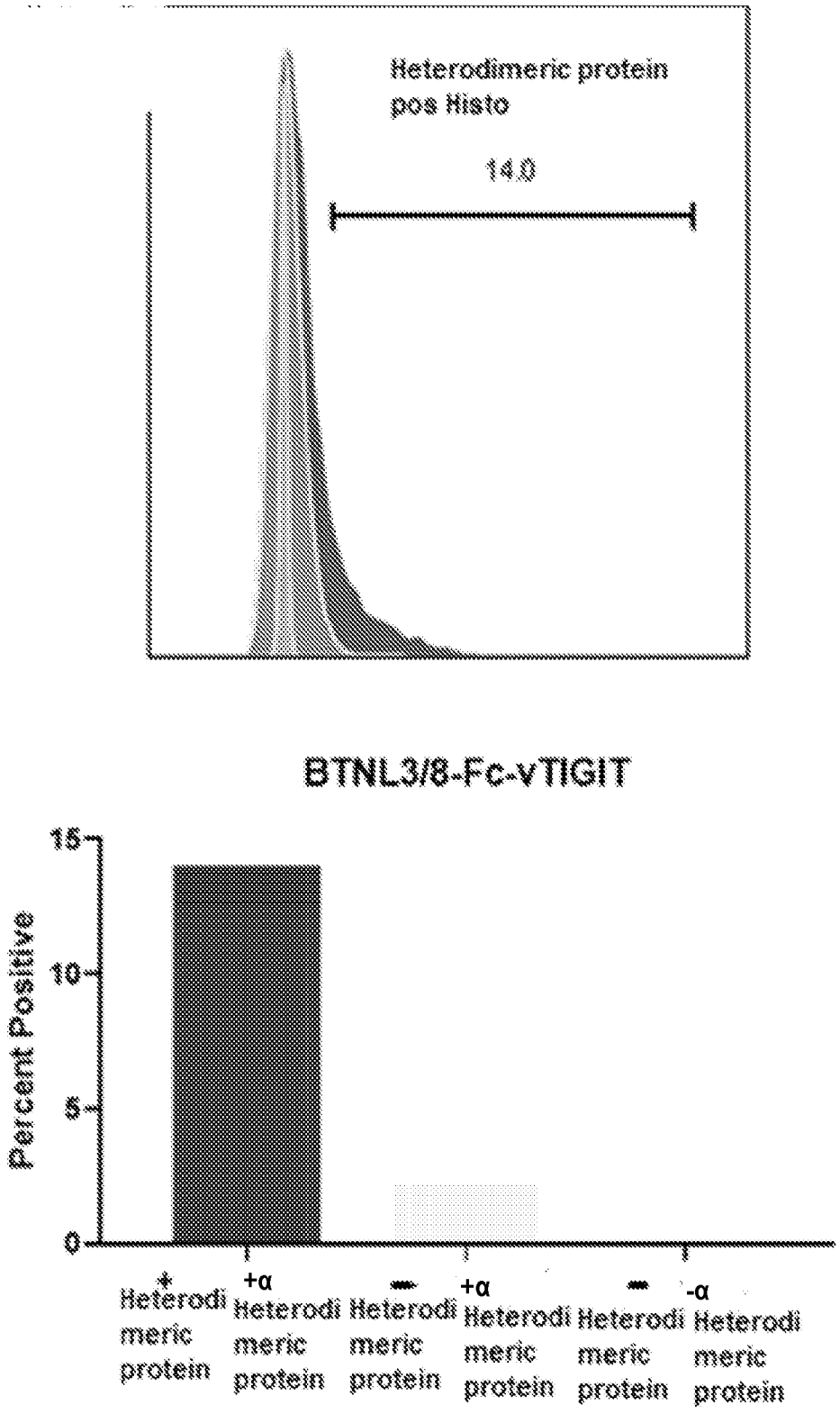
