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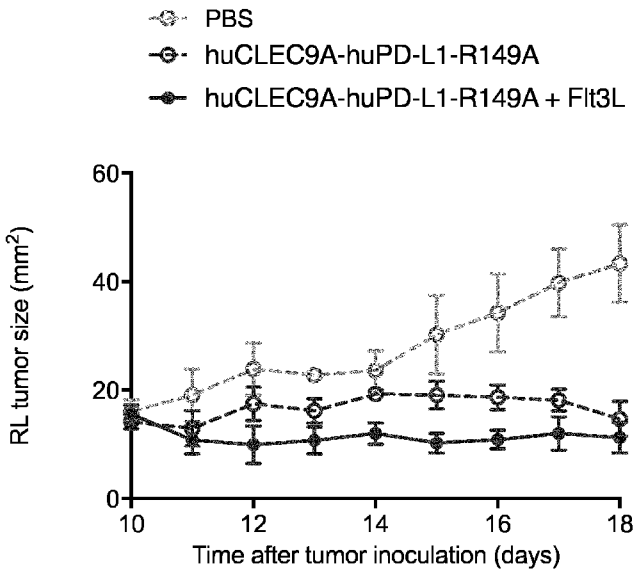
[Continued on next page]

(54) Title: BISPECIFIC SIGNALING AGENTS AND USES THEREOF

FIG. 18



(57) Abstract: The present invention relates, in part, to bispecific chimeric proteins that find use in various immunotherapies based on various properties, including, for example, a dual immune cell recruitment and immune signal delivery function.

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BISPECIFIC SIGNALING AGENTS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Nos. 62/291,769, filed February 5, 2016; 62/335,880, filed May 13, 2016; 62/411,805, filed October 24, 2016; 62/291,772, filed February 5, 2016; 62/291,774, filed February 5, 2016; 62/335,965, filed May 13, 2016; 62/291,776, filed February 5, 2016; 62/335,968, filed May 13, 2016; 62/335,979, filed May 13, 2016; 62/336,030, filed May 13, 2016, 62/353,607, filed June 23, 2016; and 62/291,779, filed February 5, 2016, the entire contents of all of which are herein incorporated by reference.

FIELD

The present invention relates, in part, to chimeric proteins that can recruit effector cells and deliver signaling to provide beneficial effects.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

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: ORN-009PC_Sequence_listing; date recorded: February 3, 2017; file size: 386 KB).

BACKGROUND

Cancer is a global health challenge that causes nearly 7 million deaths each year worldwide and which has, to date, proven largely untreatable despite major advances in medicine. Frustratingly, cancers appear to develop strategies to evade immune detection and destruction thereby sidestepping the body's main protection against disease.

Many recent immunotherapies have been developed to re-direct the body's immune system towards cancers. Immunotherapy provides the advantage of cell specificity that other treatment modalities, such as chemotherapy and radiation, lack. As such, methods for enhancing the efficacy of immune based therapies can be clinically beneficial. For example, costimulatory and coinhibitory molecules play a central role in the regulation of T cell immune responses. However, despite impressive patient responses to agents targeting these costimulatory and coinhibitory molecules, including, for example, clinical trials that led to the approval of YERVOY, KEYTRUDA, and OPDIVO, checkpoint inhibition therapy still fails in the overwhelming majority of patients.

Further, most cancer treatments, including immunotherapies, require complex regimens of various agents - each typically bringing a complex pattern of side effects that narrows a patient's therapeutic window for treatment and makes the patient more susceptible to other diseases.

Therefore, there remains a need for improved immunotherapeutic agents, including those that can effectively modulate the immune system in a multifunctional manner.

SUMMARY

In some aspects, the present invention relates to chimeric proteins which find use in various targeted cell methods. In some aspects, the present invention relates to chimeric proteins having two or more targeting moieties which have recognition domains (*e.g.* antigen recognition domains, including without limitation various antibody formats, inclusive of single-domain antibodies) which specifically bind to a target (*e.g.* antigen, receptor) of interest. The chimeric protein further comprises a modified (*e.g.* mutant) signaling agent (for instance, an immune-modulating agent), the modified (*e.g.* mutant) signaling agent having one or more modifications (*e.g.* mutations) that provide improved safety as compared to an unmodified (*e.g.* wild type) signaling agent. The targeting moieties and the modified (*e.g.* mutant) signaling agent are optionally connected by one or more linkers.

In various embodiments, the targeting moieties having recognition domains that specifically bind to a target (*e.g.* antigen, receptor) of interest, including those found on one or more immune cells, which can include, without limitation, T cells, cytotoxic T lymphocytes, T helper cells, natural killer (NK) cells, natural killer T (NKT) cells, anti-tumor macrophages (*e.g.* M1 macrophages), neutrophils, B cells, and dendritic cells. In some embodiments, the recognition domains specifically bind to a target (*e.g.* antigen, receptor) of interest and effectively recruit one of more immune cells. In some embodiments, the targets (*e.g.* antigens, receptors) of interest can be found on one or more tumor cells. In these embodiments, the present chimeric proteins may recruit an immune cell, *e.g.* an immune cell that can kill and/or suppress a tumor cell, to a site of action (such as, by way of non-limiting example, the tumor microenvironment).

Furthermore, not only can the present chimeric proteins directly or indirectly recruit cells that increase the likelihood of a beneficial effect, *e.g.* an anti-tumor effect, but also the present chimeric proteins may deliver important signaling to the cells via signaling agents (*e.g.* via one or more of an interferon, interleukin, TNF, EPO, etc.). Further, such signaling agents may be mutated such that the signaling is controlled and provides improved safety (*e.g.* does not come at the cost of systemic toxicity). Further still, in various embodiments, the present invention provides for various mutations to the signaling agents that can, for example, temper activity at a site of therapeutic action (*e.g.* at a therapeutic receptor) and also reduce or eliminate off-target binding at a non-therapeutic receptor. That is, in some embodiments, the signaling agents have reduced receptor promiscuity and controlled on-target action.

In various embodiments, the signaling agent can modulate one or more cells that are targeted by the targeting moieties (*e.g.* recruited cells, such as disease cells and/or effector cells). For instance, the signaling agent can modulate one or both of the targeted cells (and the targeted cells can be effector and/or disease cells), depending on whether the targeted cells express a receptor for the signaling agent.

In various embodiments, the present chimeric proteins find use in the treatment of various diseases or disorders such as cancer, infections, immune disorders, anemia, autoimmune diseases, cardiovascular diseases, wound

healing, ischemia-related diseases, neurodegenerative diseases, metabolic diseases and many other diseases and disorders, and the present invention encompasses various methods of treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows, in panel A, C57BL/6 mice were inoculated subcutaneously (50 µl) with $6x10^5$ B16mCD20cl1 melanoma tumor cells. Perilesional treatment with 120 µg of an anti-PD-L1 VHH (120 µl) or a fusion of an anti-PD-L1 VHH to human IFNalpha, Q124R mutant was started when tumors reached a size of ± 10 mm^2 as measured by caliper. The curves are in the same order (top to bottom) as in the figure legend identifying the treatment types (*e.g.* PBS is the top curve, anti-PD-L1 is the middle curve, and the bottom curve is the fusion of an anti-PD-L1 VHH to human IFNalpha, Q124R mutant). In panel B: C57BL/6 littermates were inoculated subcutaneously (50 µl) with $6x10^5$ B16mCD20cl1 melanoma tumor cells. Perilesional treatment with 30 µg of the indicated constructs (100 µl) was started when tumors reached a size of ± 10 mm^2 as measured by caliper. Black arrows indicate the treatment-schedule. The curves are in the same order (top to bottom) as in the figure legend identifying the treatment types (*e.g.* top curve is PBS, middle curve is a fusion of an anti-CD20 VHH to human IFNalpha, Q124R mutant, and the bottom curve is a bi-specific construct: anti-CD20 VHH-human IFNalpha, Q124R mutant-anti-PD-L1 VHH).

FIG. 2 shows an experiment in which C57BL/6 mice were inoculated subcutaneously (50 µl) with $6x10^5$ B16mCD20cl1 melanoma tumor cells. Perilesional treatment with 30 µg of the indicated chimeras (100 µl) was started when tumors reached a size of ± 10 mm^2 as measured by caliper. Top row of arrows indicate the treatment-schedule. Bottom row of arrows indicate perilesional injection of 120 µg of anti-PD-L1 VHH (120 µl). The curves are in the same order (top to bottom) as in the figure legend identifying the treatment types. "PD-L1 Q124R" is a fusion of an anti-PD-L1 VHH to human IFNalpha, Q124R mutant; "Clec9A-Q124R" is a fusion of an anti-Clec9A VHH to human IFNalpha, Q124R mutant; and "Clec9A-Q124R-PD-L1" is a bi-specific construct: anti-Clec9A VHH-human IFNalpha, Q124R mutant-anti-PD-L1 VHH.

FIG. 3 shows various safety parameters of the bi-specific mCD20-Q124R-PD-L1 chimera. In panels B-G, the order of histograms is, left to right, PBS, "CD20-Q124R," a fusion of an anti-CD20 VHH to human IFNalpha Q124R mutant, and a bi-specific of anti-CD20 VHH-human IFNalpha, Q124R mutant-anti-PD-L1 VHH ("CD20-Q124R-PD-L1").

FIG. 4 shows various safety parameters of the bi-specific Clec9A-Q124R-PD-L1 chimera. In panels B-G, the order of histograms is, left to right, PBS, anti-PD-L1, "Clec9A-Q124R," a fusion of an anti-Clec9A VHH to human IFNalpha Q124R mutant, a bi-specific of anti-Clec9A VHH-human IFNalpha, Q124R mutant-anti-PD-L1 VHH ("Clec9A-Q124R-PD-L1"), and "PD-L1-Q124R," a fusion of an anti-PD-L1 VHH to human IFNalpha Q124R mutant.

FIG. 5 shows a mouse tumor growth study in which C57BL/6 mice were inoculated subcutaneously (50 µl) with $6x10^5$ B16 melanoma tumor cells. Perilesional treatment with 30 µg of the indicated treatment agents (100 µl)

was started when tumors reached a size of ± 10 mm^2 as measured by caliper. Graph shows the evolution of tumor size over the indicated time.

FIG. 6 shows a mouse tumor growth study in which C57BL/6 mice were inoculated subcutaneously with B16 melanoma tumor cells. Perilesional treatment with the indicated treatment agents was started when tumors reached certain size as measured by caliper. Graph shows the evolution of tumor size over the indicated time.

FIG. 7 shows a mouse tumor growth study in which Batf3 knockout mice that lack cross-presenting dendritic cells were inoculated subcutaneously with B16 melanoma tumor cells. Perilesional treatment with the indicated treatment agents was started when tumors reached certain size as measured by caliper. Graph shows the evolution of tumor size over the indicated time.

FIG. 8, panels A-C, show a mouse tumor growth study in which mice were inoculated with 4T1 mammary tumor cells. The mice were treated with the indicated agents. Graphs show the evolution of tumor size over the indicated time.

FIG. 9 shows a mouse tumor growth study in which mice were inoculated with either B16 melanoma tumor cells (panel A) or 4T1 mammary tumor cells (panels B and C). The mice were then treated with the indicated agents with or without doxorubicin. Graphs show the evolution of tumor size over the indicated time.

FIG. 10 shows a mouse tumor growth study in which mice were inoculated with 4T1 mammary tumor cells. Tumor free mice from Fig. 9, panel B were rechallenged with 10^5 4T1 cells, and tumor growth was monitored. Graph shows the evolution of tumor size over the indicated time.

FIG. 11 shows the efficacy of bispecific chimeras using an experimental autoimmune encephalomyelitis (EAE) model. Panel A shows a schematic of the experimental design. Panel B shows the clinical score of mice treated with 100 IU of the indicated agents. Panel C shows the clinical score of mice treated with 1000 IU of the indicated agents. Panel D shows that the Clec9A-CD20-hIFNa2-Q124R bispecific chimera was safe and did not induce lymphopenia. The histogram bars represent, from left to right, treatment with PBS, a monospecific fusion of anti-Clec9A to modified human IFN alpha Q124R, a monospecific fusion of anti-mCD20 to modified human IFN alpha Q124R, a monospecific fusion of anti-Bcll10 to modified human IFN alpha Q124R, wild type IFN, a combination of the monospecific fusion of anti-Clec9A to modified human IFN alpha Q124R and the monospecific fusion of anti-mCD20 to modified human IFN alpha Q124R, the Clec9A-hIFNa2-Q124R-CD20 bispecific chimera, and the Clec9A-CD20-hIFNa2-Q124R bispecific chimera. Panel E compares the efficacy of the Clec9A-hIFNa2-Q124R-CD20 bispecific chimera at 100 IU and 1000 IU dosages. Panel F compares the efficacy of the Clec9A-CD20-hIFNa2-Q124R bispecific chimera at 100 IU and 1000 IU dosages.

FIG. 12 shows the efficacy of the Clec9A-CD20-hIFNa2-Q124R bispecific chimera at the 5000 IU dosage. Panel A shows the effect of the bispecific chimera on clinical score. Panel B shows the effect of the bispecific chimera on weight. Panel C shows the effect of the bispecific chimera on the incidence and onset of disease.

FIG. 13 shows an evaluation of tumor-antigen specific $CD8^+$ T cell proliferation and activation both in tumor-draining lymph nodes (panels A and B) as well as in spleen (panels C and D), two prime organs in the induction of anti-tumor immunity. B16-OVA bearing mice were injected once with PBS, bispecific chimera of anti-Clec9A VHH/ anti-PD-L1 VHH /human IFN Q124R or the same bispecific chimera in combination with doxorubicin one day after adoptive transfer of CFSE-labeled $CD8^+$ OT-I cells carrying a CD8 TCR recognizing the model antigen ovalbumin (OVA), present on B16-OVA tumor cells. Proliferation and activation of CFSE-labeled $CD8^+$ OT-I cells was evaluated 4 days later by flow cytometry, showing enhanced proliferation and activation status of the proliferated cells in mice receiving the bispecific chimera of anti-Clec9A VHH/ anti-PD-L1 VHH /human IFN Q124R or the same bispecific chimera in combination with doxorubicin as compared to perilesional injection with PBS (panels A and C). Data show the percentage of OT-I $CD8^+$ cells having undergone at least one division. One representative flow cytometric profile is included. In addition, as shown in panels B and D, flow cytometric analysis of CD44 and CD62L expression was performed on $CD8^+$ $CFSE^+$ T cells showing activated and memory T cell phenotype upon delivery of bispecific chimera of anti-Clec9A VHH/ anti-PD-L1 VHH /human IFN Q124R either alone or in combination with doxorubicin. * P<0.05, ** P<0,01, *** P<0.001 and **** P<0.0001 compared with PBS treated animals by one-way ANOVA with Dunnett's multiple comparison test.

FIG. 14 shows B16 cells stimulated with 100 ng/ml chimeras (or were left unstimulated) and stained for phospho STAT1. Data are plotted as mean fluorescent intensities. Chimeras analyzed are: anti-Sirp1α VHH/ anti-PD-L1 VHH /human IFN Q124R, anti-DNAM VHH/ anti-PD-L1 VHH /human IFN Q124R; and a monospecific fusion of anti-Bcll10 VHH to modified human IFN alpha Q124R (non-specific control).

FIG. 15 shows, in panels A, B, and C, human CD8 targeting of mono-specific chimeras (anti-human CD8 VHH/human IFN R149A). Zebra-plot of CD8 versus pSTAT1 staining of stimulated PBMCs is shown in panel A. Panels B and C: mean fluorescent intensities (MFI) of pSTAT1 staining of CD8-positive (panel B) or CD8-negative (panel C) cells are plotted. **FIG. 15** shows, in panels D, E, and F human CD8 targeting of bi-specific chimeras (anti-human CD8 VHH/anti-human PD-L1 VHH/human IFN R149A).

FIG. 16 shows an experiment in which MDA-MB-321 cells were stimulated with a serial dilution of chimeras and stained for phospho STAT1. Data are plotted as mean fluorescent intensities (MFI). Chimeras studied were anti-human PD-L1 VHH/human IFN R149A; anti-human PD-L1 VHH/human IFN R33A/E120R; anti-human Clec9A VHH/anti-human PD-L1 VHH/human IFN R149A; anti-human Clec9A VHH/ anti-human PD-L1 VHH/human IFN R33A/E120R; and anti-human Bcl10 VHH/human IFN R149A. For reference, this is the order of the constructs in **FIG. 16**, from top to bottom, when viewing the 4 ng/ml point on the X axis.

FIG. 17 shows, Daudi, Jurkat and Wish cells were stimulated with a serial dilution scFv chimeras and stained for phospho STAT1. Data are plotted as mean fluorescent intensities (MFI). Chimeras studied were anti-human CD20 scFv/anti-human CD3 scFv/IFN R149A. When viewing the graphs at point 1000 on the x axis, the order of the curves is Jurkat, Daudi, and Wish cells.

FIG. 18 shows the antitumor effects of an anti-human Clec9a VHH/anti-human PDL1 VHH/human IFN-R149A bi-specific chimera on a human tumor (RL) grown in humanized mice. With reference to day 18 on the X axis, the constructs studied were, from top to bottom: PBS (control); anti-human Clec9a VHH/anti-human PDL1 VHH/human IFN-R149A bi-specific chimera; and anti-human Clec9a VHH/anti-human PDL1 VHH/human IFN-R149A bi-specific chimera plus FMS-like tyrosine kinase 3 ligand (FLT3L).

FIG. 19 shows a human dendritic cell pSTAT1 signaling assay. Chimeras studied were anti-human Clec9A VHH/anti-human PD-L1 VHH/human IFN R149A and anti-human Clec9A VHH/ anti-human PD-L1 VHH/human IFN R33A/E120R. Two doses of the agents were studied: 100 ng/ml and 500 ng/ml. PBS was the control and the data are expressed as a fold change of the percentage of $pSTAT1^+$ dendritic cells (data is an average of a triplicate data set).

DETAILED DESCRIPTION

The present invention is based, in part, on the surprising discovery that bi-specific chimeric proteins with signaling agents bearing therapeutically beneficial mutations provide beneficial therapeutic properties and reduced side effects. In various embodiments, these chimeric proteins may recruit the appropriate cells (*e.g.* immune cells) to site of in need of therapeutic action (*e.g.* a tumor cell) via the dual targeting moieties and, in addition, delivery one or more important signals to effect a therapeutic signal (*e.g.* via one or more modified signaling agents). The present invention provides pharmaceutical compositions comprising the chimeric proteins and their use in the treatment of various diseases. Administration of the chimeric proteins and pharmaceutical compositions of the invention achieves significantly reduced side effects compared to the wild type soluble agent.

Chimeric Proteins

In various embodiments, the present invention relates to a bi-specific or multifunctional chimeric protein having two or more targeting moieties having recognition domains that specifically bind to a target (*e.g.* antigen, receptor) of interest, which optionally, and directly or indirectly, recruit cells of relevance and/or modulate the function of the recruited cells, and a modified signaling agent, which bears one or more mutations that render the signaling agent suitable for pharmaceutical use with minimal side effects (*e.g.* minimal cytokine storm-like effects, flu-like symptoms, suicidal thoughts, off-target side effects, among others).

In various embodiments, the chimeric protein, among other features, directly or indirectly recruits one or more effector cells to a disease cell, *e.g.* via the targeting moieties, and further delivers a signal to the effector cell to modulate the disease cell in a therapeutically-relevant manner. In various embodiments, the chimeric protein, among other features, directly or indirectly recruits one or more effector cells to a disease cell, *e.g.* via the targeting moieties, and further delivers a signal to the disease cell to modulate the effector cell in a therapeutically-relevant manner. In various embodiments, the chimeric protein, among other features, directly or indirectly recruits one or more effector cells to a disease cell, *e.g.* via the targeting moieties, and further delivers a signal to the effector cell to modulate the effector cell in a therapeutically-relevant manner. In various embodiments, the chimeric protein, among other features, directly or indirectly recruits one or more effector cells

to a disease cell, *e.g.* via the targeting moieties, and further delivers a signal to the disease cell to modulate the disease cell in a therapeutically-relevant manner. In some embodiments, the signaling agent effects effector cell and/or the disease cell.

In various embodiments, the present chimeric protein provides at least two therapeutic benefits when used pharmaceutically. For instance, the present chimeric protein may effectively recruit proper cells to a site of required therapy (by way of non-limiting example, immune effector cells to a tumor, *e.g.* the tumor microenvironment) and deliver one or more signals to the cells - *e.g.* immune effector cells and/or tumor cells - to promote a cancer reducing or eliminating effect (*e.g.* provide immune cell stimulation from the signaling agent, provide immune co-stimulatory signals via the targeting domains, provide reduction or silencing of immune co-inhibitory signals via the targeting domains, etc.). Accordingly, as described herein, the present chimeric protein provides a platform of therapeutically-relevant options for the effective treatment of diseases via the immune system, including, without limitation, the treatment of cancer.

Furthermore, the present chimeric protein, in various embodiments, has pharmacodynamic and pharmacokinetic properties that make it particularly suited for use in therapies. For example, in various embodiments, including embodiments in which smaller antibody-based formats are used for targeting (as described elsewhere herein), the present chimeric protein has a molecular weight that allows avoidance of renal excretion to allow for therapeutic effects at a low dose (*e.g.* without loss of the therapeutic via the kidney). For instance, the present chimeric protein, in various embodiments, is engineered to have a molecular mass of about 50 KDa, or about 60 KDa, or about 70 KDa, or about 80 KDa, or about 90 KDa, or about 100 KDa. In various embodiments, the present constructs evade kidney filtration such that they do not require external methods of half-life extension.

In various embodiments, each of the individual chimeric 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 chimeric 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 some embodiments, the chimeric protein may be fused or conjugated with an antibody or an antibody fragment such as an Fc fragment. For example, the chimeric protein may be fused to either the N-terminus or the C-terminus of the Fc domain of human immunoglobulin (Ig) G. In various embodiments, each of the individual chimeric 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.

As described herein, the present chimeric protein may have improved safety due to one of more modifications, *e.g.* mutations. In various embodiments, improved safety means that the present chimeric protein provides lower toxicity (*e.g.* systemic toxicity and/or tissue/organ-associated toxicities); and/or lessened or substantially eliminated side effects; and/or increased tolerability, lessened or substantially eliminated adverse events; and/or reduced or substantially eliminated off-target effects; and/or an increased therapeutic window.