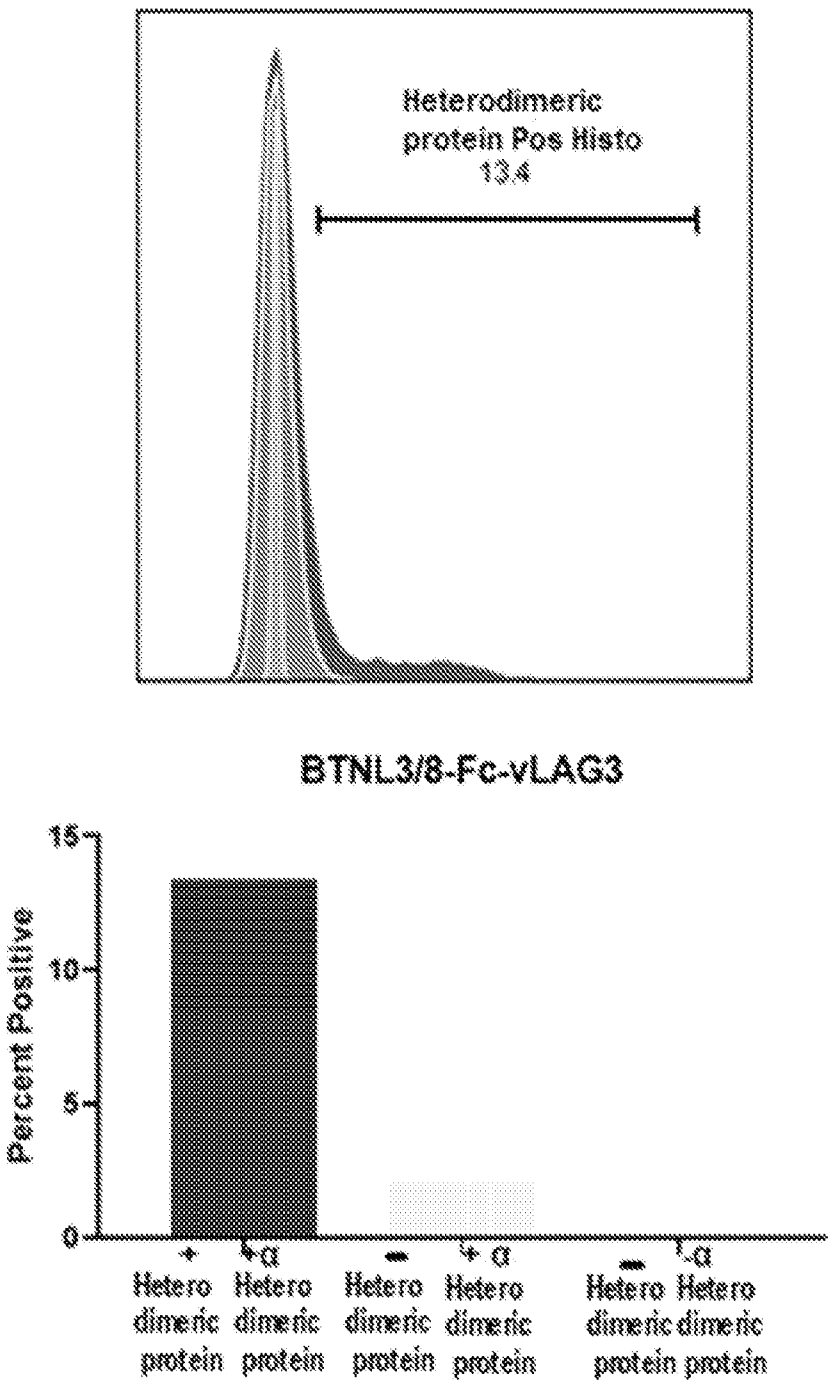