In some embodiments, the present chimeric protein increases the therapeutic index (*e.g.*, by about 2-fold, about 5-fold, about 10-fold, about 25-fold, about 50-fold, about 100-fold, about 200-fold, or more) of any protein therapeutic (*e.g.*, based on a naturally occurring or engineered protein or fragment thereof) by reducing the general toxicity of the protein and maintaining or increasing the selective effect of the protein on target cells or tissues. For instance, this increase in therapeutic index (*e.g.*, by about 2-fold, about 5-fold, about 10-fold, about 25-fold, about 50-fold, about 100-fold, about 200-fold, or more) may be of the modified signaling agent (*e.g.* relative to the wild type signaling agent, or relative to a wild type signaling agent in the context of a fusion protein with a single targeting moiety, or relative to a wild type signaling agent in the context of a fusion protein with more than one targeting moieties).

In some embodiments, the present chimeric protein allows for efficient binding of the targeting moieties and the signaling agent to their receptors. For instance, in some embodiments, the chimeric protein allows for efficient binding of one of the targeting moieties and the signaling agent to receptors on the same cell (*e.g.*, different receptors) as well as the efficient binding of the other targeting moiety to another cell. As described elsewhere herein, in various embodiments, the signaling agent is mutated to provide attenuated activity, and the binding of the targeting moiety and the signaling agent to receptors on the same cell is sequential, *e.g.* targeting moiety/receptor binding preceding signaling agent/receptor binding. For instance, in some embodiments the signaling agent by itself is significantly less active in its mutated form (*e.g.* relative to wild type) because it cannot efficiently bind to its receptor(s). Accordingly, chimeric proteins of the invention are useful to avoid unwanted side effects caused by the signaling agent binding to its natural receptor on non-target cells. However, the signaling agent is active on target cells because the targeting moiety(ies) compensates for the missing/insufficient binding (*e.g.*, without limitation and/or avidity) required for substantial activation. In various embodiments, the chimeric proteins of the present invention have a modified (*e.g.* mutant) signaling agent which causes the signaling agent to be inactive *en route* to the site of therapeutic activity (*e.g.* in contact with a target cell, including a tumor cell) through the body and to have its effect substantially on specifically targeted cell types which greatly reduces undesired side effects

In various embodiments, the present chimeric proteins have selective bioactivity, *e.g.* therapeutically-relevant bioactivity, towards targeted cells (*e.g.* tumor cells), but not towards cells that are not targeted (*e.g.* normal, non-tumor cells).

In various embodiments, the present chimeric proteins provide synergistic activity and/or therapeutic effects. In such embodiments, the activity and/or therapeutic effects of the chimeric proteins have improved therapeutic effects, *e.g.* synergistically greater, than the therapeutic effects of the individual components (*i.e.*, the targeting moieties and the signaling agent) administered alone or in combination via co-administration.

In various embodiments, the present chimeric proteins have two or more targeting moieties and the signaling agent connected together via different configurations. In an embodiment, one of the targeting moieties is linked to the amino-terminus of the signaling agent and the other targeting moiety is linked to the carboxy-terminus of

the signaling agent. In another embodiment, both targeting moieties are linked to the amino-terminus of the signaling agent. For example, the amino-terminus of the signaling agent may be linked to the carboxy-terminus of one of the targeting moieties, which in turn is linked to the other targeting moiety (*e.g.*, via its amino-terminus). In a further embodiment, both targeting moieties are linked to the carboxy-terminus of the signaling agent. For example, the carboxy-terminus of the signaling agent may be linked to the amino-terminus of one of the targeting moieties, which in turn is linked to the other targeting moiety (*e.g.*, via its carboxy-terminus).

In various embodiments, the present chimeric proteins are in the form of a fusion protein having the components described herein.

In various embodiments, the present chimeric proteins have two or more targeting moieties that target different antigens or receptors, and one targeting moiety may be attenuated for its antigen or receptor, *e.g.* the targeting moiety binds its antigen or receptor with a low affinity or avidity (including, for example, at an affinity or avidity that is less than the affinity or avidity the other targeting moiety has for its for its antigen or receptor, for instance the difference between the binding affinities may be about 10-fold, or 25-fold, or 50-fold, or 100-fold, or 300-fold, or 500-fold, or 1000-fold, or 5000-fold; for instance the lower affinity or avidity targeting moiety may bind its antigen or receptor at a K_D in the mid- to high-nM or low- to mid-μM range while the higher affinity or avidity targeting moiety may bind its antigen or receptor at a K_D in the mid- to high-pM or low- to mid-nM range). For instance, in some embodiments, the present chimera comprises an attenuated targeting moiety that is directed against a promiscuous antigen or receptor, which may improve targeting to a cell of interest (*e.g.* via the other targeting moiety) and prevent effects across multiple types of cells, including those not being targeted for therapy (*e.g.* by binding promiscuous antigen or receptor at a higher affinity than what is provided in these embodiments).

In various embodiments, the present invention provides for an isolated nucleic acid encoding a chimeric protein as described herein.

Targeting Moiety Cellular Recruitment

In various embodiments, the chimeric proteins of the present invention have targeting moieties having recognition domains which specifically bind to a target (*e.g.* antigen, receptor) of interest. In various embodiments, the chimeric proteins of the present invention have one or more targeting moieties which target different cells (*e.g.* to make a synapse) or the same cell (*e.g.* to get a more concentrated signaling agent or therapeutic effect). The target (*e.g.* antigen, receptor) of interest can be found on one or more immune cells, which can include, without limitation, 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, dendritic cells, or subsets thereof. In some embodiments, the recognition domains specifically bind to a target (*e.g.* antigen, receptor) of interest and effectively, directly or indirectly, recruit one of more immune cells. In some embodiments, the target (*e.g.* antigen, receptor) of interest can be found on one or more tumor cells. In these embodiments, the present chimeric proteins may directly or indirectly recruit an immune cell, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). In some

embodiments, the present chimeric proteins may directly or indirectly recruit an immune cell, *e.g.* an immune cell that can kill and/or suppress a tumor cell, to a site of action (such as, by way of non-limiting example, the tumor microenvironment).

In various embodiments, the chimeric proteins have targeting moieties having recognition domains which specifically bind to a target (*e.g.* antigen, receptor) which is part of a non-cellular structure. In some embodiments, the antigen or receptor is not an integral component of an intact cell or cellular structure. In some embodiments, the antigen or receptor is an extracellular antigen or receptor. In some embodiments, the target is a non-proteinaceous, non-cellular marker, including, without limitation, nucleic acids, inclusive of DNA or RNA, such as, for example, DNA released from necrotic tumor cells or extracellular deposits such as cholesterol.

In some embodiments, the target (*e.g.* antigen, receptor) of interest is part of the non-cellular component of the stroma or the extracellular matrix (ECM) or the markers associated therewith. As used herein, stroma refers to the connective and supportive framework of a tissue or organ. Stroma may include a compilation of cells such as fibroblasts/myofibroblasts, glial, epithelia, fat, immune, vascular, smooth muscle, and immune cells along with the extracellular matrix (ECM) and extracellular molecules. In various embodiments, the target (*e.g.* antigen, receptor) of interest is part of the non-cellular component of the stroma such as the extracellular matrix and extracellular molecules. As used herein, the ECM refers to the non-cellular components present within all tissues and organs. The ECM is composed of a large collection of biochemically distinct components including, without limitation, proteins, glycoproteins, proteoglycans, and polysaccharides. These components of the ECM are usually produced by adjacent cells and secreted into the ECM via exocytosis. Once secreted, the ECM components often aggregate to form a complex network of macromolecules. In various embodiments, the chimeric protein of the invention comprises a targeting moiety that recognizes a target (*e.g.*, an antigen or receptor or non-proteinaceous molecule) located on any component of the ECM. Illustrative components of the ECM include, without limitation, the proteoglycans, the non-proteoglycan polysaccharides, fibers, and other ECM proteins or ECM non-proteins, *e.g.* polysaccharides and/or lipids, or ECM associated molecules (*e.g.* proteins or non-proteins, *e.g.* polysaccharides, nucleic acids and/or lipids).

In some embodiments, the targeting moiety recognizes a target (*e.g.* antigen, receptor) on ECM proteoglycans. Proteoglycans are glycosylated proteins. The basic proteoglycan unit includes a core protein with one or more covalently attached glycosaminoglycan (GAG) chains. Proteoglycans have a net negative charge that attracts positively charged sodium ions (Na+), which attracts water molecules via osmosis, keeping the ECM and resident cells hydrated. Proteoglycans may also help to trap and store growth factors within the ECM. Illustrative proteoglycans that may be targeted by the chimeric proteins of the invention include, but are not limited to, heparan sulfate, chondroitin sulfate, and keratan sulfate. In an embodiment, the targeting moiety recognizes a target (*e.g.* antigen, receptor) on non-proteoglycan polysaccharides such as hyaluronic acid.

In some embodiments, the targeting moiety recognizes a target (*e.g.* antigen, receptor) on ECM fibers. ECM fibers include collagen fibers and elastin fibers. In some embodiments, the targeting moiety recognizes one or

more epitopes on collagens or collagen fibers. Collagens are the most abundant proteins in the ECM. Collagens are present in the ECM as fibrillar proteins and provide structural support to resident cells. In one or more embodiments, the targeting moiety recognizes and binds to various types of collagens present within the ECM including, without limitation, fibrillar collagens (types I, II, III, V, XI), facit collagens (types IX, XII, XIV), short chain collagens (types VIII, X), basement membrane collagens (type IV), and/or collagen types VI, VII, or XIII. Elastin fibers provide elasticity to tissues, allowing them to stretch when needed and then return to their original state. In some embodiments, the target moiety recognizes one or more epitopes on elastins or elastin fibers.

In some embodiments, the targeting moiety recognizes one or more ECM proteins including, but not limited to, a tenascin, a fibronectin, a fibrin, a laminin, or a nidogen/entactin.

In an embodiment, the targeting moiety recognizes and binds to tenascin. The tenascin (TN) family of glycoproteins includes at least four members, tenascin-C, tenascin-R, tenascin-X, and tenascin W. The primary structures of tenascin proteins include several common motifs ordered in the same consecutive sequence: amino-terminal heptad repeats, epidermal growth factor (EGF)-like repeats, fibronectin type III domain repeats, and a carboxyl-terminal fibrinogen-like globular domain. Each protein member is associated with typical variations in the number and nature of EGF-like and fibronectin type III repeats. Isoform variants also exist particularly with respect to tenascin-C. Over 27 splice variants and/or isoforms of tenascin-C are known. In a particular embodiment, the targeting moiety recognizes and binds to tenascin-CA1. Similarly, tenascin-R also has various splice variants and isoforms. Tenascin-R usually exists as dimers or trimers. Tenascin-X is the largest member of the tenascin family and is known to exist as trimers. Tenascin-W exists as trimers. In some embodiments, the targeting moiety recognizes one or more epitopes on a tenascin protein. In some embodiments, the targeting moiety recognizes the monomeric and/or the dimeric and/or the trimeric and/or the hexameric forms of a tenascin protein.

In an embodiment, the targeting moieties recognize and bind to fibronectin. Fibronectins are glycoproteins that connect cells with collagen fibers in the ECM, allowing cells to move through the ECM. Upon binding to integrins, fibronectins unfolds to form functional dimers. In some embodiments, the targeting moiety recognizes the monomeric and/or the dimeric forms of fibronectin. In some embodiments, the targeting moiety recognizes one or more epitopes on fibronectin. In illustrative embodiments, the targeting moiety recognizes fibronectin extracellular domain A (EDA) or fibronectin extracellular domain B (EDB). Elevated levels of EDA are associated with various diseases and disorders including psoriasis, rheumatoid arthritis, diabetes, and cancer. In some embodiments, the targeting moiety recognizes fibronectin that contains the EDA isoform and may be utilized to target the chimeric protein to diseased cells including cancer cells. In some embodiments, the targeting moiety recognizes fibronectin that contains the EDB isoform. In various embodiments, such targeting moieties may be utilized to target the chimeric protein to tumor cells including the tumor neovasculature.

In an embodiment, the targeting moiety recognizes and binds to fibrin. Fibrin is another protein substance often found in the matrix network of the ECM. Fibrin is formed by the action of the protease thrombin on fibrinogen

which causes the fibrin to polymerize. In some embodiments, the targeting moiety recognizes one or more epitopes on fibrin. In some embodiments, the targeting moiety recognizes the monomeric as well as the polymerized forms of fibrin.

In an embodiment, the targeting moiety recognizes and binds to laminin. Laminin is a major component of the basal lamina, which is a protein network foundation for cells and organs. Laminins are heterotrimeric proteins that contain an α-chain, a β-chain, and a γ-chain. In some embodiments, the targeting moiety recognizes one or more epitopes on laminin. In some embodiments, the targeting moiety recognizes the monomeric, the dimeric as well as the trimeric forms of laminin.

In an embodiment, the targeting moiety recognizes and binds to a nidogen or entactin. Nidogens/entactins are a family of highly conserved, sulfated glycoproteins. They make up the major structural component of the basement membranes and function to link laminin and collagen IV networks in basement membranes. Members of this family include nidogen-1 and nidogen-2. In various embodiments, the targeting moiety recognizes an epitope on nidogen-1 and/or nidogen-2.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes an epitope present on any of the targets (*e.g.*, ECM proteins) described herein. In an embodiment, the antigen-recognition domain recognizes one or more linear epitopes present on the protein. As used herein, a linear epitope refers to any continuous sequence of amino acids present on the protein. In another embodiment, the antigen-recognition domain recognizes one or more conformational epitopes present on the protein. As used herein, a conformation epitope refers to one or more sections of amino acids (which may be discontinuous) which form a three-dimensional surface with features and/or shapes and/or tertiary structures capable of being recognized by an antigen recognition domain.

In various embodiments, the targeting moiety may bind to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants of any of the targets (*e.g.*, ECM proteins) described herein. In various embodiments, the targeting moiety may bind to any forms of the proteins described herein, including monomeric, dimeric, trimeric, tetrameric, heterodimeric, multimeric and associated forms. In various embodiments, the targeting moiety may bind to any post-translationally modified forms of the proteins described herein, such as glycosylated and/or phosphorylated forms.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes extracellular molecules such as DNA. In some embodiments, the targeting moiety comprises an antigen recognition domain that recognizes DNA. In an embodiment, the DNA is shed into the extracellular space from necrotic or apoptotic tumor cells or other diseased cells.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes one or more non-cellular structures associated with atherosclerotic plaques. Two types of atherosclerotic plaques are known. The fibro-lipid (fibro-fatty) plaque is characterized by an accumulation of lipid-laden cells underneath the

intima of the arteries. Beneath the endothelium there is a fibrous cap covering the atheromatous core of the plaque. The core includes lipid-laden cells (macrophages and smooth muscle cells) with elevated tissue cholesterol and cholesterol ester content, fibrin, proteoglycans, collagen, elastin, and cellular debris. In advanced plaques, the central core of the plaque usually contains extracellular cholesterol deposits (released from dead cells), which form areas of cholesterol crystals with empty, needle-like clefts. At the periphery of the plaque are younger foamy cells and capillaries. A fibrous plaque is also localized under the intima, within the wall of the artery resulting in thickening and expansion of the wall and, sometimes, spotty localized narrowing of the lumen with some atrophy of the muscular layer. The fibrous plaque contains collagen fibers (eosinophilic), precipitates of calcium (hematoxylinophilic) and lipid-laden cells. In some embodiments, the targeting moiety recognizes and binds to one or more of the non-cellular components of these plaques such as the fibrin, proteoglycans, collagen, elastin, cellular debris, and calcium or other mineral deposits or precipitates. In some embodiments, the cellular debris is a nucleic acid, *e.g.* DNA or RNA, released from dead cells.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes one or more non-cellular structures found in the brain plaques associated with neurodegenerative diseases. In some embodiments, the targeting moiety recognizes and binds to one or more non-cellular structures located in the amyloid plaques found in the brains of patients with Alzheimer's disease. For example, the targeting moiety may recognize and bind to the peptide amyloid beta, which is a major component of the amyloid plaques. In some embodiments, the targeting moiety recognizes and binds to one or more non-cellular structures located in the brains plaques found in patients with Huntington's disease. In various embodiments, the targeting moiety recognizes and binds to one or more non-cellular structures found in plaques associated with other neurodegenerative or musculoskeletal diseases such as Lewy body dementia and inclusion body myositis.

In some embodiments, the chimeric proteins of the invention may have two or more targeting moieties that bind to non-cellular structures. In some embodiments, there are two targeting moieties and one targets a cell while the other targets a non-cellular structure.In various embodiments, the targeting moieties can directly or indirectly recruit cells, such as disease cells and/or effector cells. In various embodiments, the signaling agent can modulate one or more cells that are targeted by the targeting moieties (*e.g.* recruited cells, such as disease cells and/or effector cells). For instance, the signaling agent can modulate one or both of the targeted cells (and the targeted cells can be effector and/or disease cells), depending on whether the targeted cells express a receptor for the signaling agent.

In some embodiments, the present chimeric proteins are capable of, or find use in methods involving, shifting the balance of immune cells in favor of immune attack of a tumor. For instance, the present chimeric proteins can shift the ratio of immune cells at a site of clinical importance in favor of cells that can kill and/or suppress a tumor (*e.g.* 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, dendritic cells, or subsets thereof) and in opposition to cells that protect tumors (*e.g.* myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs); tumor associated

neutrophils (TANs), M2 macrophages, tumor associated macrophages (TAMs), or subsets thereof). In some embodiments, the present chimeric protein is capable of increasing a ratio of effector T cells to regulatory T cells.

For example, in some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with T cells. In some embodiments, the targeting moiety comprises an antigen recognition domain that directly or indirectly recruits T cells. In an embodiment, the targeting moiety comprises an antigen recognition domain that specifically binds to effector T cells. In some embodiments, the targeting moiety comprises an antigen recognition domain that directly or indirectly recruits effector T cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative effector T cells include cytotoxic T cells (*e.g.* αβ TCR, $CD3^+$, $CD8^+$, $CD45RO^+$); $CD4^+$ effector T cells (*e.g.* αβ TCR, $CD3^+$, $CD4^+$, $CCR7^+$, CD62Lhi, $IL\text{-}7R/CD127^+$); $CD8^+$ effector T cells (*e.g.* αβ TCR, $CD3^+$, $CD8^+$, $CCR7^+$, CD62Lhi, $IL\text{-}7R/CD127^+$); effector memory T cells (*e.g.* CD62Llow, $CD44^+$, TCR, $CD3^+$, $IL\text{-}7R/CD127^+$, $IL\text{-}15R^+$, CCR7low); central memory T cells (*e.g.* $CCR7^+$, $CD62L^+$, $CD27^+$; or CCR7hi, $CD44^+$, CD62Lhi, TCR, $CD3^+$, $IL\text{-}7R/CD127^+$, $IL\text{-}15R^+$); $CD62L^+$ 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, $ICOS^+$ effector T cells; $CD4^+CD45RO^+CCR7(^-)$ effector T cells; and effector T cells secreting IL-2, IL-4 and/or IFN-γ.

Illustrative T cell antigens of interest include, for example (and inclusive of the extracellular domains, where applicable): CD8, CD3, SLAMF4, IL-2Rα, 4-1BB/TNFRSF9, IL-2 R β, ALCAM, B7-1, IL-4 R, B7-H3, BLAME/SLAMFS, CEACAM1, IL-6 R, CCR3, IL-7 Rα, CCR4, CXCRl/IL-S RA, CCR5, CCR6, IL-10R α, CCR 7, IL-l 0 R β, CCRS, IL-12 R β 1, CCR9, 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/CDIS, KIR/CD15S, CD27/TNFRSF7, KIR2DL1, CD2S, KIR2DL3, CD30/TNFRSFS, KIR2DL4/CD15Sd, CD31/PECAM-1, KIR2DS4, CD40 Ligand/TNFSF5, 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, SLAMF7 (CS1), PD-1, CRTAM, PSGL-1, CTLA-4, RANK/TNFRSF11A, CX3CR1, CX3CL1, L-Selectin, CXCR3, SIRP β 1, CXCR4, SLAM, CXCR6, TCCR/WSX-1, DNAM-1, Thymopoietin, EMMPRIN/CD147, TIM-1, EphB6, TIM-2, Fas/TNFRSF6, TIM-3, Fas Ligand/TNFSF6, TIM-4, Fcγ RIII/CD16, TIM-6, TNFR1/TNFRSF1A, Granulysin, TNF RIII/TNFRSF1B, TRAIL Rl/TNFRSFlOA, ICAM-1/CD54, TRAIL R2/TNFRSF10B, ICAM-2/CD102, TRAILR3/TNFRSF10C,IFN-γR1, TRAILR4/TNFRSF10D, IFN-γ R2,

TSLP, IL-1 R1 and TSLP R. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these illustrative T cell antigens.

In an exemplary embodiment, the present chimeric protein comprises a targeting moiety directed against CD8. In various embodiments, the targeting moiety directed against CD8 is a protein-based agent capable of specific binding to CD8 without functional modulation (*e.g.* partial or complete neutralization) of CD8. CD8 is a heterodimeric type I transmembrane glycoprotein, whose α and β chains are both composed of an immunoglobulin (Ig)-like extracellular domain connected by an extended O-glycosylated stalk to a single-pass transmembrane domain and a short cytoplasmic tail (Li *et al.*, 2013). The cytoplasmic region of the α-chain contains two cysteine motifs that serve as a docking site for src tyrosine kinase p56lck (Lck). In contrast, this Lck binding domain appears to be absent from the β chain, suggesting that the CD8 β chain is not involved in downstream signaling (Artyomov *et al.*, 2010). CD8 functions as a co-receptor for the T-cell receptor with its principle role being the recruitment of Lck to the TCR-pMHC complex following co-receptor binding to MHC (Turner *et al.*, 1990, Veillette *et al.*, 1988). The increase in the local concentration of this kinase activates a signaling cascade that recruits and activates ζ-chain-associated protein kinase 70 (ZAP-70), subsequently leading to the amplification or enhancement of T-cell activation signals (Purbhoo *et al.*, 2001, Laugel *et al.*, 2007a).

In various embodiments, the chimeric protein of the invention comprises a targeting moiety having an antigen recognition domain that recognizes an epitope present on the CD8 α and/or β chains. In an embodiment, the antigen-recognition domain recognizes one or more linear epitopes on the CD8 α and/or β chains. As used herein, a linear epitope refers to any continuous sequence of amino acids present on the CD8 α and/or β chains. In another embodiment, the antigen-recognition domain recognizes one or more conformational epitopes present on the CD8 α and/or β chains. As used herein, a conformation epitope refers to one or more sections of amino acids (which may be discontinuous) which form a three-dimensional surface with features and/or shapes and/or tertiary structures capable of being recognized by an antigen recognition domain.