Heterodimeric protein
pos Histo
14.0
BTNL3/8-Fc-vTIGIT
Percent Positive
15
10
5
0
+
Heterodi meric protein
+α
Heterodi meric protein
-
Heterodi meric protein
+α
Heterodi meric protein
-
Heterodi meric protein
-α
Heterodi meric protein

FIG. 28


Heterodimeric protein Pos Histo 13.4
BTNL3/8-Fc-vLAG3
Percent Positive
15
10
5
0
+
+ α
-
- α
- α
Hetero dimeric protein
Hetero dimeric protein
Hetero dimeric protein
Hetero dimeric protein
Hetero dimeric protein
Hetero dimeric protein

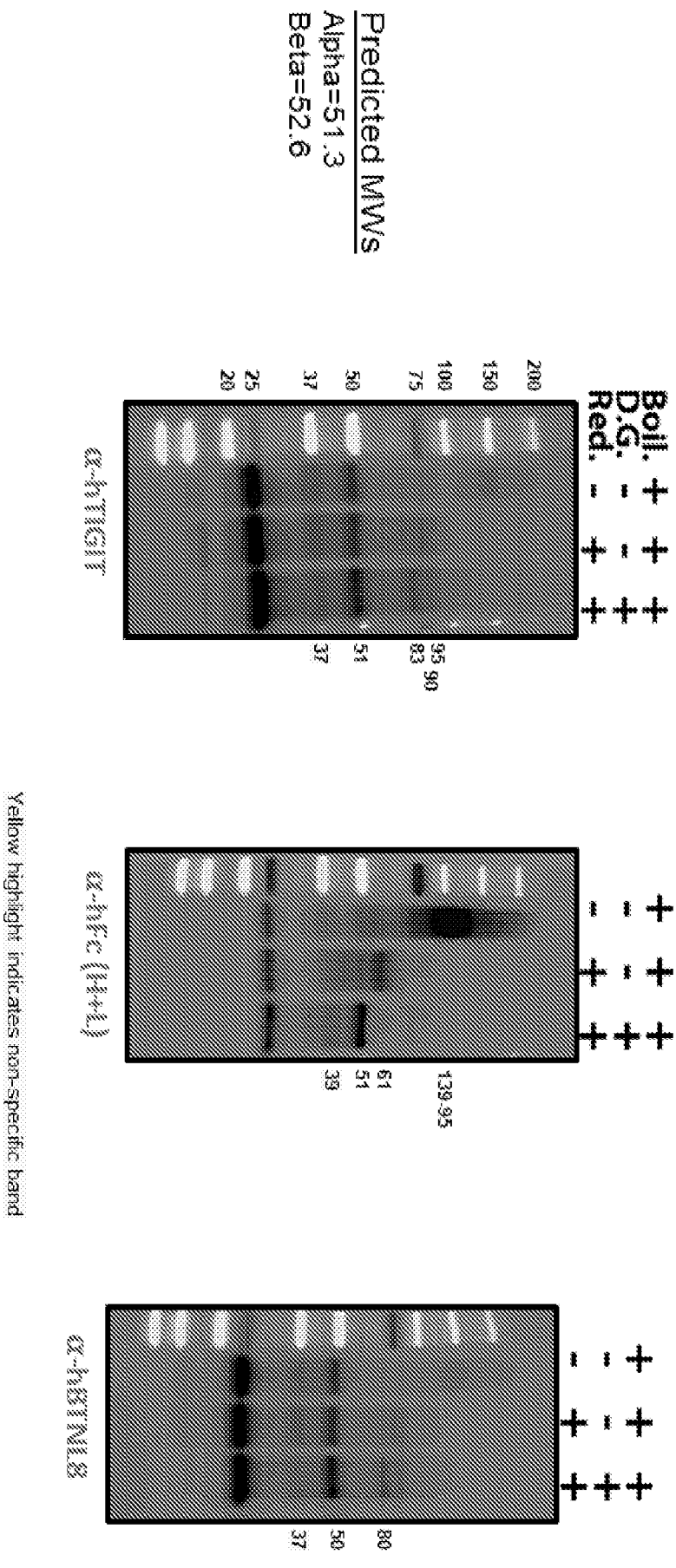
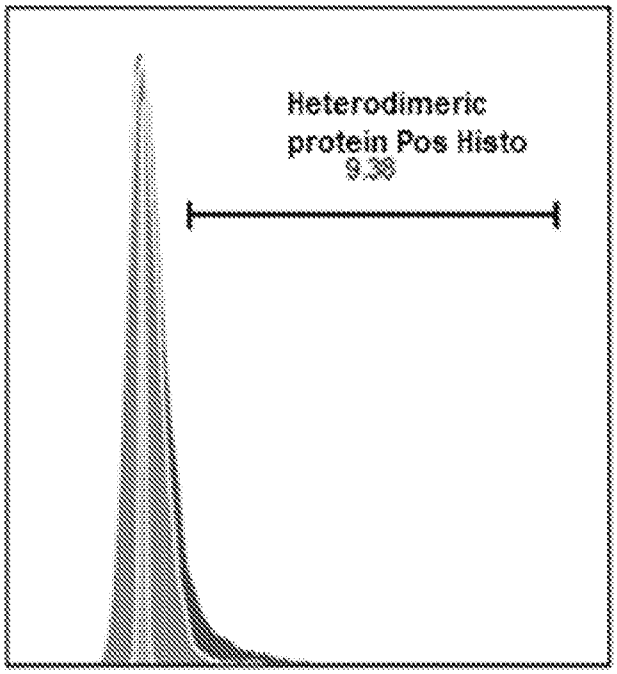

Predicted MWs
Alpha=51.3
Beta=52.6
Boil.
D.G.
Red.
200
150
100
75
50
37
25
20
95
90
83
51
37
α-hTIGIT
61
51
39
139-95
α-hFc (H+L)
80
50
37
α-hBTNL8
Yellow highlight indicates non-specific band

FIG. 29

FIG. 30


Heterodimeric
protein Pos Histo
9.3%

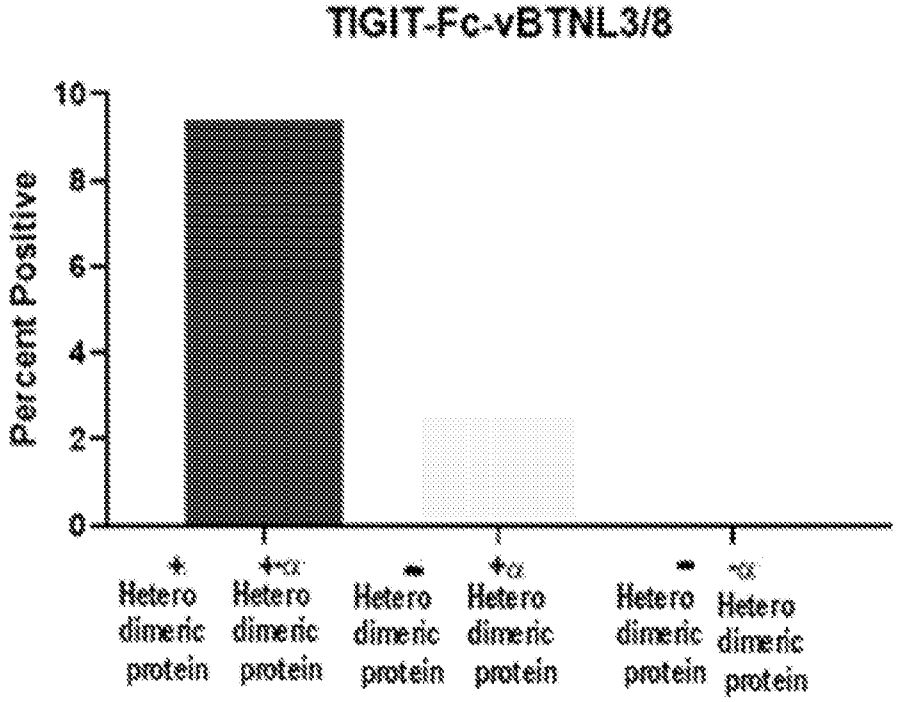
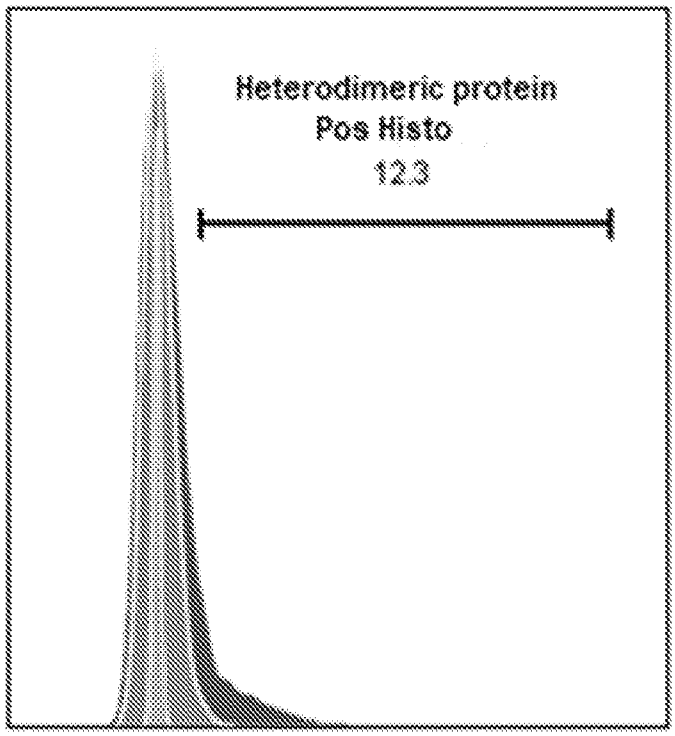
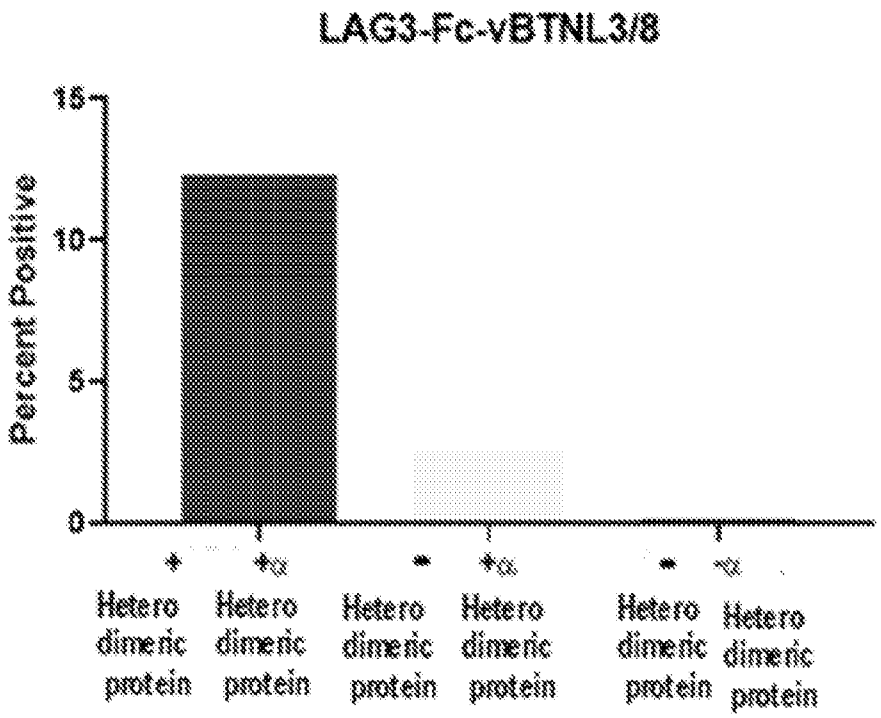

TIGIT-Fc-vBTNL3/8
Percent Positive
10
8
6
4
2
0
+
Hetero dimeric protein
+
Hetero dimeric protein
-
Hetero dimeric protein
+
Hetero dimeric protein
-
Hetero dimeric protein
Hetero dimeric protein