In various embodiments, the present chimeric protein may comprise a targeting moiety that may bind to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants of human CD8 α and/or β chains. In various embodiments, the targeting moiety directed against CD8 may bind to any forms of the human CD8 α and/or β chains, including monomeric, dimeric, heterodimeric, multimeric and associated forms. In an embodiment, the targeting moiety directed against CD8 may bind to the monomeric form of CD8 α chain or CD8 β chain. In another embodiment, the targeting moiety directed against CD8 may bind to a homodimeric form comprised of two CD8 α chains or two CD8 β chains. In a further embodiment, the targeting moiety directed against CD8 may bind to a heterodimeric form comprised of one CD8 α chain and one CD8 β chain.

In an embodiment, the present chimeric protein comprises a targeting moiety with an antigen recognition domain that recognizes one or more epitopes present on the human CD8 α chain. In an embodiment, the human CD8 α chain comprises the amino acid sequence of:

Isoform 1 (SEQ ID NO: 1)

MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPT
SGCSWLFQPRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLT
LSDFRRENEGYYFCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIA
SQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLY
CNHRNRRRVCKCPRPVVKSGDKPSLSARYV.

In an embodiment, the human CD8 α chain comprises the amino acid sequence of:

Isoform 2 (SEQ ID NO: 2)

MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPT
SGCSWLFQPRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLT
LSDFRRENEGYYFCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIA
SQPLSLRPEACRPAAGGAGNRRRVCKCPRPVVKSGDKPSLSARYV.

In an embodiment, the human CD8 α chain comprises the amino acid sequence of:

Isoform 3 (SEQ ID NO: 3)

MRNQAPGRPKGATFPPRRPTGSRAPPLAPELRAKQRPGERVMALPVTALL
LPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQ
PRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENE
GYYFCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPE
ACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRRR
VCKCPRPVVKSGDKPSLSARYV.

In an embodiment, the present chimeric protein comprises a targeting moiety with an antigen recognition domain that recognizes one or more epitopes present on the human CD8 β chain. In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 1 (SEQ ID NO: 4)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFMKQ
FYK.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 2 (SEQ ID NO: 5)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFMKQ
LRLHPLEKCSRMDY.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 3 (SEQ ID NO: 6)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGRRRRARLRFMKQPQGEGISGTFVPQCLHGYYSNTTTSQKL
LNPWILKT.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 4 (SEQ ID NO: 7)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFMKQ
KFNIVCLKISGFTTCCCFQILQISREYGFGVLLQKDIGQ.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 5 (SEQ ID NO: 8)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFMKQ
PQGEGISGTFVPQCLHGYYSNTTTSQKLLNPWILKT.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 6 (SEQ ID NO: 9)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL

TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGRRRRARLRFMKQFYK.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 7 (SEQ ID NO: 10)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKDFTNKQRIGFWCPATKRHRSVMSTMWKNERRDTFNPGEFN
GC.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 8 (SEQ ID NO: 11)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGLKGKVYQEPLSPNACMDTTAILQPHRSCLTHGS.

In various embodiments, the present chimeric protein comprises a targeting moiety capable of specific binding. In various embodiments, the chimeric protein comprises a targeting moiety having an antigen recognition domain such as an antibody or derivatives thereof. In an embodiment, the CD8 binding agent comprises a targeting moiety which is an antibody. In various embodiments, the antibody is a full-length multimeric protein that includes two heavy chains and two light chains as described elsewhere herein. In some embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is a humanized antibody.

In some embodiments, the present chimeric protein comprises a targeting moiety directed against CD8 which is a single-domain antibody, such as a VHH. The VHH may be derived from, for example, an organism that produces VHH antibody such as a camelid, a shark, or the VHH may be a designed VHH. VHHs are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. VHH technology is based on fully functional antibodies from camelids that lack light chains. These heavy-chain antibodies contain a single variable domain (V_HH) and two constant domains (CH2 and CH3). VHHs are commercially available under the trademark of NANOBODY or NANOBODIES. In an embodiment, the present chimeric protein comprises a VHH.

In some embodiments, the present chimeric protein comprises a targeting moiety directed against CD8 which is a VHH comprising a single amino acid chain having four “framework regions” or FRs and three “complementary determining regions” or CDRs. As used herein, “framework region” or “FR” refers to a region in the variable domain which is located between the CDRs. As used herein, “complementary determining region” or “CDR”

refers to variable regions in VHHs that contains the amino acid sequences capable of specifically binding to antigenic targets.

In various embodiments, the targeting moiety directed against CD8 comprises a VHH having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences.

In some embodiments, the CDR1 sequence is selected from:

GFTFDDYAMS (SEQ ID NO:12) or

GFTFDDYAIG (SEQ ID NO:13).

In some embodiments, the CDR2 sequence is selected from:

TINWNGGSAEYAEPVKG (SEQ ID NO:14) or

CIRVSDGSTYYADPVKG (SEQ ID NO:15).

In some embodiments, the CDR3 sequence is selected from:

KDADLVWYNLS (SEQ ID NO:16) or

KDADLVWYNLR (SEQ ID NO:17) or

AGSLYTCVQSIVVVPARPYYDMDY (SEQ ID NO:18).

In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:12, SEQ ID NO:14, and SEQ ID NO:16. In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:12, SEQ ID NO:14, and SEQ ID NO:17. In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:12, SEQ ID NO:14, and SEQ ID NO:18.

In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO:16. In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO:17. In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO:18.

In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:16. In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:17. In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:18.

In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:13, SEQ ID NO:15, and SEQ ID NO:16. In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:13, SEQ ID NO:15, and SEQ ID NO:17. In various embodiments, the CD8 targeting moiety comprises SEQ ID NO:13, SEQ ID NO:15, and SEQ ID NO:18.

In various embodiments, the CD8 targeting moiety comprises an amino acid sequence selected from the following sequences:

R3HCD27 (SEQ ID NO:19)

QVQLQESGGGSVQPGGSLRLSCAASGFTFDDYAMSWVRQVPGKGLEWV
STINWNGGSAEYAEPVKGRFTISRDNAKNTVYLQMNSLKLEDTAVYYCAK
DADLVWYNLSTGQGTQVTVSSAAAYPYDVPDYGS

or

R3HCD129 (SEQ ID NO:20)

QVQLQESGGGLVQPGGSLRLSCAASGFTFDDYAMSWVRQVPGKGLEWV
STINWNGGSAEYAEPVKGRFTISRDNAKNTVYLQMNSLKLEDTAVYYCAK
DADLVWYNLRTGQGTQVTVSSAAAYPYDVPDYGS

or

R2HCD26 (SEQ ID NO:21)

QVQLQESGGGLVQAGGSLRLSCAASGFTFDDYAIGWFRQAPGKEREGVS
CIRVSDGSTYYADPVKGRFTISSDNAKNTVYLQMNSLKPEDAAVYYCAAGS
LYTCVQSIVVVPARPYYDMDYWGKGTQVTVSSAAAYPYDVPDYGS.

In various embodiments, the targeting moiety comprises an amino acid sequence described in US Patent Publication No. 2014/0271462, the entire contents of which are incorporated by reference. In various embodiments, the the CD8 targeting moiety comprises an amino acid sequence described in Table 0.1, Table 0.2, Table 0.3, and/or Figures 1A-12I of US Patent Publication No. 2014/0271462, the entire contents of which are incorporated by reference. In various embodiments, the the CD8 targeting moiety comprises a HCDR1 of a HCDR1 of SEQ ID NO: 22 or 23 and/or a HCDR2 of HCDR1 of SEQ ID NO: 22 or 23 and/or a HCDR3 of HCDR1 of SEQ ID NO: 22 or 23 and/or a LCDR1 of LCDR1 of SEQ ID NO: 24 and/or a LCDR2 of LCDR1 of SEQ ID NO: 24 and/or a LCDR3 of LCDR1 of SEQ ID NO: 24, as provided below.

SEQ ID NO: 22:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu
Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr Tyr Ile His Trp Val
Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Arg Ile Asp Pro Ala Asn
Asp Asn Thr Leu Tyr Ala Ser Lys Phe Gln Gly Arg Ala Thr Ile Ser Ala Asp
Thr Ser Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr
Ala Val Tyr Tyr Cys Gly Arg Gly Tyr Gly Tyr Tyr Val Phe Asp His Trp Gly Gln
Gly Thr Leu Val Thr Val Ser Ser.

SEQ ID NO: 23:

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Thr Val Lys
Ile Ser Cys Lys Val Ser Gly Phe Asn Ile Lys Asp Thr Tyr Ile His Trp Val Gln

Gln Ala Pro Gly Lys Gly Leu Glu Trp Met Gly Arg Ile Asp Pro Ala Asn Asp Asn Thr Leu Tyr Ala Ser Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Asp Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Gly Tyr Gly Tyr Tyr Val Phe Asp His Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser.

SEQ ID NO: 24:

Asp Val Gln Ile Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Thr Ser Arg Ser Ile Ser Gln Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Val Pro Lys Leu Leu Ile Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Val Ala Thr Tyr Tyr Cys Gln Gln His Asn Glu Asn Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys.

In various embodiments, the present invention contemplates the use of any natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as "analogs") of the targeting moiety directed against CD8 as described herein. In various embodiments, the amino acid sequence of the targeting moiety that targets CD8 further includes an amino acid analog, an amino acid derivative, or other non-classical amino acids.

In various embodiments, the targeting moiety of the invention may comprise an amino acid sequence that targets CD8 which is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical with any one of the sequences disclosed herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or 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%, about 99% or about 100% sequence identity with any one of the sequences disclosed herein).

In various embodiments, the targeting moiety of the invention comprises one or more mutations that do not substantially reduce the present CD8 targeting moiety's capability to specifically bind to CD8. In various

embodiments, the mutations do not substantially reduce the targeting moiety's capability to specifically bind to CD8 without functionally modulating CD8.

In various embodiments, the binding affinity of the CD8 targeting moiety for the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants (including monomeric, dimeric, heterodimeric, multimeric and/or associated forms) of human CD8 α and/or β chains may be described by the equilibrium dissociation constant (K_D). In various embodiments, the present chimeric protein comprises a targeting moiety that binds to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants (including monomeric, dimeric, heterodimeric, multimeric and/or associated forms) of human CD8 α and/or β chains with a K_D of less than about 1 uM, about 900 nM, about 800 nM, about 700 nM, about 600 nM, about 500 nM, about 400 nM, about 300 nM, about 200 nM, about 100 nM, about 90 nM, about 80 nM, about 70 nM, about 60 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, or about 5 nM, or about 1 nM.

In various embodiments, the present chimeric protein comprises a targeting moiety that binds but does not functionally modulate the antigen of interest, *i.e.*, CD8. For instance, in various embodiments, the targeting moiety simply targets the antigen but does not substantially functionally modulate the antigen, *e.g.*it does not substantially inhibit, reduce or neutralize a biological effect that the antigen has. In various embodiments, the targeting moiety agent binds an epitope that is physically separate from an antigen site that is important for its biological activity (*e.g.* an antigen's active site).

Such non-functionally modulating (*e.g.* non-neutralizing) binding finds use in various embodiments of the present invention, including methods in which the present chimeric protein is used to directly or indirectly recruit active immune cells to a site of need via an effector antigen. For example, in various embodiments, the present chimeric protein may be used to directly or indirectly recruit cytotoxic T cells via CD8 to a tumor cell in a method of reducing or eliminating a tumor (*e.g.* the chimeric protein may comprise a targeting moiety having an anti-CD8 antigen recognition domain and a targeting moiety having a recognition domain (*e.g.* an antigen recognition domain) directed against a tumor antigen or receptor). In such embodiments, it is desirable to directly or indirectly recruit CD8-expressing cytotoxic T cells but not to neutralize the CD8 activity. In these embodiments, CD8 signaling is an important piece of the tumor reducing or eliminating effect.

By way of non-limiting example, in various embodiments, the present chimeric protein has a targeting moiety directed against a checkpoint marker expressed on a T cell, *e.g.* one or more of PD-1, CD28, CTLA4, ICOS, BTLA, KIR, LAG3, CD137, OX40, CD27, CD40L, TIM3, and A2aR.

For example, in some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with B cells. In some embodiments, the recognition domains directly or indirectly recruit B cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative B cell antigens of

interest include, for example, CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD37, CD38, CD39, CD40, CD70, CD72, CD73, CD74, CDw75, CDw76, CD77, CD78, CD79a/b, CD80, CD81, CD82, CD83, CD84, CD85, CD86, CD89, CD98, CD126, CD127, CDw130, CD138, CDw150, and B-cell maturation antigen (BCMA). In various embodiments, a targeting moiety of the chimeric protein binds one or more of these illustrative B cell antigens.

By way of further example, in some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with Natural Killer cells. In some embodiments, the recognition domains directly or indirectly recruit Natural Killer cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative Natural Killer cell antigens of interest include, for example TIGIT, 2B4/SLAMF4, KIR2DS4, CD155/PVR, KIR3DL1, CD94, LMIR1/CD300A, CD69, LMIR2/CD300c, CRACC/SLAMF7, LMIR3/CD300LF, Kir1alpha, DNAM-1, LMIR5/CD300LB, Fc-epsilon RII, LMIR6/CD300LE, Fc-γ RI/CD64, MICA, Fc-γ RIIB/CD32b, MICB, Fc-γ RIIC/CD32c, MULT-1, Fc-γ RIIA/CD32a, Nectin-2/CD112, Fc-γ RIII/CD16, NKG2A, FcRH1/IRTA5, NKG2C, FcRH2/IRTA4, NKG2D, FcRH4/IRTA1, NKp30, FcRH5/IRTA2, NKp44, Fc-Receptor-like 3/CD16-2, NKp46/NCR1, NKp80/KLRF1, NTB-A/SLAMF6, Rae-1, Rae-1 α, Rae-1 β, Rae-1 delta, H60, Rae-1 epsilon, ILT2/CD85j, Rae-1 γ, ILT3/CD85k, TREM-1, ILT4/CD85d, TREM-2, ILT5/CD85a, TREM-3, KIR/CD158, TREML1/TLT-1, KIR2DL1, ULBP-1, KIR2DL3, ULBP-2, KIR2DL4/CD158d and ULBP-3. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these illustrative NK cell antigens.

Also, in some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with macrophages/monocytes. In some embodiments, the recognition domains directly or indirectly recruit macrophages/monocytes, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative macrophages/monocyte antigens of interest include, for example SIRP1a, B7-1/CD80, ILT4/CD85d, B7-H1, ILT5/CD85a, Common β Chain, Integrin α 4/CD49d, BLAME/SLAMF8, Integrin α X/CDIIc, CCL6/C10, Integrin β 2/CD18, CD155/PVR, Integrin β 3/CD61, CD31/PECAM-1, Latexin, CD36/SR-B3, Leukotriene B4 R1, CD40/TNFRSF5, LIMPIIISR-B2, CD43, LMIR1/CD300A, CD45, LMIR2/CD300c, CD68, LMIR3/CD300LF, CD84/SLAMF5, LMIR5/CD300LB, CD97, LMIR6/CD300LE, CD163, LRP-1, CD2F-10/SLAMF9, MARCO, CRACC/SLAMF7, MD-1, ECF-L, MD-2, EMMPRIN/CD147, MGL2, Endoglin/CD105, Osteoactivin/GPNMB, Fc-γ RI/CD64, Osteopontin, Fc-γ RIIB/CD32b, PD-L2, Fc-γ RIIC/CD32c, Siglec-3/CD33, Fc-γ RIIA/CD32a, SIGNR1/CD209, Fc-γ RIII/CD16, SLAM, GM-CSF R α, TCCR/WSX-1, ICAM-2/CD102, TLR3, IFN-γ Rl, TLR4, IFN-gannna R2, TREM-l, IL-l RII, TREM-2, ILT2/CD85j, TREM-3, ILT3/CD85k, TREML1/TLT-1, 2B4/SLAMF 4, IL-10 R α, ALCAM, IL-10 R β, AminopeptidaseN/ANPEP, ILT2/CD85j, Common β Chain, ILT3/CD85k, Clq R1/CD93, ILT4/CD85d, CCR1, ILT5/CD85a, CCR2, Integrin α 4/CD49d, CCR5, Integrin α M/CDII b, CCR8, Integrin α X/CDIIc, CD155/PVR, Integrin β 2/CD18, CD14, Integrin β 3/CD61, CD36/SR-B3, LAIR1, CD43, LAIR2, CD45, Leukotriene B4-R1, CD68, LIMPIIISR-B2, CD84/SLAMF5, LMIR1/CD300A, CD97, LMIR2/CD300c, CD163, LMIR3/CD300LF, Coagulation Factor III/Tissue Factor, LMIR5/CD300LB, CX3CR1,

CX3CL1, LMIR6/CD300LE, CXCR4, LRP-1, CXCR6, M-CSF R, DEP-1/CD148, MD-1, DNAM-1, MD-2, EMMPRIN/CD147, MMR, Endoglin/CD105, NCAM-L1, Fc-γ RI/CD64, PSGL-1, Fc-γ RIIIICD16, RP105, G-CSF R, L-Selectin, GM-CSF R α, Siglec-3/CD33, HVEM/TNFRSF14, SLAM, ICAM-1/CD54, TCCR/WSX-1, ICAM-2/CD102, TREM-I, IL-6 R, TREM-2, CXCRI/IL-8 RA, TREM-3 and TREMLI/TLT-1. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these illustrative macrophage/monocyte antigens.

Also, in some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with dendritic cells. In some embodiments, the recognition domains directly or indirectly recruit dendritic cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative dendritic cell antigens of interest include, for example, CLEC9A, XCR1, RANK, CD36/SRB3, LOX-1/SR-E1, CD68, MARCO, CD163, SR-A1/MSR, CD5L, SREC-1, CL-PI/COLEC12, SREC-II, LIMPIIISRB2, RP105, TLR4, TLR1, TLR5, TLR2, TLR6, TLR3, TLR9, 4-IBB Ligand/TNFSF9, IL-12/IL-23 p40, 4-Amino-1,8-naphthalimide, ILT2/CD85j, CCL21/6Ckine, ILT3/CD85k, 8-oxo-dG, ILT4/CD85d, 8D6A, ILT5/CD85a, A2B5, lutegrin α 4/CD49d, Aag, Integrin β 2/CD18, AMICA, Langerin, B7-2/CD86, Leukotriene B4 RI, B7-H3, LMIR1/CD300A, BLAME/SLAMF8, LMIR2/CD300c, Clq R1/CD93, LMIR3/CD300LF, CCR6, LMIR5/CD300LB CCR7, LMIR6/CD300LE, CD40/TNFRSF5, MAG/Siglec-4-a, CD43, MCAM, CD45, MD-1, CD68, MD-2, CD83, MDL-1/CLEC5A, CD84/SLAMF5, MMR, CD97, NCAMLI, CD2F-10/SLAMF9, Osteoactivin GPNMB, Chern 23, PD-L2, CLEC-1, RP105, CLEC-2, CLEC-8, Siglec-2/CD22, CRACC/SLAMF7, Siglec-3/CD33, DC-SIGN, Siglec-5, DC-SIGNR/CD299, Siglec-6, DCAR, Siglec-7, DCIR/CLEC4A, Siglec-9, DEC-205, Siglec-10, Dectin-1/CLEC7A, Siglec-F, Dectin-2/CLEC6A, SIGNR1/CD209, DEP-1/CD148, SIGNR4, DLEC, SLAM, EMMPRIN/CD147, TCCR/WSX-1, Fc-γ R1/CD64, TLR3, Fc-γ RIIB/CD32b, TREM-1, Fc-γ RIIC/CD32c, TREM-2, Fc-γ RIIA/CD32a, TREM-3, Fc-γ RIII/CD16, TREML1/TLT-1, ICAM-2/CD102, DEC205, and Vanilloid R1. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these illustrative DC antigens.

In an exemplary embodiment, the present chimeric protein comprises a targeting moiety directed against Clec9A. In various embodiments, the targeting moiety directed against Clec9A is a protein-based agent capable of specific binding to Clec9A. In various embodiments, the targeting moiety is a protein-based agent capable of specific binding to Clec9A without neutralization of Clec9A. Clec9A is a group V C-type lectin-like receptor (CTLR) expressed on the surface of a subset of dendritic cells (*i.e.*, $BDCA_3$+ dendritic cells) specialized for the uptake and processing of materials from dead cells. Clec9A recognizes a conserved component within nucleated and nonnucleated cells, exposed when cell membranes are damaged. CLEC9A is expressed at the cell surface as a glycosylated dimer and can mediate endocytosis, but not phagocytosis. CLEC9A possesses a cytoplasmic immunoreceptor tyrosine-based activation-like motif that can recruit Syk kinase and induce proinflammatory cytokine production (see Huysamen *et al.* (2008), JBC, 283:16693-701).

In various embodiments, the present chimeric protein comprises a targeting moiety having an antigen recognition domain that recognizes an epitope present on Clec9A. In an embodiment, the antigen-recognition domain recognizes one or more linear epitopes present on Clec9A. As used herein, a linear epitope refers to any

continuous sequence of amino acids present on Clec9A. In another embodiment, the antigen-recognition domain recognizes one or more conformational epitopes present on Clec9A. As used herein, a conformation epitope refers to one or more sections of amino acids (which may be discontinuous) which form a three-dimensional surface with features and/or shapes and/or tertiary structures capable of being recognized by an antigen recognition domain.

In various embodiments, the present chimeric protein comprises a targeting moiety that may bind to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants of human Clec9A. In various embodiments, the targeting moiety may bind to any forms of the human Clec9A, including monomeric, dimeric, heterodimeric, multimeric and associated forms. In an embodiment, the targeting moiety binds to the monomeric form of Clec9A. In another embodiment, the targeting moiety binds to a dimeric form of Clec9A. In a further embodiment, the targeting moiety binds to glycosylated form of Clec9A, which may be either monomeric or dimeric.

In an embodiment, the present chimeric protein comprises a targeting moiety with an antigen recognition domain that recognizes one or more epitopes present on human Clec9A. In an embodiment, the human Clec9A comprises the amino acid sequence of:

MHEEEIYTSLQWDSPAPDTYQKCLSSNKCSGACCLVMVISCVFCMGLLTA
SIFLGVKLLQVSTIAMQQQEKLIQQERALLNFTEWKRSCALQMKYCQAFMQ
NSLSSAHNSSPCPNNWIQNRESCYYVSEIWSIWHTSQENCLKEGSTLLQIE
SKEEMDFITGSLRKIKGSYDYWVGLSQDGHSGRWLWQDGSSPSPGLLPA
ERSQSANQVCGYVKSNSLLSSNCSTWKYFICEKYALRSSV (SEQ ID NO: 25).

In various embodiments, the present chimeric protein comprises a targeting moiety capable of specific binding. In various embodiments, the chimeric protein comprises a targeting moiety having an antigen recognition domain such as an antibody or derivatives thereof. In an embodiment, the chimeric protein comprises a targeting moiety which is an antibody. In various embodiments, the antibody is a full-length multimeric protein that includes two heavy chains and two light chains as described elsewhere herein. In some embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is a humanized antibody.