FIG. 31


Heterodimeric protein
Pos Histo
123


LAG3-Fc-vBTNL3/8
Percent Positive
15
10
5
0
Hetero dimeric protein
Hetero dimeric protein
Hetero dimeric protein
Hetero dimeric protein
Hetero dimeric protein
Hetero dimeric protein

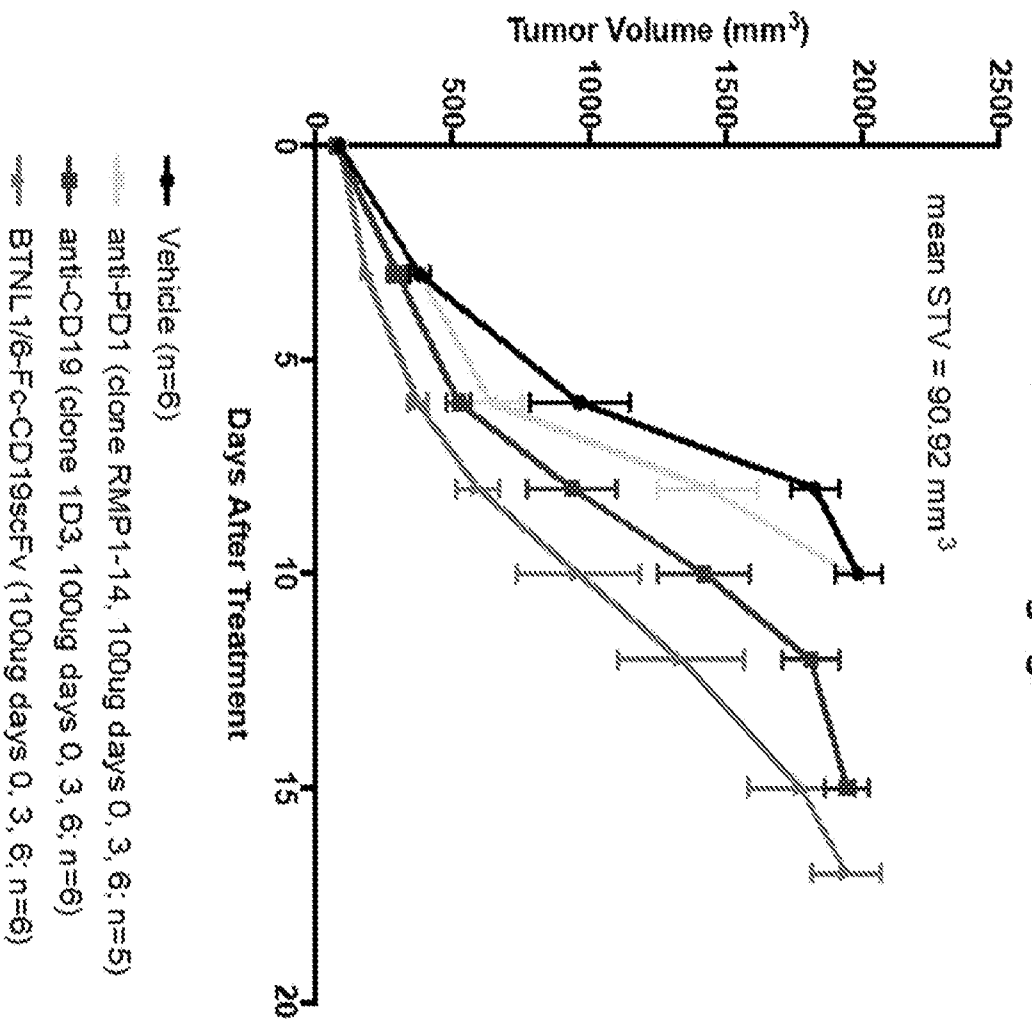

WEHI3 average growth
Tumor Volume (mm3)
2500
2000
1500
1000
500
0
mean STV = 90.92 mm3
0
5
10
15
20
Days After Treatment
Vehicle (n=6)
anti-PD1 (clone RMP1-14, 100ug days 0, 3, 6; n=5)
anti-CD19 (clone 1D3, 100ug days 0, 3, 6; n=6)
BTNL1/6-Fc-CD19scFv (100ug days 0, 3, 6; n=6)

FIG. 32A

FIG. 32B

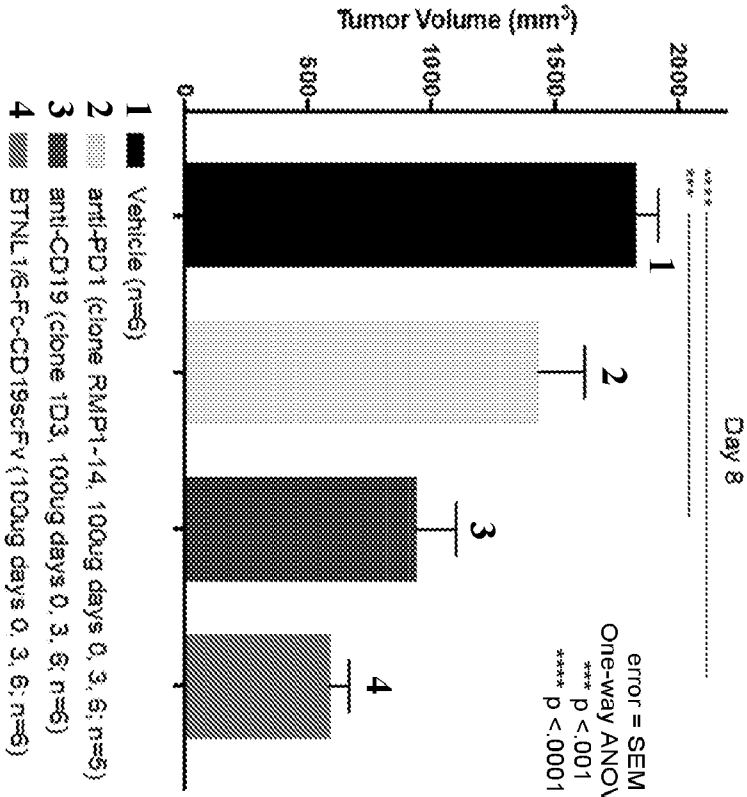
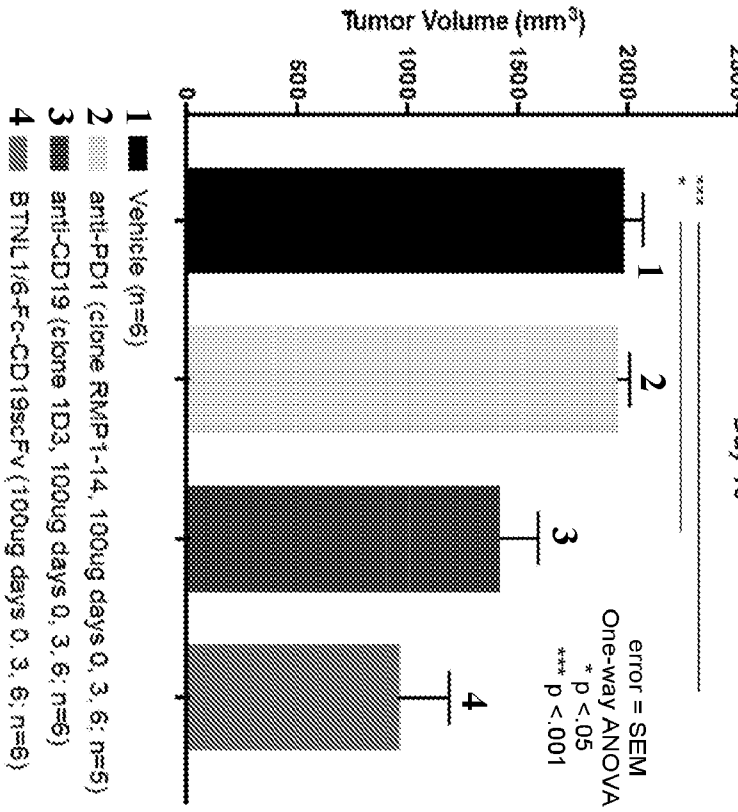