In some embodiments, the present chimeric protein comprises a targeting moiety which is a single-domain antibody, such as a VHH (which is commercially available under the trademark of NANOBODIES) as described elsewhere herein. In an embodiment, the present chimeric protein comprises a VHH.

In some embodiments, the present chimeric protein comprises a targeting moiety which is a VHH comprising a single amino acid chain having four “framework regions” or FRs and three “complementary determining regions” or CDRs. As used herein, “framework region” or “FR” refers to a region in the variable domain which is located between the CDRs. As used herein, “complementary determining region” or “CDR” refers to variable regions in VHHs that contains the amino acid sequences capable of specifically binding to antigenic targets.

In various embodiments, the present chimeric protein comprises a VHH having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences.

In some embodiments, the CDR1 sequence is selected from:

GSISSINVMG (SEQ ID NO: 26);

GSFSSINVMG (SEQ ID NO: 27);

GSISSINIMG (SEQ ID NO: 28);

GSISSINIMG (SEQ ID NO: 29);

VSIFSINAMG (SEQ ID NO: 30);

GSIFSLNAMG (SEQ ID NO: 31);

GRTISNYDMA (SEQ ID NO: 32);

GRTFTTSLMQ (SEQ ID NO: 33);

ERNLRIYDMA (SEQ ID NO: 34);

ERNLRSYDMA (SEQ ID NO: 35);

GLTFSNYHMG (SEQ ID NO: 36);

GLTFSSYHMG (SEQ ID NO: 37);

GLTFSRYHMG (SEQ ID NO: 38);

GLTLSSYYIA (SEQ ID NO: 39);

GLTFSSYYTG (SEQ ID NO: 40);

GLTLSSYHMG (SEQ ID NO: 41);

GRTSSPYVTG (SEQ ID NO: 42);

GFTFSGYVMS (SEQ ID NO: 43);

GFTFSGYVMT (SEQ ID NO: 44); or

GFTFSGYLMS (SEQ ID NO: 45).

In some embodiments, the CDR2 sequence is selected from:

RITNLGLPNYADWLKD (SEQ ID NO: 46);

RITNLGLPNYADSVTG (SEQ ID NO: 47);

RITNIGLPNYADSVKG (SEQ ID NO: 48);

RITNLGLPNYADSVEG (SEQ ID NO: 49);

AITSGGRVVYSDSVKG (SEQ ID NO: 50);

AITSGGRTAYADSVKG (SEQ ID NO: 51);

HITSDGRIVYADPVKG (SEQ ID NO: 52);

RISGSGDRTDYADSVKG (SEQ ID NO: 53);

SITWSTGNTHYADSVKG (SEQ ID NO: 54);

VISSSGDSTHYSDFVKG (SEQ ID NO: 55);

VITSSGDSTHYSDFVKG (SEQ ID NO: 56);

QITWSDASIYYAGSVKG (SEQ ID NO: 57);

QITWSDTSIYYAGSVKG (SEQ ID NO: 58);

QITWSDGTTYYPGSVKG (SEQ ID NO: 59);

QIRWSDDSTYYPGSVKG (SEQ ID NO: 60);

QISWSDDSTYYADSVKG (SEQ ID NO: 61);

TVSWGGVTYYADSVKG (SEQ ID NO: 62);

SIGSGGGYPSYTDSVEG (SEQ ID NO: 63);

SIGSGGGYPSYTGSVEG (SEQ ID NO: 64);

HIGSGGGYPSYTDSVQG (SEQ ID NO: 65);

HIGSGGGHATYTDSVEG (SEQ ID NO: 66); or

TIGSGGGITSYADSVKG (SEQ ID NO: 67).

In some embodiments, the CDR3 sequence is selected from:

VALSAEY (SEQ ID NO: 68);

VALKAEY (SEQ ID NO: 69);

VGLKAEY (SEQ ID NO: 70);

KTKSAVLFGGMDY (SEQ ID NO: 71);

YIRGEDY (SEQ ID NO: 72);

KHYASNY (SEQ ID NO: 73);

QDFGSPSF (SEQ ID NO: 74);

QDFRSPDF (SEQ ID NO: 75);

QIFGSPNF (SEQ ID NO: 76);

LAIHGDY (SEQ ID NO: 77);

NQIRQWP (SEQ ID NO: 78);

NSIRQWP (SEQ ID NO: 79);

NAIRQWP (SEQ ID NO: 80);

RKVGGPDY (SEQ ID NO: 81);

NTFGNVY (SEQ ID NO: 82);

LGR; or

VIK.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:26, SEQ ID NO:46, and SEQ ID NO:68.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:27, SEQ ID NO:47, and SEQ ID NO:69.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:28, SEQ ID NO:48, and SEQ ID NO:69.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:26, SEQ ID NO:49, and SEQ ID NO:70.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:29, SEQ ID NO:50, and SEQ ID NO:71.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:30, SEQ ID NO:51, and SEQ ID NO:72.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:31, SEQ ID NO:52, and SEQ ID NO:73.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:32, SEQ ID NO:53, and SEQ ID NO:74.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:32, SEQ ID NO:53, and SEQ ID NO:75.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:32, SEQ ID NO:53, and SEQ ID NO:76.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:33, SEQ ID NO:54, and SEQ ID NO:77.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:34, SEQ ID NO:55, and SEQ ID NO:78.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:34, SEQ ID NO:55, and SEQ ID NO:79.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:35, SEQ ID NO:56, and SEQ ID NO:80.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:36, SEQ ID NO:57, and SEQ ID NO:81.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:37, SEQ ID NO:58, and SEQ ID NO:81.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:38, SEQ ID NO:59, and SEQ ID NO:81.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:39, SEQ ID NO:60, and SEQ ID NO:81.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:40, SEQ ID NO:61, and SEQ ID NO:81.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:41, SEQ ID NO:61, and SEQ ID NO:81.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:42, SEQ ID NO:62, and SEQ ID NO:82.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:43, SEQ ID NO:63, andLGR.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:43, SEQ ID NO:64, and LGR.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:43, SEQ ID NO:65, and LGR.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:44, SEQ ID NO:66, and LGR.

In some embodiments, the Clec9A targeting moiety comprises SEQ ID NO:45, SEQ ID NO:67, andVIK.

In various embodiments, the Clec9A targeting moiety comprises an amino acid sequence selected from the following sequences:

R2CHCL8 (SEQ ID NO: 83)

QVQLVESGGGLVHPGGSLRLSCAASGSISSINVMGWYRQAPGKERELVAR
ITNLGLPNYADWLKDRFTISRDNAKNTVYLQMNSLKPEDTAVYYCYLVALS
AEYWGQGTQVTVSS;

R1CHCL50 (SEQ ID NO: 84)

QVQLVESGGGLVHPGGSLRLSCAASGSFSSINVMGWYRQAPGKERELVA
RITNLGLPNYADSVTGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCYLVALK
AEYWGQGTQVTVSS;

R1CHCL21 (SEQ ID NO: 85)

QVQLVESGGGLVHRGGSLRLSCAASGSISSINIMGWYRQAPGKERELVARI
TNIGLPNYADSVKGRFTISRDNAKSTVYLQMNSLNAEDTAVYYCYLVALKA
EYWGQGTQVTVSS;

R2CHCL87 (SEQ ID NO: 86)

QVQLVESGGGLVQPGGSLRLSCAASGSISSINVMGWYRQAPGKERELVA
RITNLGLPNYADSVEGRFTISRDKDENTVYLEMNTLKPEDTAVYYCYLVGLK
AEYWGQGTQVTVSS;

R2CHCL24 (SEQ ID NO: 87)

QVQLVESGGGLVQPGGSLRLSCAASGSSDSINAMGWYRQAPGKERELVA
AITSGGRVVYSDSVKGRGTISRDNAKNTVYLQIASLKPEDTAVYYCNVKTK
SAVLFGGMDYWGKGTQVTVSS;

R2CHCL38 (SEQ ID NO: 88)

QVQLVESGGGLVQPGGSLRLSCAASVSIFSINAMGWYRQAPGKERELVAA
ITSGGRTAYADSVKGRFTISRDNSKNTVYLQMDSLKPEDTDVYYCKAYIRG
EDYWGKGTQVTVSS;

R1CHCL16 (SEQ ID NO: 89)

DVQLVESGGGLVQPGGSLRLSCAASGSIFSLNAMGWYRQAPGKERELVA
HITSDGRIVYADPVKGRFTISRVDGKNMVTLQMNSLKPEDTAVYYCNAKHY
ASNYWGQGTQVTVSS;

R2CHCL10 (SEQ ID NO: 90)

QVQLVESGGGSVQAGGSLRLSCAASGRTISNYDMAWSRQAPGKEREFVA
RISGSGDRTDYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCQIQD
FGSPSFSGQGTQVTVSS;

R1CHCL34 (SEQ ID NO: 91)

DVQLVESGGGSVQAGGSLRLSCAASGRTISNYDMAWSRQAPGKEREFVA
RISGSGDRTDYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCQIQD
FRSPDFWSQGTQVTVSS;

R1CHCL82 (SEQ ID NO: 92)

QVQLVESGGESVQAGGSLRLSCAASGRTISNYDMAWSRQAPGKEREFVA
RISGSGDRTDYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYNCQTQI
FGSPNFSGQGTQVTVSS;

R2CHCL3 (SEQ ID NO: 93)

QVQLVESGGGLVQAGDSLRLSCAASGRTFTTSLMQWHRQAPGKEREFVA
SITWSTGNTHYADSVKGRFTISRDNARNTVYLQMNSLKPEDTAIYTCRVLAI
HGDYWGQGTQVTVSS;

R2CHCL69 (SEQ ID NO: 94)

DVQLVESGGGLVQAGDSLRLSCAASERNLRIYDMAWYRQAPGKEREYVA
VISSSGDSTHYSDFVKGRFTISRDNAKNTVSLQMDSLKPEDTAFYYCNVNQ
IRQWPWGQGTQVTVSS;

R1CHCL56 (SEQ ID NO: 95)

QVQLVESGGGLVQAGDSLRLSCAASERNLRIYDMAWYRQAPGKEREYVA
VISSSGDSTHYSDFVKGRFTISRDNAKNTVSLQMDSLKPEDTAFYYCNVNS
IRQWPWGQGTQVTVSS;

R2CHCL32 (SEQ ID NO: 96)

QVQLVESGGGLVQAGDSLRLSCTASERNLRSYDMAWWRQAPGKEREYV
AVITSSGDSTHYSDFVKGRFTISRDNAKNTVSLQMDSLKPEDTASYYCNVN
AIRQWPWGQGTQVTVSS;

R2CHCL49 (SEQ ID NO: 97)

DVQLVESGGGSVQAGGSLRLSCAISGLTFSNYHMGWYRQAPGREREFVA
QITWSDASIYYAGSVKGRFTISRDNVKNIVYLQIDNLKPEDTAIYYCDARKVG
GPDYWGQGTQVTVSS;

R2CHCL53 (SEQ ID NO: 98)

QVQLVESGGGLVQAGGSLTLSCAISGLTFSSYHMGWYRQAPGREREFVA
QITWSDTSIYYAGSVKGRFTISRDNVKNIVYLQIDNLKPEDTAIYYCDARKVG
GPDYWGQGTQVTVSS;

R2CHCL22 (SEQ ID NO: 99)

DVQLVESGGGLVQAGGSLRLSCAISGLTFSRYHMGWYRQAPGREREFVA
QITWSDGTTYYPGSVKGRFTISRDNARNTVYLQIDNLKPEDTAIYYCDARKV
GGPDYWGQGTQVTVSS;

R2CHCL25 (SEQ ID NO: 100)

QVQLVESGGGLVQAGGSLRLSCATSGLTLSSYYIAWYRQAPGREREFVAQ
IRWSDDSTYYPGSVKGRFTISRDNARNTVYLRMDNLKPEDTARYYCDARK
VGGPDYWGQGTQVTVSS;

R2CHCL18 (SEQ ID NO: 101)

DVQLVESGGGLVQAGGSLRLSCATSGLTFSSYYTGWYRQAPGREREFVA
QISWSDDSTYYADSVKGRFTISRDNARNTVYLQMNNLKPGDTAIYYCDARK
VGGPDYWGQGTQVTVSS;

R1CHCL23 (SEQ ID NO: 102)

DVQLVESGGGLVQAGGSLRLSCATSGLTLSSYHMGWYRQAPGREREFVA
QISWSDDSTYYADSVKGRFTISRDNARNTVYLQMNNLKPEDTAIYYCDARK
VGGPDYWGQGTQVTVSS;

R1CHCL27 (SEQ ID NO: 103)

DVQLVESGGGLVQAGGSLRLSCAASGRTSSPYVTGWYRQTPGKEREPVA
TVSWGGVTYYADSVKGRFTISRDNAKNTVYLQMNALKPEDTAIYYCNVNTF
GNVYWGQGTQVTVSS;

R2CHCL13 (SEQ ID NO: 104)

QVQLVESGGGLVQPGGSLRLSCAASGFTFSGYVMSWVRQAPGKGLEWV
ASIGSGGGYPSYTDSVEGRFTISRDNAKNTLYLLMDNLKPDDTAVYYCEML
GRRGQGTQVTVSS;

R2CHCL14 (SEQ ID NO: 105)

QVQLVESGGGLVQPGGSLRLSCAASGFTFSGYVMSWVRQAPGKGLEWV
ASIGSGGGYPSYTDSVEGRFTISRDNAKNTLYLQMNNLKPDDTAVYYCEM
LGRRGQGTQVTVSS;

R2CHCL42 (SEQ ID NO: 106)

QVQLVESGGGLVQPGGSLRLSCAASGFTFSGYVMSWVRQAPGKGLEWV
ASIGSGGGYPSYTGSVEGRFTISRDNAKNTLYLLMNNLKPDDTAVYYCEML
GRRGQGTQVTVSS;

R2CHCL41 (SEQ ID NO: 107)

QVQLVESGGGLVQPGGSLRLSCAASGFTFSGYVMSWVRQAPGKGLEWV
AHIGSGGGYPSYTDSVQGRFTISRDNAKNTLYLQMNNLKPEDTAVYYCEM
LGRRGQGTQVTVSS;

R2CHCL94 (SEQ ID NO: 108)

QVQLVESGGGLVQPGGSLRLSCAASGFTFSGYVMTWVRQAPGKGLEWV
AHIGSGGGHATYTDSVEGRFTISRDNAKNTLYLQMNNLKAEDTAVYYCEFL
GRRGQGTQVTVSS; or

R2CHCL27(SEQ ID NO: 109)

QVQLVESGGGLVQPGGSLRLSCAASGFTFSGYLMSWVRQAPGKGLEWV
ATIGSGGGITSYADSVKGRFTISRDNAKNTLYLQMNNLKHEDTAVYYCETVI
KRGQGTQVTVSS.

In various embodiments, the present invention contemplates the use of any natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as "analogs") of the targeting moiety directed against Clec9A as described herein. In various embodiments, the amino acid sequence of the targeting moiety directed against Clec9A further includes an amino acid analog, an amino acid derivative, or other non-classical amino acids.

In various embodiments, the present chimeric protein comprises a targeting moiety comprising an amino acid sequence that is at least 60% identical to any one of the sequences disclosed herein. For example, the chimeric protein may comprise a targeting moiety comprising an amino acid sequence that is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to any one of the sequences discloses herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or 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%, about 99% or about 100% sequence identity to any one of the sequences disclosed herein).

In various embodiments, the targeting moiety of the invention comprises one or more mutations that do not substantially reduce the Clec9A targeting moiety's capability to specifically bind to Clec9A. In various embodiments, the mutations do not substantially reduce the targeting moiety's capability to specifically bind to Clec9A without neutralizing Clec9A.

In various embodiments, the binding affinity of the Clec9A targeting moiety for the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or monomeric and/or dimeric forms and/or any other naturally occurring or synthetic analogs, variants, or mutants (including monomeric and/or dimeric forms) of human Clec9A may be described by the equilibrium dissociation constant (K_D). In various embodiments, the present chimeric protein comprises a targeting moiety that binds to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants (including monomeric and/or dimeric forms) of human Clec9A with a K_D of less than about 1 uM, about 900 nM, about 800 nM, about 700 nM, about 600 nM, about 500 nM, about 400 nM, about 300 nM, about 200 nM, about 100 nM, about 90 nM, about 80 nM, about 70 nM, about 60 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, or about 5 nM, or about 1 nM.

In various embodiments, the present chimeric protein comprises a targeting moiety that binds but does not functionally modulate the antigen of interest, *e.g.*, Clec9A. For instance, in various embodiments, the Clec9A targeting moiety simply targets the antigen but does not substantially functionally modulate (*e.g.* substantially inhibit, reduce or neutralize) a biological effect that the antigen has. In various embodiments, the Clec9A targeting moiety binds an epitope that is physically separate from an antigen site that is important for its biological activity (*e.g.* an antigen's active site).

Such binding without significant function modulation finds use in various embodiments of the present invention, including methods in which the present chimeric protein is used to directly or indirectly recruit active immune cells to a site of need via an effector antigen. For example, in various embodiments, the present chimeric protein may be used to directly or indirectly recruit dendritic cells via Clec9A to a tumor cell in a method of reducing or eliminating a tumor (*e.g.* the chimeric protein may comprise a targeting moiety having an anti-Clec9A antigen recognition domain and a targeting moiety having a recognition domain (*e.g.* antigen recognition domain) directed against a tumor antigen or receptor). In such embodiments, it is desirable to directly or indirectly recruit dendritic cells but not to functionally modulate the Clec9A activity. In these embodiments, Clec9A signaling is an important piece of the tumor reducing or eliminating effect.

In some embodiments, the present chimeric protein enhances antigen-presentation by dendritic cells. For example, in various embodiments, the present chimeric protein may directly or indirectly recruits dendritic cells via Clec9A to a tumor cell, where tumor antigens are subsequently endocytosed and presented on the dendritic cell for induction of potent humoral and cytotoxic T cell responses.

In some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) on immune cells selected from, but not limited to, megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils, or subsets thereof. In some embodiments, the recognition domains directly or indirectly recruit megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils, or subsets thereof, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect).

In some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with megakaryocytes and/or thrombocytes. Illustrative megakaryocyte and/or thrombocyte antigens of interest include, for example, GP IIb/IIIa, GPIb, vWF, PF4, and TSP. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these illustrative megakaryocyte and/or thrombocyte antigens.

In some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with erythrocytes. Illustrative erythrocyte antigens of interest include, for example, CD34, CD36, CD38, CD41a (platelet glycoprotein IIb/IIIa), CD41b (GPIIb), CD71 (transferrin receptor), CD105, glycophorin A, glycophorin C, c-kit, HLA-DR, H2 (MHC-II), and Rhesus antigens. In In various embodiments, a targeting moiety of the chimeric protein binds one or more of these illustrative erythrocyte antigens.

In some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with mast cells. Illustrative mast cells antigens of interest include, for example, SCFR/CD117, $Fc_{\varepsilon}RI$, CD2, CD25, CD35, CD88, CD203c, C5R1, CMAl, FCERlA, FCER2, TPSABl. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these mast cell antigens.

In some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with basophils. Illustrative basophils antigens of interest include, for example, $Fc_{\varepsilon}RI$, CD203c, CD123, CD13, CD107a, CD107b, and CD164. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these basophil antigens.

In some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with neutrophils. Illustrative neutrophils antigens of interest include, for example, 7D5, CD10/CALLA, CD13, CD16 (FcRIII), CD18 proteins (LFA-1, CR3, and p150, 95), CD45, CD67, and CD177. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these neutrophil antigens.

In some embodiments, the targeting moiety comprises an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with eosinophils. Illustrative eosinophils antigens of interest include, for example, CD35, CD44 and CD69. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these eosinophil antigens.

In various embodiments, the targeting moiety may comprise an antigen recognition domain may bind to any appropriate target, antigen, receptor, or cell surface markers known by the skilled artisan. In some embodiments, the antigen or cell surface marker is a tissue-specific marker. Illustrative tissue-specific markers include, but are not limited to, endothelial cell surface markers such as ACE, CD14, CD34, CDH5, ENG, ICAM2, MCAM, NOS3, PECAMl, PROCR, SELE, SELP, TEK, THBD, VCAMl, VWF; smooth muscle cell surface markers such as ACTA2, MYHIO, MYHl 1, MYH9, MYOCD; fibroblast (stromal) cell surface markers such as ALCAM, CD34, COLlAl, COL1A2, COL3A1, FAP, PH-4; epithelial cell surface markers such as CDID, K6IRS2, KRTIO, KRT13,

KRT17, KRT18, KRT19, KRT4, KRT5, KRT8, MUCl, TACSTDl; neovasculature markers such as CD13, TFNA, Alpha-v beta-3 ($\alpha_V\beta_3$), E-selectin; and adipocyte surface markers such as ADIPOQ, FABP4, and RETN. In various embodiments, a targeting moiety of the chimeric protein binds one or more of these antigens. In various embodiments, a targeting moiety of the chimeric protein binds one or more of cells having these antigens.

In some embodiments, the recognition domains specifically bind to a target (*e.g.* antigen, receptor) associated with tumor cells. In some embodiments, the recognition domains directly or indirectly recruit tumor cells. For instance, in some embodiments, the direct or indirect recruitment of the tumor cell is to one or more effector cell (*e.g.* an immune cell as described herein) that can kill and/or suppress the tumor cell.

Tumor cells, or cancer cells refer to an uncontrolled growth of cells or tissues and/or an abnormal increased in cell survival and/or inhibition of apoptosis which interferes with the normal functioning of bodily organs and systems. For example, tumor cells include benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases. Illustrative tumor cells include, but are not limited to cells of: 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.

Tumor cells, or cancer cells also include, but are not limited to, carcinomas, *e.g.* various subtypes, including, for example, adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma), sarcomas (including, for example, bone and soft tissue), leukemias (including, for example, acute myeloid, acute lymphoblastic, chronic myeloid, chronic lymphocytic, and hairy cell), lymphomas and myelomas (including, for example, Hodgkin and non-Hodgkin lymphomas, light chain, non-secretory, MGUS, and plasmacytomas), and central nervous system cancers (including, for example, brain (*e.g.* gliomas (*e.g.* astrocytoma,

oligodendroglioma, and ependymoma), meningioma, pituitary adenoma, and neuromas, and spinal cord tumors (*e.g.* meningiomas and neurofibroma).

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, prostate-specific membrane antigen (PSMA), 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, Imp-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, CD20, CD22, CD30, CD33, CD37, CD56, CD70, CD74, CD138, AGS16, MUC1, GPNMB, Ep-CAM, PD-L1, PD-L2, PMSA, and BCMA (TNFRSF17). In various embodiments, a targeting moiety of the chimeric protein binds one or more of these tumor antigens.