FIG. 32C



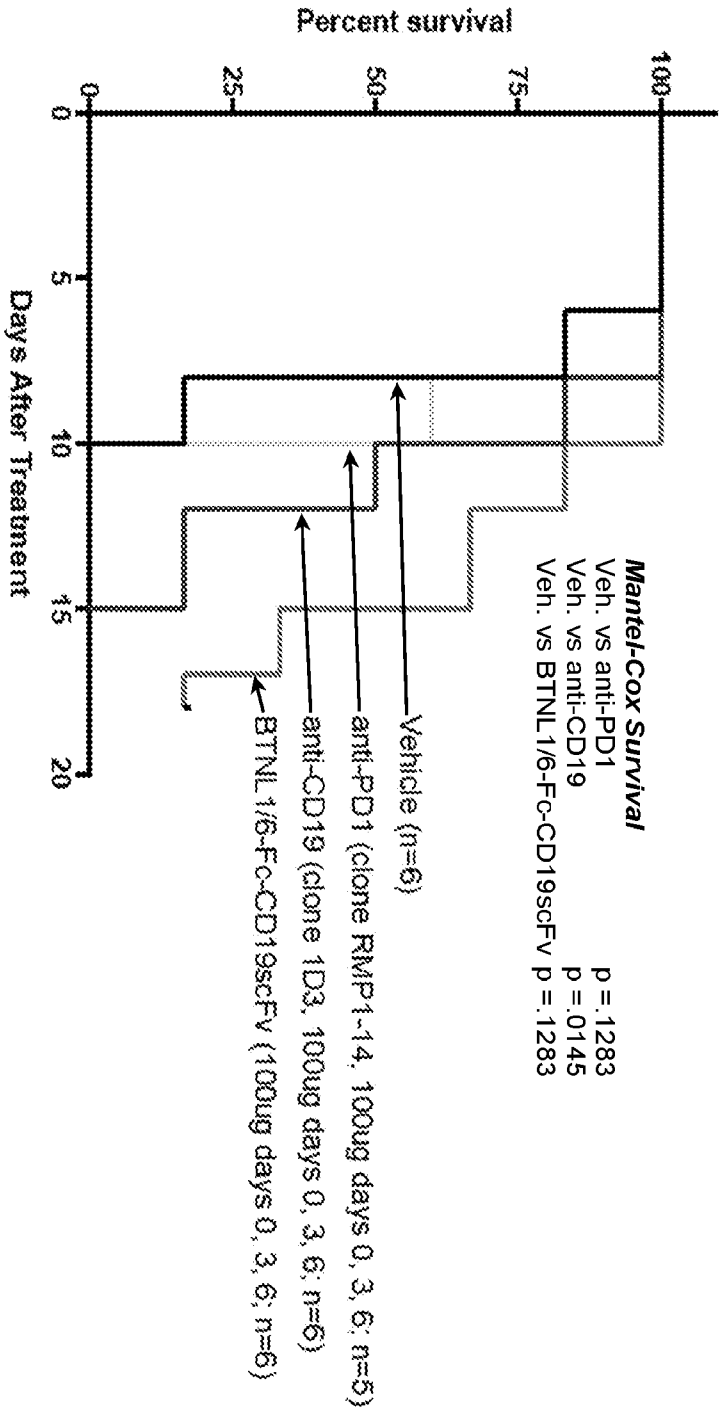

WEHI3 Survival
Percent survival
0
25
50
75
100
Days After Treatment
0
5
10
15
20
Vehicle (n=6)
anti-PD1 (clone RMP1-14, 100ug days 0, 3, 6; n=5)
anti-CD19 (clone 1D3, 100ug days 0, 3, 6; n=6)
BTNL1/6-Fc-CD19scFv (100ug days 0, 3, 6; n=6)
Mantel-Cox Survival
Veh. vs anti-PD1 p =.1283
Veh. vs anti-CD19 p =.0145
Veh. vs BTNL1/6-Fc-CD19scFv p =.1283

FIG. 33

FIG. 34A

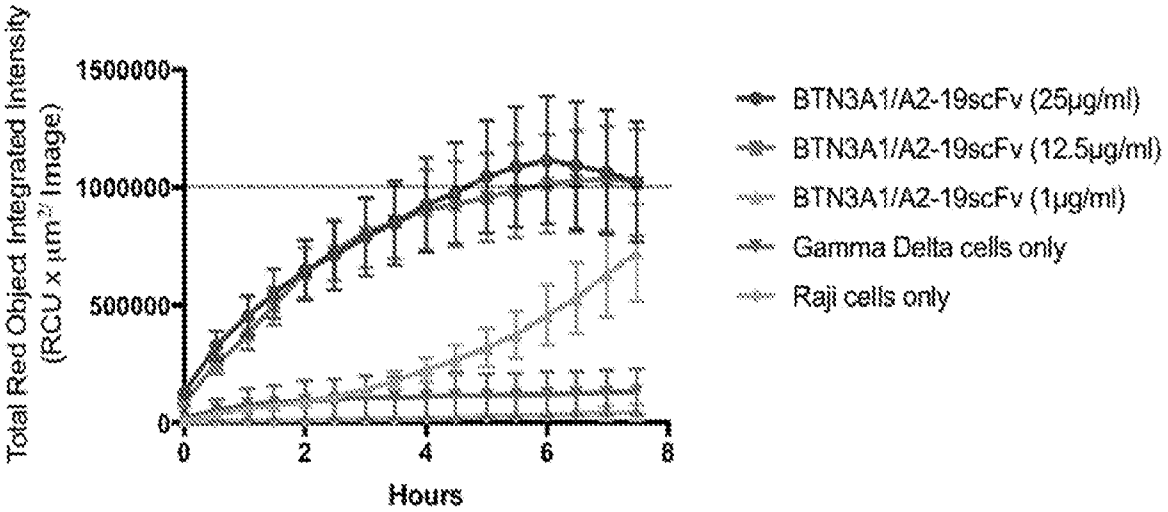


FIG. 34B

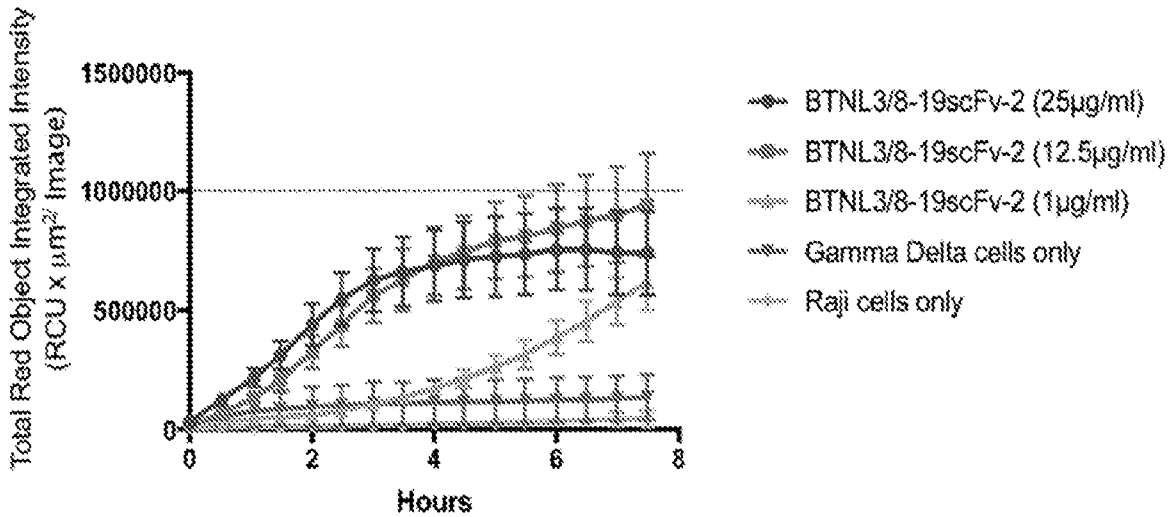
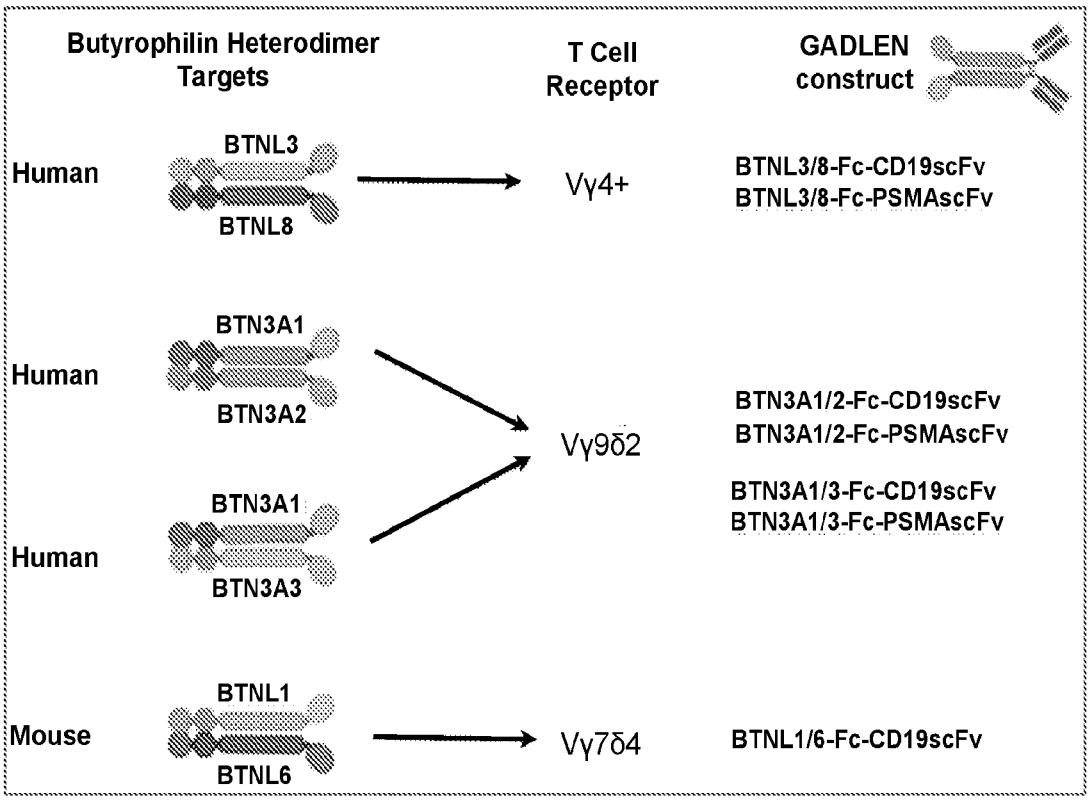


FIG. 1



Other Human Butyrophilin Targets	Potential Cancer Antigens Targeted with scFv Scaffolds
BTN1A1, BTN2A1, BTN2A2, BTN2A3, BTN3A3, BTNL2, BTNL9, BTNL10, SKINT	GD2, PSCA, BCMA, CD123, B7-H3, CD20, CD30, CD33, CD38, CEA, CLEC12A, DLL3, EGFRvIII, EpCAM, CD307, FLT3, GPC3, gpA33, GPCR5D, HER2, MUC16, P-cadherin, SSTR2, mesothelin, etc.