In some embodiments, the present chimeric protein has (i) one or more of the targeting moieties which is directed against an immune cell selected from a T cell, a B cell, a dendritic cell, a macrophage, a NK cell, or subsets thereof and (ii) one or more of the targeting moieties which is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a T cell (including, without limitation an effector T cell) and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a B cell and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a dendritic cell and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a macrophage and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a NK cell and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

By way of non-limiting example, in various embodiments, the present chimeric protein has (i) a targeting moiety directed against a T cell, for example, mediated by targeting to CD8, SLAMF4, IL-2 R α, 4-1BB/TNFRSF9, IL-2 R

β, ALCAM, B7-1, IL-4 R, B7-H3, BLAME/SLAMFS, CEACAM1, IL-6 R, CCR3, IL-7 Rα, CCR4, CXCRl/IL-S RA, CCR5, CCR6, IL-10R α, CCR 7, IL-l 0 R β, CCRS, IL-12 R β 1, CCR9, 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, CD27/TNFRSF7, KIR2DL1, CD2S, KIR2DL3, CD30/TNFRSFS, KIR2DL4/CD15Sd, CD31/PECAM-1, KIR2DS4, CD40 Ligand/TNFSF5, 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, RANK/TNFRSF11A, CX3CR1, CX3CL1, L-Selectin, CXCR3, SIRP β 1, CXCR4, SLAM, CXCR6, TCCR/WSX-1, DNAM-1, Thymopoietin, EMMPRIN/CD147, TIM-1, EphB6, TIM-2, Fas/TNFRSF6, TIM-3, Fas Ligand/TNFSF6, TIM-4, Fcγ RIII/CD16, TIM-6, TNFR1/TNFRSF1A, Granulysin, TNF RIII/TNFRSF1B, TRAIL Rl/TNFRSFlOA, ICAM-1/CD54, TRAIL R2/TNFRSF10B, ICAM-2/CD102, TRAILR3/TNFRSF10C,IFN-γR1, TRAILR4/TNFRSF10D, IFN-γ R2, TSLP, IL-1 R1, or TSLP R; and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

By way of non-limiting example, in various embodiments, the present chimeric protein has a targeting moiety directed against (i) a checkpoint marker expressed on a T cell, *e.g.* one or more of PD-1, CD28, CTLA4, ICOS, BTLA, KIR, LAG3, CD137, OX40, CD27, CD40L, TIM3, and A2aR and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In various embodiments, the present chimeric protein has one or more targeting moieties directed against PD-1. In some embodiments, the chimeric protein has one or more targeting moieties which selectively bind a PD-1 polypeptide. In some embodiments, the chimeric protein comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a PD-1 polypeptide.

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody pembrolizumab (aka MK-3475, KEYTRUDA), or fragments thereof. Pembrolizumab and other humanized anti-PD-1 antibodies are disclosed in Hamid, et al. (2013) New England Journal of Medicine 369 (2): 134-44, US 8,354,509, and WO 2009/114335, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, pembrolizumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

QVQLVQSGVEVKKPGASVKVSCKASGYTFTNYYMYWVRQAPGQGLEWMGGINPSNGGTNF
NEKFKNRVTLTTDSSTTTAYMELKSLQFDDTAVYYCARRDYRFDMGFDYWGQGTTVTVSS
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFLGGPSV
FLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG
NVFSCSVMHEALHNHYTQKSLSLSLGK (SEQ ID NO: 110) ;

and/or a light chain comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWYQQKPGQAPRLLIYLASYLES
GVPARFSGSGSGTDFTLTISSLEPEDFAVYYCQHSRDLPLTFGGGTKVEIKRTVAAPSVF
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 111).

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody, nivolumab (aka BMS-936558, MDX-1106, ONO-4538, OPDIVO), or fragments thereof. Nivolumab (clone 5C4) and other human monoclonal antibodies that specifically bind to PD-1 are disclosed in US 8,008,449 and WO 2006/121168, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, nivolumab or an antigen-binding fragment thereof comprises a heavy chain comprising the amino acid sequence of:

QVQLVESGGG VVQPGRSLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY
ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTLVT VSSASTKGPS
VFPLAPCSRS TSESTAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS
VVTVPSSSLG TKTYTCNVDH KPSNTKVDKR VESKYGPPCP PCPAPEFLGG PSVFLFPPKP
KDTLMISRTP EVTCVVVDVS QEDPEVQFNW YVDGVEVHNA KTKPREEQFN STYRVVSVLT
VLHQDWLNGK EYKCKVSNKG LPSSIEKTIS KAKGQPREPQ VYTLPPSQEE MTKNQVSLTC
LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SRLTVDKSRW QEGNVFSCSV
MHEALHNHYT QKSLSLSLGK (SEQ ID NO: 112);

and/or a light chain comprising the amino acid sequence of:

EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA
RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIKRTV AAPSVFIFPP
SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT
LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC (SEQ ID NO: 113).

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody pidilizumab (aka CT-011, hBAT or hBAT-1), or fragments thereof. Pidilizumab and other humanized anti-PD-I monoclonal antibodies are disclosed in US 2008/0025980 and WO 2009/101611, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the anti-PD-1 antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a light chain variable regions comprising an amino acid sequence selected from SEQ ID NOS: 15-18 of US 2008/0025980:

SEQ ID No: 15 of US 2008/0025980 (SEQ ID NO: 114):

EIVLTQSPSSLSASVGDRVTITCSARSSVSYMHWYQQKPGKAPKLLIYRTSNLASGVPSR
FSGSGSGTDFTLTINSLQPEDFATYYCQQRSSFPLTFGGGTKLEIK;

SEQ ID No: 16 of US 2008/0025980 (SEQ ID NO: 115):

EIVLTQSPSSLSASVGDRVTITCSARSSVSYMHWFQQKPGKAPKLWIYRTSNLASGVPSR
FSGSGSGTDYTLTINSLQPEDFATYYCQQRSSFPLTFGGGTKLEIK;

SEQ ID No: 17 of US 2008/0025980 (SEQ ID NO: 116):

EIVLTQSPSSLSASVGDRVTITCSARSSVSYMHWFQQKPGKAPKLWIYRTSNLASGVPSR
FSGSGSGTDYCLTINSLQPEDFATYYCQQRSSFPLTFGGGTKLEIK;

SEQ ID No: 18 of US 2008/0025980 (SEQ ID NO: 117):

EIVLTQSPSSLSASVGDRVTITCSARSSVSYMHWFQQKPGKAPKLWIYRTSNLASGVPSR
FSGSGSGTSYCLTINSLQPEDFATYYCQQRSSFPLTFGGGTKLEIK;

and/or a heavy chain comprising an amino acid sequence selected from SEQ ID NOS: 20-24 of US 2008/0025980:

SEQ ID No: 20 of US 2008/0025980 (SEQ ID NO: 118):

QVQLVQSGSELKKPGASVKISCKASGYSFSNYGMNWVRQAPGQGLQWMGWINTDSGESTY
AEEFKGRFVFSLDTSVSTAYLQITSLTAEDTGMYFCAKVGYDALDYWGQGTLVTVSS;

SEQ ID No: 21 of US 2008/0025980 (SEQ ID NO: 119):

QVQLVQSGSELKKPGASVKISCKASGYTFTNYGMNWVRQAPGQGLQWMGWINTDSGESTY
AEEFKGRFVFSLDTSVSTAYLQITSLTAEDTGMYFCAKVGYDALDYWGQGTLVTVSS;

SEQ ID No: 22 of US 2008/0025980 (SEQ ID NO: 120):

QVQLVQSGSELKKPGASVKISCKASGYTFTNYGMNWVRQAPGQGLQWMGWINTDSGESTY
AEEFKGRFVFSLDTSVNTAYLQITSLTAEDTGMYFCVRVGYDALDYWGQGTLVTVSS;

SEQ ID No: 23 of US 2008/0025980 (SEQ ID NO: 121):

QIQLVQSGSELKKPGASVKISCKASGYTFTNYGMNWVRQAPGQGLQWMGWINTDSGESTY
AEEFKGRFVFSLDTSVNTAYLQITSLTAEDTGMYFCVRVGYDALDYWGQGTLVTVSS;

SEQ ID No: 24 of US 2008/0025980 (SEQ ID NO: 122):

QIQLVQSGSELKKPGASVKISCKASGYTFTNYGMNWVKQAPGQGLKWMGWINTDSGESTY
AEEFKGRFAFSLDTSVNTAYLQITSLNAEDTGMYFCVRVGYDALDYWGQGTLVTVSS.

In an embodiment, the targeting moiety comprises a light chain comprising SEQ ID NO:18 of US 2008/0025980 and a heavy chain comprising SEQ ID NO:22 of US 2008/0025980.

In an embodiment, the targeting moiety comprises AMP-514 (aka MEDI-0680).

In an embodiment, the targeting moiety comprises the PD-L2-Fc fusion protein AMP-224, which is disclosed in WO2010/027827 and WO 2011/066342, the entire disclosures of which are hereby incorporated by reference. In such an embodiment, the targeting moiety may include a targeting domain which comprises SEQ ID NO:4 of WO2010/027827 (SEQ ID NO: 123):

LFTVTVPKELYIIEHGSNVTLECNFDTGSHVNLGAITASLQKVENDTSPHRERATLLEEQ
LPLGKASFHIPQVQVRDEGQYQCIIIYGVAWDYKYLTLKVKASYRKINTHILKVPETDEV

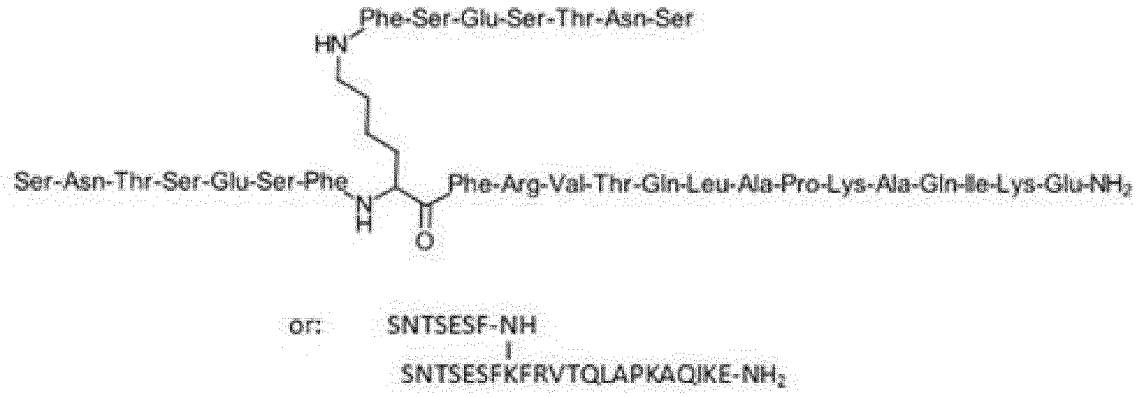
ELTCQATGYPLAEVSWPNVSVPANTSHSRTPEGLYQVTSVLRLKPPPGRNFSCVFWNTHV
RELTLASIDLQSQMEPRTHPTWLLHIFIPFCIIAFIFIATVIALRKQLCQKLYSSKDTTK
RPVTTTKREVNSAI

and/or the B7-DC fusion protein which comprises SEQ ID NO:83 of WO2010/027827 (SEQ ID NO: 124):

MIFLLLMLSLELQLHQIAALFTVTVPKELYIIEHGSNVTLECNFDTGSHVNLGAITASLQ
KVENDTSPHRERATLLEEQLPLGKASFHIPQVQVRDEGQYQCIIIYGVAWDYKYLTLKVK
ASYRKINTHILKVPETDEVELTCQATGYPLAEVSWPNVSVPANTSHSRTPEGLYQVTSVL
RLKPPPGRNFSCVFWNTHVRELTLASIDLQSQMEPRTHPTWEPKSCDKTHTCPPCPAPEL
LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK.

In an embodiment, the targeting moiety comprises the peptide AUNP 12 or any of the other peptides disclosed in US 2011/0318373 or 8,907,053. For example, the targeting moiety may comprise AUNP 12 (*i.e.*, Compound 8 or SEQ ID NO:49 of US 2011/0318373) which has the sequence of SEQ ID NO: 125:

SNTSESFK (SNTSESF)FRVTQLAPKAQIKE-NH2



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In an embodiment, the targeting moiety comprises the anti-PD-1 antibody 1E3, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1E3 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLQQSGPV LVKPGASVKM SCKASGYTFT DYYMNWVKQS HGKSLEWIGN
INPYNGGTTY NQKFKGKATL TVDKSSRTAY MEINSLTSED SAVYYCARGR
IYDGSLDYWG QGTALTVSS (SEQ ID NO: 126);

and/or a light chain variable region comprising the amino acid sequence of:

DIQMTQFPSS LCASQGGKVT VTCKASQDIN NYMAWYQHKP GKGPRLLIHY
TSTLLSGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNLWTFGGG

TKLEIK (SEQ ID NO: 127).

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody 1E8, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1E8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLQQSGAE LAKPGASVRL SCKASGYTFT NYWMHWVKQR PGQGLEWIGH
INPSSGFTTY NQNFKDKATL TADKSSNTAY MQLSSLTYED SAVYFCARED
YDVDYWGQGT TLTVSS (SEQ ID NO: 128);

and/or a light chain variable region comprising the amino acid sequence of:

DIVMTQSQKF MSTSVGDRVS VTCKASQSVD TNVAWYQQKP GQSPKALIFS
ASYRYSGVPD RFTGSGSGTD FTLTINSVQS EDLAEYFCQQ YNSYPYTFGS
GTKLEIK (SEQ ID NO: 129).

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody 1H3, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1H3 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLVESGGG LVKPGGSLKL SCAASGFTFS DYGMHWVRQA PEKGLEWVAY
ISSGSYTIYY TDTVKGRFTI SRDNAKNTLF LQMTSLRSED TAMYYCARRG
YGSFYEYYFD YWGQGTTLTV SS (SEQ ID NO: 130;

and/or light chain variable region comprising the amino acid sequence of:

QIVLTQSPAL MSASPGEKVT MTCSASSSVS YMYWYQQKPR SSPKPWIYLT
SNLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSNPFTFGSG
TKLEIK (SEQ ID NO: 131).

In an embodiment, the targeting moiety comprises a VHH directed against PD-1 as disclosed, for example, in US 8,907,065 and WO 2008/071447, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the VHHs against PD-1 comprise SEQ ID NOS: 347-351 of US 8,907,065:

SEQ ID No: 347 of US 8,907,065 (SEQ ID NO: 132):

EVQLVESGGGLVQAGKSLRLSCAASGSIFSIHAMGWFRQAPGKEREFVAA
ITWSGGITYYEDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCAADR
AESSWYDYWGQGTQVTVSS;

SEQ ID No: 348 of US 8,907,065 (SEQ ID NO: 133):

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAV
ITWSGGITYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCAGDK
HQSSWYDYWGQGTQVTVSS;

SEQ ID No: 349 of US 8,907,065 (SEQ ID NO: 134):

EVQLVESGGGLVQAGGSLRLSCAASGSISSIHAMGWFRQAPGKEREFVAA
ITWSGGITYYADSLKGRFTISRDNAKNTGYLQMNSLKPEDTAIYYCAADR
AQSSWYDYWGQGTQVTVSS;

SEQ ID No: 350 of US 8,907,065 (SEQ ID NO: 135):

EVQLVESGGGLVQAGGSLGLSCAASGSIFSINAMAWFRQAPGKEREFVAL
ISWSGGSTYYEDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCAADR
VDSNWYDYWGQGTQVTVSS;

SEQ ID No: 351 of US 8,907,065 (SEQ ID NO: 136):

EVQLVESGGGLVQAGGSLRLSCAASGRAFSSGTMGWFRRAPGKEREFVA
SIPWSGGRIYYADSVKGRFTISRDNAQNTVYLQMNSLKPEDTAVYYCAVK
ERSTGWDFASWGQCTQVTVSS.

In an embodiment, the targeting moiety comprises any one of the anti-PD-1 antibodies, or fragments thereof, as disclosed in US2011/0271358 and WO2010/036959, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID NOS: 25-29 of US2011/0271358:

SEQ ID No: 25 of US2011/0271358 (SEQ ID NO: 137):

QVQLVQSGAELKQPGASVKMSCKASGYSFTSSWIHWVKQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATLTADKSTSTAYMELSSLRSEDSAVYYCARWRDSSGYHAMDYWGQGTSVTVS
S;

SEQ ID No: 26 of US2011/0271358 (SEQ ID NO: 138):

QVQLVQSGAEVKQPGASVKMSCKASGYSFTSSWIHWVKQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATLTADKSTSTAYMELSSLRSEDTAVYY3/d10CARWRDSSGYHAMDYWGQGTSVTVS
S;

SEQ ID No: 27 of US2011/0271358 (SEQ ID NO: 139):

QVQLVQSGHEVKQPGASVKMSCKASGYSFTSSWIHWVKQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATLTADKSTSTAYMELSSLRSEDTAVYYCARWRDSSGYHAMDYWGQGTLVTVS
S;

SEQ ID No: 28 of US2011/0271358 (SEQ ID NO: 140):

QVQLVQSGHEVKQPGASVKMSCKASGYSFTSSWIHWVRQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATLTADKSTSTAYMELSSLRSEDTAVYYCARWRDSSGYHAMDYWGQGTLVTVS
S;

SEQ ID No: 29 of US2011/0271358 (SEQ ID NO: 141):

QVQLVQSGHEVKQPGASVKVSCKASGYSFTSSWIHWVRQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATITADKSTSTAYMELSSLRSEDTAVYYCARWRDSSGYHAMDYWGQGTLVTVS
S;

and/or a light chain comprising an amino acid sequence selected from SEQ ID NOS: 30-33 of US2011/0271358:

SEQ ID No: 30 of US2011/0271358 (SEQ ID NO: 142):

DIVLTQSPASLTLSPGQRLTISCRASQSVSTSGYSYMHWYQQKPDQSPKLLIKFGSNLES
GIPARFSGSGSGTDFTLTISSLEEEDFATYYCQHSWEIPYTFGQGTKLEIK;

SEQ ID No: 31 of US2011/0271358 (SEQ ID NO: 143):

DIVLTQSPATLSLSPGQRLTISCRASQSVSTSGYSYMHWYQQKPDQSPKLLIKFGSNLES
GIPARFSGSGSGTDFTLTISSLEPEDFATYYCQHSWEIPYTFGQGTKLEIK;

SEQ ID No: 32 of US2011/0271358 (SEQ ID NO: 144):

EIVLTQSPATLSLSPGQRLTISCRASQSVSTSGYSYMHWYQQKPDQSPKLLIKFGSNLES
GIPARFSGSGSGTDFTLTISSLEPEDFATYYCQHSWEIPYTFGQGTKLEIK;

SEQ ID No: 33 of US2011/0271358 (SEQ ID NO: 145):

DIVLTQSPATLSLSPGQRLTISCRASQSVSTSGYSYMHWYQQKPDQSPKLLIKFGSNLES
GIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQHSWEIPYTFGQGTKLEIK.

In various embodiments, the present chimeric protein comprises one or more antibodies directed against PD-1, or antibody fragments thereof, selected from TSR-042 (Tesaro, Inc.), REGN2810 (Regeneron Pharmaceuticals, Inc.), PDR001 (Novartis Pharmaceuticals), and BGB-A317 (BeiGene Ltd.)

In various embodiments, the present chimeric protein has one or more targeting moieties directed against PD-L1. In some embodiments, the chimeric protein has one or more targeting moieties which selectively bind a PD-L1 polypeptide. In some embodiments, the chimeric protein comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a PD-L1 polypeptide.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody MEDI4736 (aka durvalumab), or fragments thereof. MEDI4736 is selective for PD-L1 and blocks the binding of PD-L1 to the PD-1 and CD80 receptors. MEDI4736 and antigen-binding fragments thereof for use in the methods provided herein comprises a heavy chain and a light chain or a heavy chain variable region and a light chain variable region. The sequence of MEDI4736 is disclosed in WO/2016/06272, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, MEDI4736 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

EVQLVESGGG LVQPGGSLRL SCAASGFTFS RYWMSWVRQA PGKGLEWVAN
IKQDGSEKYY VDSVKGRFTI SRDNAKNSLY LQMNSLRAED TAVYYCAREG
GWFGELAFDY WGQGTLVTVS SASTKGPSVF PLAPSSKSTS GGTAALGCLV
KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTQ
TYICNVNHKP SNTKVDKRVE PKSCDKTHTC PPCPAPEFEG GPSVFLFPPK

PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY
NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPASIEKTI SKAKGQPREP
QVYTLPPSRE EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP
VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG
K (SEQ ID NO: 146);

and/or a light chain comprising the amino acid sequence of:

EIVLTQSPGT LSLSPGERAT LSCRASQRVS SSYLAWYQQK PGQAPRLLIY
DASSRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSLPWTFG
QGTKVEIKRT VAAPSVFIFP PSDEQLKSGT ASVVCLLNNF YPREAKVQWK
VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
GLSSPVTKSF NRGEC (SEQ ID NO: 147).

In illustrative embodiments, the MEDI4736 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:4 of WO/2016/06272 (SEQ ID NO: 148):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYWMSWVRQAPGKGLEWVANIKQDGSEKYY
VDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAREGGWFGELAFDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO:3 of WO/2016/06272 (SEQ ID NO: 149):

EIVLTQSPGTLSLSPGERATLSCRASQRVSSSYLAWYQQKPGQAPRLLIYDASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSLPWTFGQGTKVEIK

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody atezolizumab (aka MPDL3280A, RG7446), or fragments thereof. In illustrative embodiments, atezolizumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWVAWISPYGGSTYYADSVKGRF
TISADTSKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSSASTKGPSVFPLAPSSKST
SGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN
HKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY
SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 150);

and/or a light chain comprising the amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSG
TDFTLTISSLQPEDFATYYCQQYLYHPATFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP
VTKSFNRGEC (SEQ ID NO: 151).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody avelumab (aka MSB0010718C), or fragments thereof. In illustrative embodiments, avelumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

EVQLLESGGG LVQPGGSLRL SCAASGFTFS SYIMMWVRQA PGKGLEWVSS
IYPSGGITFY ADTVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARIK
LGTVTTVDYW GQGTLVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK
DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT
YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP
KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN
STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV
LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK (SEQ ID NO: 152);

and/or a light chain comprising the amino acid sequence of:

QSALTQPASV SGSPGQSITI SCTGTSSDVG GYNYVSWYQQ HPGKAPKLMI
YDVSNRPSGV SNRFSGSKSG NTASLTISGL QAEDEADYYC SSYTSSSTRV
FGTGTKVTVL GQPKANPTVT LFPPSSEELQ ANKATLVCLI SDFYPGAVTV
AWKADGSPVK AGVETTKPSK QSNNKYAASS YLSLTPEQWK SHRSYSCQVT
HEGSTVEKTV APTECS (SEQ ID NO: 153).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody BMS-936559 (aka 12A4, MDX-1105), or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, BMS-936559 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKTSGDTFSTYAISWVRQAPGQGLEWMGGIIPIFGKAHY
AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYFCARKFHFVSGSPFGMDVWGQGTTVT
VSS (SEQ ID NO: 154);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGQGTKVEIK (SEQ ID NO: 155).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3G10, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3G10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYGFSWVRQAPGQGLEWMGWITAYNGNTNY
AQKLQGRVTMTTDTSTSTVYMELRSLRSDDTAVYYCARDYFYGMDVWGQGTTVTVSS (SEQ ID NO: 156);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLVWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPRTFGQGTKVEIK (SEQ ID NO: 157).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 10A5, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 10A5 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYDVHWVRQAPGQRLEWMGWLHADTGITKF SQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYCARERIQLWFDYWGQGTLVTVSS (SEQ ID NO: 158);

and/or a light chain variable region comprising the amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPS RFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK (SEQ ID NO: 159).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 5F8, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 5F8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKVSGGIFSTYAINWVRQAPGQGLEWMGGIIPIFGTANH AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARDQGIAAALFDYWGQGTLVTVSS (SEQ ID NO: 160);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIP DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIK (SEQ ID NO: 161).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 10H10, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 10H10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLVESGGGLVQPGRSLRLSCAVSGFTFDDYVVHWVRQAPGKGLEWVSGISGNSGNIGY ADSVKGRFTISRDNAKNSLYLQMNSLRAEDTALYYCAVPFDYWGQGTLVTVSS (SEQ ID NO: 162);

and/or a light chain variable region comprising the amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPS RFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK (SEQ ID NO: 163).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 1B12, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1B12 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKTSGDTFSSYAISWVRQAPGQGLEWMGGIIPIFGRAHY
AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYFCARKFHFVSGSPFGMDVWGQGTTVT
VSS (SEQ ID NO: 164);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGQGTKVEIK (SEQ ID NO: 165).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 7H1, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 7H1 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKTSGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGKAHY
AQKFQGRVTITADESTTTAYMELSSLRSEDTAVYYCARKYDYVSGSPFGMDVWGQGTTVT
VSS (SEQ ID NO: 166);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGQGTKVEIK (SEQ ID NO: 167).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 11E6, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 11E6 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAINWVRQAPGQGLEWMGGIIPIFGSANY
AQKFQDRVTITADESTSAAYMELSSLRSEDTAVYYCARDSSGWSRYYMDVWGQGTTVTVS
S (SEQ ID NO: 168);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPFGGGTKVEIK (SEQ ID NO: 169).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 12B7, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 12B7 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKEPGSSVKVSCKASGGTFNSYAISWVRQAPGQGLEWMGGIIPLFGIAHY
AQKFQGRVTITADESTNTAYMDLSSLRSEDTAVYYCARKYSYVSGSPFGMDVWGQGTTVT
VSS (SEQ ID NO: 170);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGQGTRLEIK (SEQ ID NO: 171).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 13G4, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 13G4 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLVESGGGLVQPGRSLRLSCAASGITFDDYGMHWVRQAPGKGLEWVSGISWNRGRIEY
ADSVKGRFTISRDNAKNSLYLQMNSLRAEDTALYYCAKGRFRYFDWFLDYWGQGTLVTVS
S (SEQ ID NO: 172);

and/or a light chain variable region comprising the amino acid sequence of:

AIQLTQSPSSLSASVGDRVTITCRASQGISSALAWYQQKPGKAPKLLIYDASSLESGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQFNSYPFTFGPGTKVDIK (SEQ ID NO: 173).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 1E12, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1E12 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVKLQESGPS LVKPSQTLSL TCSVTGYSIT SDYWNWIRKF PGNKLEYVGY
ISYTGSTYYN PSLKSRISIT RDTSKNQYYL QLNSVTSEDT ATYYCARYGG
WLSPFDYWGQ GTTLTVSS (SEQ ID NO: 174);

and/or a light chain variable region comprising the amino acid sequence of:

DIVMTQSHKL MSTSVGDRVS ITCKASQDVG TAVAWYQQKP GQSPKLLIYW
ASTRHTGVPD RFTGSGSGTD FTLTISNVQS EDLADYFCQQ DSSYPLTFGA
GTKVELK (SEQ ID NO: 175).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 1F4, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1F4 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLQESGPG LVAPSQSLSI TCTVSGFSLT TYSINWIRQP PGKGLEWLGV
MWAGGGTNSN SVLKSRLIIS KDNSKSQVFL KMNSLQTDDT ARYYCARYYG
NSPYYAIDYW GQGTSVTVSS (SEQ ID NO: 176);

and/or a light chain variable region comprising the amino acid sequence of:

DIVTTQSHKL MSTSVGDRVS ITCKASQDVG TAVAWYQQKP GQSPKLLIYW
ASTRHTGVPD RFTGSGSGTD FTLTISNVQS EDLADYFCQQ DSSYPLTFGA
GTKVELK (SEQ ID NO: 177).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2G11, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2G11 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVKLQESGPS LVKPSQTLSL TCSVTGYSII SDYWNWIRKF PGNKLEYLGY ISYTGSTYYN PSLKSRISIT RDTSKNQYYL QLNSVTTEDT ATYYCARRGG WLLPFDYWGQ GTTLTVSS (SEQ ID NO: 178);

and/or a light chain variable region comprising the amino acid sequence of:

DIVMTQSPSS LAVSVGEKVS MGCKSSQSLL YSSNQKNSLA WYQQKPGQSP KLLIDWASTR ESGVPDRFTG SGSGTDFTLT ISSVKAEDLA VYYCQQYYGY PLTFGAGTKL ELK (SEQ ID NO: 179).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3B6, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3B6 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVKLQESGPS LVKPGASVKL SCKASGYTFT SYDINWVKQR PGQGLEWIGW IFPRDNNTKY NENFKGKATL TVDTSSTTAY MELHSLTSED SAVYFCTKEN WVGDFDYWGQ GTTLTLSS (SEQ ID NO: 180);

and/or a light chain variable region comprising the amino acid sequence of:

DIVMTQSPAI MSASPGEKVT MTCSASSSIR YMHWYQQKPG TSPKRWISDT SKLTSGVPAR FSGSGSGTSY ALTISSMEAE DAATYYCHQR SSYPWTFGGG TKLEIK (SEQ ID NO: 181).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3D10, or fragments thereof, as disclosed in US 2014/0044738 and WO2012/145493, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3D10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLQQSGPD LVTPGASVRI SCQASGYTFP DYYMNWVKQS HGKSLEWIGD IDPNYGGTTY NQKFKGKAIL TVDRSSSTAY MELRSLTSED SAVYYCARGA LTDWGQGTSL TVSS (SEQ ID NO: 182);

and/or a light chain variable region comprising the amino acid sequence of:

QIVLSQSPAI LSASPGEKVT MTCRASSSVS YIYWFQQKPG SSPKPWIYAT FNLASGVPAR FSGSGSGTSY SLTISRVETE DAATYYCQQW SNNPLTFGAG TKLELK (SEQ ID NO: 183).

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in US2011/0271358 and WO2010/036959, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided

herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 34-38 of US2011/0271358:

SEQ ID No: 34 of US2011/0271358 (SEQ ID NO: 184):

EVQLVQSGPELKKPGASVKMSCKASGYTFTSYVMHWVKQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATLTSDKSTSTAYMELSSLRSEDSAVYYCARQAWGYPWGQGTLVTVSS;

SEQ ID No: 35 of US2011/0271358 (SEQ ID NO: 185):

EVQLVQSGAEVKKPGASVKMSCKASGYTFTSYVMHWVKQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATLTSDKSTSTAYMELSSLRSEDTAVYYCARQAWGYPWGQGTLVTVSS;

SEQ ID No: 36 of US2011/0271358 (SEQ ID NO: 186):

EVQLVQSGAEVKKPGASVKMSCKASGYTFTSYVMHWVRQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATLTSDKSTSTAYMELSSLRSEDTAVYYCARQAWGYPWGQGTLVTVSS;

SEQ ID No: 37 of US2011/0271358 (SEQ ID NO: 187):

EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYVMHWVRQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATLTSDKSTSTAYMELSSLRSEDTAVYYCARQAWGYPWGQGTLVTVSS;

SEQ ID No: 38 of US2011/0271358 (SEQ ID NO: 188):

EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYVMHWVRQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATITSDKSTSTAYMELSSLRSEDTAVYYCARQAWGYPWGQGTLVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 39-42 of US2011/0271358:

SEQ ID No: 39 of US2011/0271358 (SEQ ID NO: 189):

DIVLTQSPASLALSPGERATLSCRATESVEYYGTSLVQWYQQKPGQPPKLLIYAASSVDS
GVPSRFSGSGSGTDFTLTINSLEEEDAAMYFCQQSRRVPYTFGQGTKLEIK;

SEQ ID No: 40 of US2011/0271358 (SEQ ID NO: 190):

DIVLTQSPATLSLSPGERATLSCRATESVEYYGTSLVQWYQQKPGQPPKLLIYAASSVDS
GVPSRFSGSGSGTDFTLTINSLEAEDAAMYFCQQSRRVPYTFGQGTKLEIK;

SEQ ID No: 41 of US2011/0271358 (SEQ ID NO: 191):

EIVLTQSPATLSLSPGERATLSCRATESVEYYGTSLVQWYQQKPGQPPKLLIYAASSVDS
GVPSRFSGSGSGTDFTLTINSLEAEDAAMYFCQQSRRVPYTFGQGTKLEIK;

SEQ ID No: 42 of US2011/0271358 (SEQ ID NO: 192):

DIVLTQSPATLSLSPGERATLSCRATESVEYYGTSLVQWYQQKPGQPPKLLIYAASSVDS
GVPSRFSGSGSGTDFTLTINSLEAEDAATYFCQQSRRVPYTFGQGTKLEIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.7A4, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.7A4 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 2 of WO 2011/066389 (SEQ ID NO: 193):

EVQLVESGGGLVKPGGSLRLSCAASGFTFSTYSMNWVRQAPGKGLEWVSSISSSGDYIYY
ADSVKGRFTISRDNAKNSLFLQMNSLKAEDTAVYYCARDLVTSMVAFDYWGQGTLVTVSS;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 7 of WO 2011/066389 (SEQ ID NO: 194):

SYELTQPPSVSVSPGQAARITCSGDALPQKYVFWYQQKSGQAPVLVIYEDSKRPSGIPER
FSGSSSGTMATLTISGAQVEDEADYYCYSTDRSGNHRVFGGGTRLTVL.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.9D10, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.9D10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 12 of WO 2011/066389 (SEQ ID NO: 195):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANIKQDGGEQYY
VDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDWNYGYYDMDVWGQGTTVTVSS;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 17 of WO 2011/066389 (SEQ ID NO: 196):

EIVLTQSPGTLSLSPGERATLSCRASQSVSSNYLAWFQQKPGQAPRLLIFGTSSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSIFTFGPGTKVDIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.14H9, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.14H9 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 22 of WO 2011/066389 (SEQ ID NO: 197):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYWMSWVRQAPGKGLEWVANIKQDGSEKYY
VDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAREGGWFGELAFDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 27 of WO 2011/066389 (SEQ ID NO: 198):

EIVLTQSPGTLSLSPGERATLSCRASQRVSSSYLAWYQQKPGQAPRLLIYDASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSLPWTFGQGTEVEIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.20A8, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.20A8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 32 of WO 2011/066389 (SEQ ID NO: 199):

EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYAMSWVRQAPGKGLEWVSAIRGSGGSTYY
ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKDLHYDSSGYLDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 37 of WO 2011/066389 (SEQ ID NO: 200):

DIQMTQSPSSVSASVGDRVTITCRASQGIRSWLAWYQQKPGKAPKLLIYAISRLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQANSFPLTFGGGTKVEIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3.15G8, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3.15G8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 42 of WO 2011/066389 (SEQ ID NO: 201):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANIKQDGGEKYY
VDSVKGRFTISRDNAKNSLFLQMNSLRAEDTAVYYCARVQLYSDYFDYWGQGTLVTVSS;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 47 of WO 2011/066389 (SEQ ID NO: 202):

DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQQKSGKAPKLLIYAASGLQSGVPS
RFSGSGSGTDFTLTISSLQPEDLATYYCQQSHSLPPTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3.18G1, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3.18G1 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 52 of WO 2011/066389 (SEQ ID NO: 203):

EVQLLESGGDLVQPGGSLRLSCAASGFTFNSYAMSWVRQAPGKGLEWVSTISGSGGFTFS
ADSVKGRFTISRDNSKNTLFLQMNSLRVEDSAVYSCAKVLVGFNNGCWDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 57 of WO 2011/066389 (SEQ ID NO: 204):

SYVLTQPPSVSVAPGQTARITCGGNNIGSKSVHWYQQKPGQAPVLVVYDDSDRPSGIPER
FSGSNSGNTATLTISRVEAGDEADYYCQVWDSSNDHVVFGGGTKLTVL.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.7A4OPT, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.7A4OPT or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 62 of WO 2011/066389 (SEQ ID NO: 205):

EVQLVESGGGLVKPGGSLRLSCAASGFTFSTYSMNWVRQAPGKGLEWVSSISSSGDYIYY
ADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDLVTSMVAFDYWGQGTLVTVSS;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 67 of WO 2011/066389 (SEQ ID NO: 206):

SYELTQPPSVSVSPGQTARITCSGDALPQKYVFWYQQKSGQAPVLVIYEDSKRPSGIPER
FSGSSSGTMATLTISGAQVEDEADYYCYSTDRSGNHRVFGGGTKLTVL.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.14H9OPT, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.14H9OPT or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 72 of WO 2011/066389 (SEQ ID NO: 207):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYWMSWVRQAPGKGLEWVANIKQDGSEKYY
VDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAREGGWFGELAFDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 77 of WO 2011/066389 (SEQ ID NO: 208):

EIVLTQSPGTLSLSPGERATLSCRASQRVSSSYLAWYQQKPGQAPRLLIYDASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSLPWTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO2016/061142, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 18, 30, 38, 46, 50, 54, 62, 70, and 78 of WO2016/061142:

SEQ ID No: 18 of WO2016/061142 (SEQ ID NO: 209):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMYWVRQATGQGLEWMGRIDPNSGSTKY
NEKFKNRFTISRDDSKNTAYLQMNSLKTEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 30 of WO2016/061142 (SEQ ID NO: 210):

EVQLVQSGAEVKKPGATVKISCKVSGYTFTSYWMYWVRQATGQGLEWMGRIDPNSGSTKY
NEKFKNRVTITADKSTSTAYMELSSLRSEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 38 of WO2016/061142 (SEQ ID NO: 211):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTSYWMYWVRQAPGQGLEWMGRIDPNSGSTKY
NEKFKNRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 46 of WO2016/061142 (SEQ ID NO: 212):

EVQLVQSGAEVKKPGATVKISCKVSGYTFTSYWMYWIRQSPSRGLEWLGRIDPNSGSTKY
NEKFKNRLTISKDTSKNQVVLTMTNMDPVDTATYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 50 of WO2016/061142 (SEQ ID NO: 213):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTSYWMYWIRQPPGKGLEWIGRIDPNSGSTKY
NEKFKNRVTITADKSTSTAYMELSSLRSEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 54 of WO2016/061142 (SEQ ID NO: 214):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMYWIRQSPSRGLEWLGRIDPNSGSTKY
NEKFKNRFTISRDDSKNTAYLQMNSLKTEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 62 of WO2016/061142: (SEQ ID NO: 215)

EVQLVQSGAEVKKPGESLRISCKGSGYTFTSYWMYWVRQARGQRLEWIGRIDPNSGSTKY
NEKFKNRLTISKDTSKNQVVLTMTNMDPVDTATYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 70 of WO2016/061142 (SEQ ID NO: 216):

QITLKESGPTLVKPTQTLTLTCTFSGYTFTSYWMYWVRQAPGKGLEWVSRIDPNSGSTKY
NEKFKNRVTITADKSTSTAYMELSSLRSEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 78 of WO2016/061142 (SEQ ID NO: 217):

EVQLVQSGAEVKKPGATVKISCKVSGYTFTSYWMYWVRQARGQRLEWIGRIDPNSGSTKY

NEKFKNRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 22, 26, 34, 42, 58, 66, 74, 82, and 86 of WO2016/061142:

SEQ ID No: 22 of WO2016/061142 (SEQ ID NO: 218):

DIVMTQTPLSLPVTPGEPASISCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGIPA
RFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 26 of WO2016/061142 (SEQ ID NO: 219):

DIQMTQSPSSLSASVGDRVTITCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 34 of WO2016/061142 (SEQ ID NO: 220):

EIVLTQSPDFQSVTPKEKVTITCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGVPD
RFSGSGSGTDFTLKISRVEAEDVGVYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 42 of WO2016/061142 (SEQ ID NO: 221):

EIVLTQSPDFQSVTPKEKVTITCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGVPS
RFSGSGSGTDFTFTISSLQPEDIATYYCQQYNSYPLTFGQGTKVEIK.

SEQ ID No: 58 of WO2016/061142 (SEQ ID NO: 222):

EIVLTQSPATLSLSPGERATLSCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGIPP
RFSGSGYGTDFTLTINNIESEDAAYYFCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 66 of WO2016/061142 (SEQ ID NO: 223):

DVVMTQSPLSLPVTLGQPASISCKASQDVGTAVAWYQQKPGQAPRLLIYWASTRHTGVPS
RFSGSGSGTEFTLTISSLQPDDFATYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 74 of WO2016/061142 (SEQ ID NO: 224):

DIQMTQSPSSLSASVGDRVTITCKASQDVGTAVAWYQQKPGQAPRLLIYWASTRHTGVPS
RFSGSGSGTDFTFTISSLQPEDIATYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 82 of WO2016/061142 (SEQ ID NO: 225):

AIQLTQSPSSLSASVGDRVTITCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGVPS
RFSGSGSGTDFTFTISSLEAEDAATYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 86 of WO2016/061142 (SEQ ID NO: 226):

EIVLTQSPDFQSVTPKEKVTITCKASQDVGTAVAWYQQKPGQAPRLLIYWASTRHTGVPS
RFSGSGSGTEFTLTISSLQPDDFATYYCQQYNSYPLTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO2016/022630, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 2, 6, 10, 14, 18, 22, 26, 30, 34, 38, 42, and 46 of WO2016/022630:

SEQ ID No: 2 of WO2016/022630 (SEQ ID NO: 227):

EVKLVESGGGLVKPGGSLKLSCAASGFIFRSYGMSWVRQTPEKRLEWVASISSGGSTYYP
DSVKGRFTISRDNARNILYLQMSSLRSEDTAMYDCARGYDSGFAYWGQGTLVTVSE;

SEQ ID No: 6 of WO2016/022630 (SEQ ID NO: 228):

EVKLVESGGGLVKPGGSLKLSCAASGFTFRSYGMSWVRQTPEKRLEWVASISSGGTTYYP
DSVKGRFIISRDNARNILYLQMSSLRSEDTAMYYCAKGYDSGFAYWGQGTLVIVSA;

SEQ ID No: 10 of WO2016/022630 (SEQ ID NO: 229):

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTTYGVHWVRQSPGKGLEWLGVIWRGVTTDYN
AAFMSRLTITKDNSKSQVFFKMNSLQANDTAIYYCARLGFYAMDYWGQGTSVTVSS;

SEQ ID No: 14 of WO2016/022630 (SEQ ID NO: 230):

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTSYGVHWVRQSPGKGLEWLGVIWSGGVTDYN
AAFISRLSISKDNSKSQVFFKMNSLQANDTAIYYCARLGFYAMDYWGQGTSVTVSS;

SEQ ID No: 18 of WO2016/022630 (SEQ ID NO: 231):

EVKLFESGGGLVQPGGSLKLSCVASGFDFSTYWMHWVRQAPGQGLEWIGQINPDSTTINY
APSLKDRFIISRDNAKNTLFLQMSKVRSEDTALYYCAKPGDYGYDFDCWGQGTTLTVSS;

SEQ ID No: 22 of WO2016/022630 (SEQ ID NO: 232):

EVQLQESGPSLVKPSQTLSLTCSVTGDSITSGYWNWIRKFPGNKLEYMGYISYSGSTYYN
PSLKSRISITRDTSKNQYYLQLNSVTTEDTATYYCARSLLWFSTGFAYWGQGTLVTVSA;

SEQ ID No: 26 of WO2016/022630 (SEQ ID NO: 233):

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTSYGVHWVRQSPGKGLEWLGVIWSGGITDYN
AAFKSRLSISKDNSKSQVFFKMNSLQANDTAIYFCARLGFYAMDYWGQGTSVTVSS;

SEQ ID No: 30 of WO2016/022630 (SEQ ID NO: 234):

EVKLVESGGGLVKPGGSLKLSCAASGFTFRSYGMSWARQIPEKRLEWVASISSGGTTYYL
GSVQGRFTISRDNARNILYLQMSSLRSEDTAMYYCARGYDAGFAYWGQGTLVSVSE;

SEQ ID No: 34 of WO2016/022630 (SEQ ID NO: 235):

EVQLQESGPSLVKPSQTLSLTCSVTGDSITSGYWTWIRKFPGNKLEYMGYISYTGSTYYN

PSLKSRISISRDTSKSQYYLQLNSVTTEDTATYYCARQRDWLGFAYWGQGTLVTVSA;

SEQ ID No: 38 of WO2016/022630 (SEQ ID NO: 236):

EEKLVESGGGLVKPGGSLKLSCAASGFSFSSYGMSWVRQTPEKRLEWVASISSGGSIYYP
DSVKGRFTISRDNARNILYLQMSSLRSEDTAMYYCARGYDAGFAFWGQGTLVTASA;

SEQ ID No: 42 of WO2016/022630 (SEQ ID NO: 237):

QITLKESGPTLVKPTQTLTLTCTVSGFSLSTYGVHWIRQPPGKALEWLGVIWRGVTTDYN
AAFMSRLTITKDNSKNQVVLTMNNMDPVDTATYYCARLGFYAMDYWGQGTLVTVSS;

SEQ ID No: 46 of WO2016/022630 (SEQ ID NO: 238):

EVQLVESGGGLVKPGGSLRLSCAASGFIFRSYGMSWVRQAPGKGLEWVASISSGGSTYYP
DSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYDCARGYDSGFAYWGQGTLVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 of WO2016/022630:

SEQ ID No: 4 of WO2016/022630 (SEQ ID NO: 239):

DIVLTQSPASLAVSLGQRATISCRASQSVSTSSSSFMHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQHSWEIPYTFGGGTKLEIKR;

SEQ ID No: 8 of WO2016/022630 (SEQ ID NO: 240):

DIVLTQSPPSLAVSLGQRATISCRASQSVSTSSSSYMHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQHSWEIPYTFGGGTKLEIK;

SEQ ID No: 12 of WO2016/022630 (SEQ ID NO: 241):

SIVMTQTPKFLLVSAGDRVTITCKASQSVSNDVAWYQQKPGQSPKLLIYYAANRYTGVPD
RFTGSGYGTDFTFTISIVQAEDLAVYFCQQDYTSPYTFGGGTKLEIK;

SEQ ID No: 16 of WO2016/022630 (SEQ ID NO: 242):

SIVMTQTPKFLLVSAGDRVTITCKASQSVSNDVGWYQQKPGQSPKLLIYYASNRYSGVPD
RFTGSGYGTDFTFTISTVQAEDLAVYFCQQDYTSPYTFGGGTKLEIK;

SEQ ID No: 20 of WO2016/022630 (SEQ ID NO: 243):

DVLMTQTPLYLPVSLGDQASISCRSSQIIVHSNANTYLEWFLQKPGQSPKLLIYKVSNRF
SGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQGSHVPYTFGGGTKLEIK;

SEQ ID No: 24 of WO2016/022630 (SEQ ID NO: 244):

QIVLTQSPAIMSASPGEKVTLTCSASSSVSSSYLYWNQQKPGSSPKVWIYNTSNLASGVP
ARFSGSGSGTSYSLTISSMEAEDAASYFCHQWRSYPPTLGAGTKLELK;

SEQ ID No: 28 of WO2016/022630 (SEQ ID NO: 245):

QIVLTQSPAIMSASPGEKVTMTCSANSSVSYMHWYQQKSGTSPKRWIYDTSKLASGVPAR
FSGSGSGTSYSLTISSMGAEDAATYYCQQWSSNPWTFGGGTKLEIK;

SEQ ID No: 32 of WO2016/022630 (SEQ ID NO: 246):

DIVLTQSPASLAVSLGQRATISCRASQSVSTSSYSYMHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQNSWEIPYTFGGGTKLEIK;

SEQ ID No: 36 of WO2016/022630 (SEQ ID NO: 247):

DIVMTQTPSSLAVSLGEKVTMSCKSSQSLLYSSNQKNSLAWYQQKPGQSPKLLIYWASNR
ESGVPDRFTGSSSGTDFTLTISSVKAEDLAVYYCQQYYSYPLTFGAGTKLELK;

SEQ ID No: 40 of WO2016/022630 (SEQ ID NO: 248):

DIVLTQSPASLAVSLGQRATISCRASQSVSTSSYSYVHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQHSWEIPYTFGGGTKLEIK;

SEQ ID No: 44 of WO2016/022630 (SEQ ID NO: 249):

DIQMTQSPSSLSASVGDRVTITCKASQSVSNDVAWYQQKPGKAPKLLIYYAANRYTGVPD
RFSGSGYGTDFTFTISSLQPEDIATYFCQQDYTSPYTFGQGTKLEIK;

SEQ ID No: 48 of WO2016/022630 (SEQ ID NO: 250):

DIVLTQSPASLAVSPGQRATITCRASQSVSTSSSSFMHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLTINPVEANDTANYYCQHSWEIPYTFGQGTKLEIK.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO2015/112900, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 38, 50, 82, and 86 of WO 2015/112900:

SEQ ID No: 38 of WO2015/112900 (SEQ ID NO: 251):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTTYWMHWVRQATGQGLEWMGNIYPGTGGSNF
DEKFKNRVTITADKSTSTAYMELSSLRSEDTAVYYCTRWTTGTGAYWGQGTTVTVSS;

SEQ ID No: 50 of WO 2015/112900 (SEQ ID NO: 252):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTTYWMHWIRQSPSRGLEWLGNIYPGTGGSNF
DEKFKNRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTRWTTGTGAYWGQGTTVTVSS;

SEQ ID No: 82 of WO 2015/112900 (SEQ ID NO: 253):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTTYWMHWIRQSPSRGLEWLGNIYPGTGGSNF
DEKFKNRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTRWTTGTGAYWGQGTTVTVSS;

SEQ ID No: 86 of WO 2015/112900 (SEQ ID NO: 254):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTTYWMHWVRQAPGQGLEWMGNIYPGTGGSNF
DEKFKNRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTRWTTGTGAYWGQGTTVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 42, 46, 54, 58, 62, 66, 70, 74, and 78 of WO 2015/112900:

SEQ ID No: 42 of WO2015/112900 (SEQ ID NO: 255):

EIVLTQSPATLSLSPGERATLSCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGVPSRFSGSGSGTEFTLTISSLQPDDFATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 46 of WO 2015/112900 (SEQ ID NO: 256):

DIQMTQSPSSLSASVGDRVTITCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGIPPRFSGSGYGTDFTLTINNIESEDAAYYFCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 54 of WO 2015/112900 (SEQ ID NO: 257):

EIVLTQSPATLSLSPGERATLSCKSSQSLLDSGNQKNFLTWYQQKPGKAPKLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 58 of WO 2015/112900 (SEQ ID NO: 258):

DIVMTQTPLSLPVTPGEPASISCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 62 of WO 2015/112900 (SEQ ID NO: 259):

EIVLTQSPATLSLSPGERATLSCKSSQSLLDSGNQKNFLTWYQQKPGKAPKLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 66 of WO 2015/112900 (SEQ ID NO: 260):

EIVLTQSPDFQSVTPKEKVTITCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 70 of WO 2015/112900 (SEQ ID NO: 261):

EIVLTQSPATLSLSPGERATLSCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 74 of WO 2015/112900 (SEQ ID NO: 262):

DIQMTQSPSSLSASVGDRVTITCKSSQSLLDSGNQKNFLTWYLQKPGQSPQLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 78 of WO 2015/112900 (SEQ ID NO: 263):

DVVMTQSPLSLPVTLGQPASISCKSSQSLLDSGNQKNFLTWYQQKPGKAPKLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO 2010/077634 and US 8,217,149, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the anti-PD-L1 antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain region comprising the amino acid sequence of:

SEQ ID No: 20 of WO 2010/077634 (SEQ ID NO: 264):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWVAWISPYGGSTYY
ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSA;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 21 of WO 2010/077634 (SEQ ID NO: 265):

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGQGTKVEIKR.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies obtainable from the hybridoma accessible under CNCM deposit numbers CNCM I-4122, CNCM I-4080 and CNCM I-4081 as disclosed in US 20120039906, the entire disclosures of which are hereby incorporated by reference.

In an embodiment, the targeting moiety comprises a VHH directed against PD-L1 as disclosed, for example, in US 8,907,065 and WO 2008/071447, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the VHHs against PD-L1 comprise SEQ ID NOS: 394-399 of US 8,907,065:

SEQ ID No: 394 of US 8,907,065 (SEQ ID NO: 266):

EVQLVESGGGLVQPGGSLRLSCAASGFTLDYYAIGWFRQAPGKEREWASS
ISSSDGSTYYADSVKGRFTISRDNAKNTVFLQMNSLKPEDTAVYSCAASQ
APITIATMMKPFYDYWGQGTQVTVSS;

SEQ ID No: 395 of US 8,907,065 (SEQ ID NO: 267):

EVQLVESGGGLVQPGGSLRLSCAASGFTLDYYAKCWFRQAPGKEREWVSC
ISSSDGSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYFCAARH
GGPLTVEYFFDYWGQGTQVTVSS:

SEQ ID No: 396 of US 8,907,065 (SEQ ID NO: 268):

EVQLVESGGGLVQPGGSLRLSCAASGFTFDYYAIGWFRQAPGKAREGVSC
ISGGDNSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCATGG
WKYCSGYDPEYIYWGQGTQVTVSS;

SEQ ID No: 397 of US 8,907,065 (SEQ ID NO: 269):

EVQLVESGGGLVQAGGSLRLSCAASGSTFSQYDVGWYRQAPGKQRELVA
FSSSGGRTIYPDSVKGRFTFSRDNTKNTVYLQMTSLKPEDTAVYYCKIDW
YLNSYWGQGTQVTVSS;

SEQ ID No: 398 of US 8,907,065 (SEQ ID NO: 270):

EVQLVESGGGLVQAGGSLRLSCAASGVDASNSAMGWYRQAPGKQREWVAR
ITGGGLIAYTDSVKGRFTISRDNAKSTVYLQMNSLEPEDTAVYYCNTINS
RDGWGQGTQVTVSS;

SEQ ID No: 399 of US 8,907,065 (SEQ ID NO: 271):

EVQLVESGGGLVQAGGSLTISCAASGITFSDSIVSWYRRARGKQREWVAG
ISNGGTTKYAESVLGRFTISRDNAKNNVYLQMNGLNPEDTAVYLCKVRQY
WGQGTQVTVSS.

In various embodiments, the present chimeric protein has one or more targeting moieties directed against PD-L2. In some embodiments, the chimeric protein has one or more targeting moieties which selectively bind a PD-L2 polypeptide. In some embodiments, the chimeric protein comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a PD-L2 polypeptide.

In an embodiment, the targeting moiety comprises a VHH directed against PD-L2 as disclosed, for example, in US 8,907,065 and WO 2008/071447, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the VHHs against PD-1 comprise SEQ ID Nos: 449-455 of US 8,907,065:

SEQ ID No: 449 of US 8,907,065 (SEQ ID NO: 272):

EVQLVESGGGLVQAGGSLRLSCAASESTVLINAMGWYRQAPGKQRELVAS
ISSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNADVY
PQDYGLGYVEGKVYYGHDYWGTGTLVTVSS;

SEQ ID No: 450 of US 8,907,065 (SEQ ID NO: 273):

EVQLVESGGGLVQAGGSLRLSCAASGSTFSNYVSNYAMGWGRQAPGTQ
RELVASISNGDTTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYY
CFEHQVAGLTWGQGTQVTVSS;

SEQ ID No: 451 of US 8,907,065 (SEQ ID NO: 274):

EVQLVESGGGLVQAGGSLRLSCVASGXALKIXVMGWYRQAPGKQRELV
AAITSGGRTNYSDSVKGRFTISGDNAXNTVYLQMNSLKSEDTAVYYCRE
WNSGYPPVDYWGQGTQVTVSS;

SEQ ID No: 452 of US 8,907,065 (SEQ ID NO: 275):

EVQLVESGGGLVQAGGSLRLSCAASGRTFSSGTMGWFRRAPGKEREFV
ASIPWSGGRTYYADSVKDRFTISRDNAQNTVFLQMNSLKPEDTAVYYCAF
KERSTGWDFASWGQGIQVTVSS;

SEQ ID No: 453 of US 8,907,065 (SEQ ID NO: 276):

EVQLVESGGGLVQTGGSLRLSCAASGFTLDYYGIGWFRQAPGKEREGVS
FISGSDGSTYYAESVKGRFTISRDKAKNTVYLQMNSLKPEDTAVYYCAAD
PWGPPSIATMTSYEYKHWGQGTQVTVSS;

SEQ ID No: 454 of US 8,907,065 (SEQ ID NO: 277):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYTMIWLRRAPGKGFEWV
STIDKDGNTNYVDSVKGRFAVSRDNTKNTLYLQMNSLKPEDTAMYYCTK
HGSSARGQGTRVTVSS;

SEQ ID No: 455 of US 8,907,065 (SEQ ID NO: 278):

EVQLVESGGGLVEPGGSLRLSCVASGFTFSSYDMSWVRQAPGKGLE
WVSTINSGGGITYRGSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYY
CENGGSSYRRGQGTQVTVSS.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L2 antibodies disclosed in US2011/0271358 and WO2010/036959, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 43-47 of US2011/0271358:

SEQ ID No: 43 of US2011/0271358 (SEQ ID NO: 279):

QVQLVQSGAELKKPGASVKMSCKASGYTFTGYTMHWVKQAPGQGLEWIGYINPRSGYTEY
NQKFKDRTTLTADKSTSTAYMELSSLRSEDSAVYYCARPWFAYWGQGTLVTVSS;

SEQ ID No: 44 of US2011/0271358 (SEQ ID NO: 280):

QVQLVQSGAEVKKPGASVKMSCKASGYTFTGYTMHWVKQAPGQGLEWIGYINPRSGYTEY
NQKFKDRTTLTADKSTSTAYMELSSLRSEDTAVYYCARPWFAYWGQGTLVTVSS;

SEQ ID No: 45 of US2011/0271358 (SEQ ID NO: 281):

QVQLVQSGAEVKKPGASVKMSCKASGYTFTGYTMHWVRQAPGQGLEWIGYINPRSGYTEY
NQKFKDRTTLTADKSTSTAYMELSSLRSEDTAVYYCARPWFAYWGQGTLVTVSS;

SEQ ID No: 46 of US2011/0271358 (SEQ ID NO: 282):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYTMHWVRQAPGQGLEWIGYINPRSGYTEY
NQKFKDRTTLTADKSTSTAYMELSSLRSEDTAVYYCARPWFAYWGQGTLVTVSS;

SEQ ID No: 47 of US2011/0271358 (SEQ ID NO: 283):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYTMHWVRQAPGQGLEWIGYINPRSGYTEY

NQKFKDRTTITADKSTSTAYMELSSLRSEDTAVYYCARPWFAYWGQGTLVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 48-51 of US2011/0271358:

SEQ ID No: 48 of US2011/0271358 (SEQ ID NO: 284):

DIVMTQSPASLTVTPGEKVTITCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTR
ESGVPDRFTGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPLTFGQGTKLEIK;

SEQ ID No: 49 of US2011/0271358 (SEQ ID NO: 285):

DIVMTQSPASLSVTPGEKVTITCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTR
ESGVPDRFTGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPLTFGQGTKLEIK;

SEQ ID No: 50 of US2011/0271358 (SEQ ID NO: 286):

DIVMTQSPAFLSVTPGEKVTITCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTR
ESGVPDRFTGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPLTFGQGTKLEIK;

SEQ ID No: 51 of US2011/0271358 (SEQ ID NO: 287):

DIVMTQSPAFLSVTPGEKVTITCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTR
ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPLTFGQGTKLEIK.

In various embodiments, the targeting moieties of the invention may comprise a sequence that targets PD-1, PD-L1, and/or PD-L2 which is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to any of the sequences disclosed herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or 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%, about 99% or about 100% sequence identity with any of the sequences disclosed herein).

In various embodiments, the targeting moieties of the invention may comprise any combination of heavy chain, light chain, heavy chain variable region, light chain variable region, complementarity determining region (CDR), and framework region sequences that target PD-1, PD-L1, and/or PD-L2 as disclosed herein.

Additional antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind or target PD-1, PD-L1 and/or PD-L2 are disclosed in WO 2011/066389, US 2008/0025980, US 2013/0034559, US 8,779,108, US 2014/0356353, US 8,609,089, US 2010/028330, US 2012/0114649, WO 2010/027827, WO 2011,/066342, US 8,907,065, WO 2016/062722, WO 2009/101611, WO2010/027827, WO 2011/066342, WO 2007/005874 , WO 2001/014556, US2011/0271358, WO 2010/036959, WO 2010/077634, US 8,217,149, US 2012/0039906, WO 2012/145493, US 2011/0318373, U.S. Patent No. 8,779,108, US 20140044738, WO 2009/089149, WO 2007/00587, WO 2016061142, WO 2016,02263, WO 2010/077634, and WO 2015/112900, the entire disclosures of which are hereby incorporated by reference.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a T cell, for example, mediated by targeting to CD8 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against CD8 on T cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a T cell, for example, mediated by targeting to CD4 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against CD4 on T cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a T cell, for example, mediated by targeting to CD3, CXCR3, CCR4, CCR9, CD70, CD103, or one or more immune checkpoint markers and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against CD3 on T cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In some embodiments, the present chimeric protein has one or more targeting moieties directed against CD3 expressed on T cells. In some embodiments, the chimeric protein has one or more targeting moieties which selectively bind a CD3 polypeptide. In some embodiments, the chimeric protein comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a CD3 polypeptide.

In an embodiment, the targeting moiety comprises the anti-CD3 antibody muromonab-CD3 (aka Orthoclone OKT3), or fragments thereof. Muromonab-CD3 is disclosed in U.S. Patent No. 4,361,549 and Wilde et al. (1996) 51:865-894, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments,

muromonab-CD3 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNY
NQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSA
KTTAPSVYPLAPVCGGTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDL
YTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRPKSCDKTHTCPPCPAPELLGG
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDE
LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 288);

and/or a light chain comprising the amino acid sequence of:

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLASGVPAH
FRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRADTAPTVSIFPPS
SEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTL
TKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID NO: 289.

In an embodiment, the targeting moiety comprises the anti-CD3 antibody otelixizumab, or fragments thereof. Otelixizumab is disclosed in U.S. Patent Publication No. 20160000916 and Chatenoud et al. (2012) 9:372-381, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, otelixizumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFPMAWVRQAPGKGLEWVSTISTSGGRTYYRDSVKGRF
TISRDNSKNTLYLQMNSLRAEDTAVYYCAKFRQYSGGFDYWGQGTLVTVSSASTKGPSVFPLAPSSKS
TSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV
NHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL
YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 290);

and/or a light chain comprising the amino acid sequence of:

DIQLTQPNSVSTSLGSTVKLSCTLSSGNIENNYVHWYQLYEGRSPTTMIYDDDKRPDGVPDRFSGSID
RSSNSAFLTIHNVAIEDEAIYFCHSYVSSFNVFGGGTKLTVLRQPKAAPSVTLFPPSSEELQANKATL

VCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGS TVEKTVAPTECS (SEQ ID NO: 291).

In an embodiment, the targeting moiety comprises the anti-CD3 antibody teplizumab (AKA MGA031 and hOKT3γ1(Ala-Ala)), or fragments thereof. Teplizumab is disclosed in Chatenoud et al. (2012) 9:372-381, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, teplizumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGKGLEWIGYINPSRGYTNYNQKVKDRF TISRDNSKNTAFLQMDSLRPEDTGVYFCARYYDDHYCLDYWGQGTPVTVSSASTKGPSVFPLAPSSKS TSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV NHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 292);

and/or a light chain comprising the amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCSASSSVSYMNWYQQTPGKAPKRWIYDTSKLASGVPSRFSGSGSGT DYTFTISSLQPEDIATYYCQQWSSNPFTFGQGTKLQITRTVAAPSVFIFPPSDEQLKSGTASVVCLLN NFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPV TKSFNRGEC (SEQ ID NO: 293).

In an embodiment, the targeting moiety comprises the anti-CD3 antibody visilizumab (AKA Nuvion®; HuM291), or fragments thereof. Visilizumab is disclosed in U.S. 5,834,597 and WO2004052397, and Cole et al., Transplantation (1999) 68:563-571, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, visilizumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGASVKVSCKASGYTFISYTMHWVRQAPGQGLEWMGYINPRSGYTHYNQKLKDKA TLTADKSASTAYMELSSLRSEDTAVYYCARSAYYDYDGFAYWGQGTLVTVSS (SEQ ID NO: 294);

and/or a light chain variable region comprising the amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCSASSSVSYMNWYQQKPGKAPKRLIYDTSKLASGVPSR FSGSGSGTDFTLTISSLQPEDFATYYCQQWSSNPPTFGGGTKVEIK (SEQ ID NO: 295).

In an embodiment, the targeting moiety comprises the anti-CD3 antibody foralumab (aka NI-0401), or fragments thereof. In various embodiments, the targeting moiety comprises any one of the anti-CD3 antibodies disclosed in US20140193399, US 7,728,114, US20100183554, and US 8,551,478, the entire disclosures of which are hereby incorporated by reference.

In illustrative embodiments, the anti-CD3 antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID Nos: 2 and 6 of US 7,728,114:

SEQ ID No: 2 of US 7,728,114 (SEQ ID NO: 296):

QVQLVESGGGVVQPGRSLRLSCAASGFKFSGYGMHWVRQAPGKGLEWVAVIWYDGSKKYY
VDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARQMGYWHFDLWGRGTLVTVSS;

SEQ ID No: 6 of US 7,728,114 (SEQ ID NO: 297):

QVQLVQSGGGVVQSGRSLRLSCAASGFKFSGYGMHWVRQAPGKGLEWVAVIWYDGSKKYY
VDSVKGRFTISRDNSKNTLYLQMNSLRGEDTAVYYCARQMGYWHFDLWGRGTLVTVSS;

and/or a light chain variable region comprising the amino acid sequence of SEQ ID NOs 4 and 8 of US 7,728,114:

SEQ ID No: 4 of US 7,728,114 (SEQ ID NO: 298):

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPPLTFGGGTKVEIK;

SEQ ID No: 8 of US 7,728,114 (SEQ ID NO: 299):

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPPLTFGGGTKVEIK;

In an embodiment, the targeting moiety comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:2 of US 7,728,114 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:4 of US 7,728,114. In an embodiment, the targeting moiety comprises any one of the anti-CD3 antibodies disclosed in US2016/0168247, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 6-9 of US2016/0168247:

SEQ ID No: 6 of US2016/0168247 (SEQ ID NO: 300):

EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYAT
YYADSVKDRFTISRDDSKSSLYLQMNNLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTL
VTVSS;

SEQ ID No: 7 of US2016/0168247 (SEQ ID NO: 301):

EVKLVESGGGLVKPGRSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYAT
YYADSVKDRFTISRDDSKSILYLQMNNLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTL
VTVSS;

SEQ ID No: 8 of US2016/0168247 (SEQ ID NO: 302):

EVKLVESGGGLVKPGRSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYAT
YYADSVKDRFTISRDDSKSILYLQMNSLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTL
VTVSS;

SEQ ID No: 9 of US2016/0168247 (SEQ ID NO: 303):

EVKLVESGGGLVKPGRSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYAT
YYADSVKDRFTISRDDSKSILYLQMNSLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTM
VTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 10-12 of US2016/0168247:

SEQ ID No: 10 of US2016/0168247 (SEQ ID NO: 304):

QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIGGTNKRAPGV
PARFSGSLIGDKAALTITGAQADDESIYFCALWYSNLWVFGGGTKLTVL;

SEQ ID No: 11 of US2016/0168247 (SEQ ID NO: 305):

QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIGGTNKRAPGV
PARFSGSILGNKAALTITGAQADDESIYFCALWYSNLWVFGGGTKLTVL;

SEQ ID No: 12 of US2016/0168247 (SEQ ID NO: 306):

QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIGGTNKRAPGV
PARFSGSILGNKAALTITGAQADDESDYYCALWYSNLWVFGGGTKLTVL.

In an embodiment, the targeting moiety comprises any one of the anti-CD3 antibodies disclosed in US2015/0175699, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID No: 9 of US2015/0175699:

SEQ ID No: 9 of US2015/0175699 (SEQ ID NO: 307):

QVQLVQSGSELKKPGASVKMSCKASGYTFTRYTMHWVRQAPGKGLEWIGYINPSRGYTNY
NQKFKDRATLTTDKSTSTAYMQLSSLRSEDTAVYYCARYYDDHYSLDYWGQGTLVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID No: 10 of US2015/0175699:

SEQ ID No: 10 of US2015/0175699 (SEQ ID NO: 308):

QIVLTQSPATLSLSPGERATMSCSASSSVSYMNWYQQKPGKAPKRWIYDTSKLASGVPSR
FRGSGSGTDYTLTISSLQPEDFATYYCQQWSSNPFTFGGGTKVEIK.

In an embodiment, the targeting moiety comprises any one of the anti-CD3 antibodies disclosed in US 8,784,821, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an

antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 2, 18, 34, 50, 66, 82, 98 and 114 of US 8,784,821:

SEQ ID No: 2 of US 8,784,821 (SEQ ID NO: 309):

ELQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVISYDGSNKYY
ADSVKGRFTISRDNSKNTLYLQMNSLRSEDTAVYYCARLSPYCTNGVCWDAFDIWGQGTM
VTVSS;

SEQ ID No: 18 of US 8,784,821 (SEQ ID NO: 310):

ELQLVESGGGLVKPGRSLRLSCTASGFTFGDYAMSWFRQAPGKGLEWVGFIRSKAYGGTT
EYAASVKGRFTISRDDSKSIAYLQMNSLKTEDTAVYYCTPQLWLLQDAFDIWGQGTMVTV
SS;

SEQ ID No: 34 of US 8,784,821 (SEQ ID NO: 311):

ELQLVESGPGLVKPSGTLSLTCAVSGGSISSRNWWSWVRQPPGKGLEWIGDIYHSGSTNY
NPSLKSRVTISVDKSKNQFSLKLSSVTAADTAVYYCASGYTSCRDAFDIWGQGTMVTVSS;

SEQ ID No: 50 of US 8,784,821 (SEQ ID NO: 312):

ELQLVEWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGSTNYN
PSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARGRGRFLGWLLGGSNWFDPWGQGT
LVTVSS;

SEQ ID No: 66 of US 8,784,821 (SEQ ID NO: 313):

ELQLVEWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGSTNYN
PSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARGPDRMGHGFDIWGQGTMVTVSS;

SEQ ID No: 82 of US 8,784,821 (SEQ ID NO: 314):

ELQLVESGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYRSKWY
NDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARDRRRIAARQYYGMDVWGQG
TTVTVSS;

SEQ ID No: 98 of US 8,784,821 (SEQ ID NO: 315):

ELQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMGWVRQAPGKGLEWVSAVSGSGGSTYY
ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAKFLGHYYGMDVWGQGTTVTVS
S;

SEQ ID No: 114 of US 8,784,821 (SEQ ID NO: 316):

ELQLVESGPVLVKPTDTLTLTCTVSGFSLNNPRMGVSWIRQPPGKTLEWLAHIFPSDAKA
HSASLKSRLTISKDTSKSQVVPTMTNMDPVDTATYYCARILGEYYPPAWFDPWGQGTLVT
VSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 10, 26, 42, 58, 74, 90, 106 and 122 of US 8,784,821:

SEQ ID No: 10 of US 8,784,821 (SEQ ID NO: 317):

ELQMTQSPSSLSASVGDRVSITCRASQTISNYLNWYQLKPGKAPKLLIYAASTLQSEVPT
RFSGSGSGTDFTLTISGLHPEDFATYYCQQFNSYPRTFGQGTKVEIK;

SEQ ID No: 26 of US 8,784,821 (SEQ ID NO: 318):

ELQMTQSPSSLSASVGDRVTITCRASQGISNYLAWYQQKPGKVPKLLIYAASTLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPPTFGQGTKLEIK;

SEQ ID No: 42 of US 8,784,821 (SEQ ID NO: 319):

ELVMTQSPSSLSASVGDRVTITCRASQGIGNYLAWYQQKPGQPPKMLIYWASIRESGVPD
RFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSNPQTFGQGTKVEIK;

SEQ ID No: 58 of US 8,784,821 (SEQ ID NO: 320):

ELVMTQSPSSLSASVGDRVTITCRASQGISNYLNWYQQKPGKAPKLLIYDASNLETGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPYTFGQGTKVDIK;

SEQ ID No: 74 of US 8,784,821 (SEQ ID NO: 321):

ELQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKSGKAPKLLIYAASSLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSSPWTFGQGTKVEIK;

SEQ ID No: 90 of US 8,784,821 (SEQ ID NO: 322):

ELVLTQSPGTLSLSPGERATLSCRASQSVSSNYLAWYQQKPGQAPRLLIYGASSRATGIP
DRFSGSGSGTDFTLTISSLQPEDVATYYCQKYNSAPLTFGGGTKVEIK;

SEQ ID No: 106 of US 8,784,821 (SEQ ID NO: 323):

ELQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNAYPYTFGQGTKVEIK;

SEQ ID No: 122 of US 8,784,821 (SEQ ID NO: 324):

ELVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIYWASTR
ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYLKIPYTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises any one of the anti-CD3 binding constructs disclosed in US20150118252, the entire contents of which are hereby incorporated by reference. In illustrative embodiments,

the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 6 and 86 of US20150118252:

SEQ ID No: 6 of US20150118252 (SEQ ID NO: 325):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTRYTMHWVRQAPGQGLEWMGYINPSRGYTNY
NQKFKDRVTMTTDTSISTAYMELSRLRSDDTAVYYCARYYDDHYCLDYWGQGTLVTVSS;

SEQ ID No: 86 of US20150118252 (SEQ ID NO: 326):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTRYTMHWVRQAPGQGLEWMGYINPSRGYTNY
NQKFKDRVTMTTDTSISTAYMELSRLRSDDTAVYYCARYYDDHYSLDYWGQGTLVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID No: 3 of US2015/0175699:

SEQ ID No: 3 of US20150118252 (SEQ ID NO: 327):

EIVLTQSPATLSLSPGERATLSCSASSSVSYMNWYQQKPGQAPRLLIYDTSKLASGVPAH
FRGSGSGTDYTLTISSLEPEDFAVYYCQQWSSNPFTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises any one of the anti-CD3 binding proteins disclosed in US2016/0039934, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 6-9 of US2016/0039934:

SEQ ID No: 6 of US2016/0039934 (SEQ ID NO: 328):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGRIRSKYNNYAT
YYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARHGNFGNSYVSWFAYWGQGTL
VTVSS;

SEQ ID No: 7 of US2016/0039934 (SEQ ID NO: 329):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGRIRSKYNNYAT
YYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARHGNFGNSYVSWFAYWGQGTL
VTVSS;

SEQ ID No: 8 of US2016/0039934 (SEQ ID NO: 330):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGRIRSKYNNYAT
YYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARHGNFGNSYVSYFAYWGQGTL
VTVSS;

SEQ ID No: 9 of US2016/0039934 (SEQ ID NO: 331):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGRIRSKYNNYAT
YYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARHGNFGNSYVSHFAYWGQGTL
VTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 1-4 of US2016/0039934:

SEQ ID No: 1 of US2016/0039934 (SEQ ID NO: 332):

DIQMTQSPSSLSASVGDRVTITCRSSTGAVTTSNYANWVQQKPGKAPKGLIGGTNKRAPG
VPSRFSGSLIGDKATLTISSLQPEDFATYYCALWYSNLWVFGQGTKVEIK;

SEQ ID No: 2 of US2016/0039934 (SEQ ID NO: 333):

DIQMTQSPSSLSASVGDRVTITCRSSTGAVTTSNYANWVQQKPGKAPKGLIGGTNKRAPG
VPARFSGSGSGTDFTLTISSLQPEDFATYYCALWYSNLWVFGQGTKVEIK;

SEQ ID No: 3 of US2016/0039934 (SEQ ID NO: 334):

DIQMTQSPSSLSASVGDRVTITCRSSTGAVTTSNYANWVQQKPGKAPKALIGGTNKRAPG
VPSRFSGSLIGDKATLTISSLQPEDFATYYCALWYSNLWVFGQGTKVEIK;

SEQ ID No: 4 of US2016/0039934 (SEQ ID NO: 335):

DIQMTQSPSSLSASVGDRVTITCRSSTGAVTTSNYANWVQQKPGKAPKGLIGGTNKRAPG
VPSRFSGSLIGDKATLTISSLQPEDFATYYCALWYSNLWVFGQGTKVEIK;

In various embodiments, the targeting moieties of the invention may comprise a sequence that targets CD3 which is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to any of the sequences disclosed herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or 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%, about 99% or about 100% sequence identity with any of the sequences disclosed herein).

In various embodiments, the targeting moieties of the invention may comprise any combination of heavy chain, light chain, heavy chain variable region, light chain variable region, complementarity determining region (CDR),

and framework region sequences that target CD3 as disclosed herein. In various embodiments, the targeting moieties of the invention may comprise any heavy chain, light chain, heavy chain variable region, light chain variable region, complementarity determining region (CDR), and framework region sequences of the CD3-specific antibodies including, but not limited to, X35-3, VIT3, BMA030 (BW264/56), CLB-T3/3, CRIS7, YTH12.5, FI 11-409, CLB-T3.4.2, TR-66, WT32, SPv-T3b, 11D8, XIII-141, XIII-46, XIII-87, 12F6, T3/RW2-8C8, T3/RW2-4B6, OKT3D, M-T301, SMC2, WT31 and F101.01. These CD3-specific antibodies are well known in the art and, *inter alia*, described in Tunnacliffe (1989), Int. Immunol. 1, 546-550, the entire disclosures of which are hereby incorporated by reference.

Additional antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind or target CD3 are disclosed in US Patent Publication No. 2016/0000916, US Patent Nos. 4,361,549, 5,834,597, 6,491,916, 6,406,696, 6,143,297, 6,750,325 and International Publication No. WO 2004/052397, the entire disclosures of which are hereby incorporated by reference.

In some embodiments, the CD3 targeting moiety is a scFv. In some embodiments, the CD3 targeting moiety is:

QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLEWIGYIN
PSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYS
LDYWGQGTTLTVSSGSTGGGGSGGGGSGGGGSDIVLTQSPAIMSASPGEKVT
MTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSL
TISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINR (SEQ ID NO: 365).

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a T cell, for example, mediated by targeting to PD-1 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

By way of non-limiting example, in various embodiments, the present chimeric protein has (i) a targeting moiety directed against a B cell, for example, mediated by targeting to CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD37, CD38, CD39, CD40, CD70, CD72, CD73, CD74, CDw75, CDw76, CD77, CD78, CD79a/b, CD80, CD81, CD82, CD83, CD84, CD85, CD86, CD89, CD98, CD126, CD127, CDw130, CD138, or CDw150; and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a B cell, for example, mediated by targeting to CD19, CD20 or CD70 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a B cell, for example, mediated by targeting to CD20 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a

targeting moiety directed against CD20 on B cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In some embodiments, the CD20 targeting moiety is a scFv. In some embodiments, the CD20 targeting moiety is:

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNL
ASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIK
RGSTGGGGSGGGGSGGGGSQVQLQQPGAELVKPGASVKMSCKASGYTFTS
YNMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQ
LSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVSS (SEQ ID NO: 366).

By way of non-limiting example, in various embodiments, the present chimeric protein has (i) a targeting moiety directed against a NK cell, for example, mediated by targeting to 2B4/SLAMF4, KIR2DS4, CD155/PVR, KIR3DL1, CD94, LMIR1/CD300A, CD69, LMIR2/CD300c, CRACC/SLAMF7, LMIR3/CD300LF, DNAM-1, LMIR5/CD300LB, Fc-epsilon RII, LMIR6/CD300LE, Fc-γ RI/CD64, MICA, Fc-γ RIIB/CD32b, MICB, Fc-γ RIIC/CD32c, MULT-1, Fc-γ RIIA/CD32a, Nectin-2/CD112, Fc-γ RIII/CD16, NKG2A, FcRH1/IRTA5, NKG2C, FcRH2/IRTA4, NKG2D, FcRH4/IRTA1, NKp30, FcRH5/IRTA2, NKp44, Fc-Receptor-like 3/CD16-2, NKp46/NCR1, NKp80/KLRF1, NTB-A/SLAMF6, Rae-1, Rae-1 α, Rae-1 β, Rae-1 delta, H60, Rae-1 epsilon, ILT2/CD85j, Rae-1 γ, ILT3/CD85k, TREM-1, ILT4/CD85d, TREM-2, ILT5/CD85a, TREM-3, KIR/CD158, TREML1/TLT-1, KIR2DL1, ULBP-1, KIR2DL3, ULBP-2, KIR2DL4/CD158d, or ULBP-3; and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a NK cell, for example, mediated by targeting to Kir1alpha, DNAM-1 or CD64 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a NK cell, for example, mediated by targeting to KIR1 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against KIR1 on NK cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a NK cell, for example, mediated by targeting to TIGIT or KIR1 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against TIGIT on NK cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

By way of non-limiting example, in various embodiments, the present chimeric protein has (i) a targeting moiety directed against a dendritic cell, for example, mediated by targeting to CLEC-9A, XCR1, RANK, CD36/SRB3,

LOX-1/SR-E1, CD68, MARCO, CD163, SR-A1/MSR, CD5L, SREC-1, CL-PI/COLEC12, SREC-II, LIMPIIISRB2, RP105, TLR4, TLR1, TLR5, TLR2, TLR6, TLR3, TLR9, 4-IBB Ligand/TNFSF9, IL-12/IL-23 p40, 4-Amino-1,8-naphthalimide, ILT2/CD85j, CCL21/6Ckine, ILT3/CD85k, 8-oxo-dG, ILT4/CD85d, 8D6A, ILT5/CD85a, A2B5, lutegrin α 4/CD49d, Aag, Integrin β 2/CD18, AMICA, Langerin, B7-2/CD86, Leukotriene B4 Rl, B7-H3, LMIR1/CD300A, BLAME/SLAMF8, LMIR2/CD300c, Clq R1/CD93, LMIR3/CD300LF, CCR6, LMIR5/CD300LB CCR7, LMIR6/CD300LE, CD40/TNFRSF5, MAG/Siglec-4-a, CD43, MCAM, CD45, MD-1, CD68, MD-2, CD83, MDL-1/CLEC5A, CD84/SLAMF5, MMR, CD97, NCAMLl, CD2F-10/SLAMF9, Osteoactivin GPNMB, Chern 23, PD-L2, CLEC-1, RP105, CLEC-2, Siglec-2/CD22, CRACC/SLAMF7, Siglec-3/CD33, DC-SIGN, Siglec-5, DC-SIGNR/CD299, Siglec-6, DCAR, Siglec-7, DCIR/CLEC4A, Siglec-9, DEC-205, Siglec-10, Dectin-1/CLEC7A, Siglec-F, Dectin-2/CLEC6A, SIGNR1/CD209, DEP-1/CD148, SIGNR4, DLEC, SLAM, EMMPRIN/CD147, TCCR/WSX-1, Fc-γ R1/CD64, TLR3, Fc-γ RIIB/CD32b, TREM-1, Fc-γ RIIC/CD32c, TREM-2, Fc-γ RIIA/CD32a, TREM-3, Fc-γ RIII/CD16, TREML1/TLT-1, ICAM-2/CD102, or Vanilloid R1; and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a dendritic cell, for example, mediated by targeting to CLEC-9A, DC-SIGN, CD64, CLEC4A, or DEC205 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against CLEC9A on dendritic cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a dendritic cell, for example, mediated by targeting to CLEC9A and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against CLEC9A on dendritic cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a dendritic cell, for example, mediated by targeting to XCR1 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against XCR1 on dendritic cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a dendritic cell, for example, mediated by targeting to RANK and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against RANK on dendritic cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

By way of non-limiting example, in various embodiments, the present chimeric protein has (i) a targeting moiety directed against a monocyte/macrophage, for example, mediated by targeting to SIRP1a, B7-1/CD80,

ILT4/CD85d, B7-H1, ILT5/CD85a, Conmmon β Chain, Integrin α 4/CD49d, BLAME/SLAMF8, Integrin α X/CDIIc, CCL6/C10, Integrin β 2/CD18, CD155/PVR, Integrin β 3/CD61, CD31/PECAM-1, Latexin, CD36/SR-B3, Leukotriene B4 R1, CD40/TNFRSF5, LIMPIIISR-B2, CD43, LMIR1/CD300A, CD45, LMIR2/CD300c, CD68, LMIR3/CD300LF, CD84/SLAMF5, LMIR5/CD300LB, CD97, LMIR6/CD300LE, CD163, LRP-1, CD2F-10/SLAMF9, MARCO, CRACC/SLAMF7, MD-1, ECF-L, MD-2, EMMPRIN/CD147, MGL2, Endoglin/CD105, Osteoactivin/GPNMB, Fc-γ RI/CD64, Osteopontin, Fc-γ RIIB/CD32b, PD-L2, Fc-γ RIIC/CD32c, Siglec-3/CD33, Fc-γ RIIA/CD32a, SIGNR1/CD209, Fc-γ RIII/CD16, SLAM, GM-CSF R α, TCCR/WSX-1, ICAM-2/CD102, TLR3, IFN-γ Rl, TLR4, IFN-gannna R2, TREM-l, IL-l RII, TREM-2, ILT2/CD85j, TREM-3, ILT3/CD85k, TREML1/TLT-1, 2B4/SLAMF 4, IL-10 R α, ALCAM, IL-10 R β, AminopeptidaseN/ANPEP, ILT2/CD85j, Common β Chain, ILT3/CD85k, Clq R1/CD93, ILT4/CD85d, CCR1, ILT5/CD85a, CCR2, CD206, Integrin α 4/CD49d, CCR5, Integrin α M/CDll b, CCR8, Integrin α X/CDIIc, CD155/PVR, Integrin β 2/CD18, CD14, Integrin β 3/CD61, CD36/SR-B3, LAIR1, CD43, LAIR2, CD45, Leukotriene B4-R1, CD68, LIMPIIISR-B2, CD84/SLAMF5, LMIR1/CD300A, CD97, LMIR2/CD300c, CD163, LMIR3/CD300LF, Coagulation Factor III/Tissue Factor, LMIR5/CD300LB, CX3CR1, CX3CL1, LMIR6/CD300LE, CXCR4, LRP-1, CXCR6, M-CSF R, DEP-1/CD148, MD-1, DNAM-1, MD-2, EMMPRIN/CD147, MMR, Endoglin/CD105, NCAM-L1, Fc-γ RI/CD64, PSGL-1, Fc-γ RIIIICD16, RP105, G-CSF R, L-Selectin, GM-CSF R α, Siglec-3/CD33, HVEM/TNFRSF14, SLAM, ICAM-1/CD54, TCCR/WSX-1, ICAM-2/CD102, TREM-l, IL-6 R, TREM-2, CXCRl/IL-8 RA, TREM-3, or TREMLl/TLT-1; and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a monocyte/macrophage, for example, mediated by targeting to B7-H1, CD31/PECAM-1, CD163, CCR2, or Macrophage Mannose Receptor CD206 and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a monocyte/macrophage, for example, mediated by targeting to SIRP1a and (ii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against SIRP1a on macrophage cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In various embodiments, the present chimeric protein has one or more targeting moieties directed against a checkpoint marker, *e.g.* one or more of PD-1/PD-L1 or PD-L2, CD28/CD80 or CD86, CTLA4/ CD80 or CD86, ICOS/ICOSL or B7RP1, BTLA/HVEM, KIR, LAG3, CD137/CD137L, OX40/OX40L, CD27, CD40L, TIM3/Gal9, and A2aR. In one embodiment, the present chimeric protein has (i) a targeting moiety directed against a checkpoint marker on a T cell, for example, PD-1 and (ii) a targeting moiety directed against a tumor cell, for example, PD-L1 or PD-L2, along with any of the modified (*e.g.* mutant) signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against PD-1 on T cells and a second targeting moiety directed against PD-L1 on tumor cells. In another embodiment, the present chimeric protein has

a targeting moiety directed against PD-1 on T cells and a second targeting moiety directed against PD-L2 on tumor cells.

In some embodiments, the present chimeric protein comprises two or more targeting moieties directed to the same or different immune cells. In some embodiments, the present chimeric protein has (i) one or more targeting moieties directed against an immune cell selected from a T cell, a B cell, a dendritic cell, a macrophage, a NK cell, or subsets thereof and (ii) one or more targeting moieties directed against either the same or another immune cell selected from a T cell, a B cell, a dendritic cell, a macrophage, a NK cell, or subsets thereof, along with any of the modified (*e.g.* mutant) signaling agents described herein (*e.g.*, modified IFN-β).

In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a T cell and one or more targeting moieties directed against the same or another T cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a T cell and one or more targeting moieties directed against a B cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a T cell and one or more targeting moieties directed against a dendritic cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties against a T cell and one or more targeting moieties directed against a macrophage. In one embodiment, the present chimeric protein comprises one or more targeting moieties against a T cell and one or more targeting moieties directed against a NK cell. For example, in an illustrative embodiment, the chimeric protein may include a targeting moiety against CD8 and a targeting moiety against Clec9A. In another illustrative embodiment, the chimeric protein may include a targeting moiety against CD8 and a targeting moiety against CD3. In another illustrative embodiment, the chimeric protein may include a targeting moiety against CD8 and a targeting moiety against PD-1.

In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a B cell and one or more targeting moieties directed against the same or another B cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a B cell and one or more targeting moieties directed against a T cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a B cell and one or more targeting moieties directed against a dendritic cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties against a B cell and one or more targeting moieties directed against a macrophage. In one embodiment, the present chimeric protein comprises one or more targeting moieties against a B cell and one or more targeting moieties directed against a NK cell.

In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a dendritic cell and one or more targeting moieties directed against the same or another dendritic cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a dendritic cell and one or more targeting moieties directed against a T cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a dendritic cell and one or more targeting moieties directed against a B cell. In one embodiment, the present chimeric protein comprises one or more

targeting moieties against a dendritic cell and one or more targeting moieties directed against a macrophage. In one embodiment, the present chimeric protein comprises one or more targeting moieties against a dendritic cell and one or more targeting moieties directed against a NK cell.

In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a macrophage and one or more targeting moieties directed against the same or another macrophage. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a macrophage and one or more targeting moieties directed against a T cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against a macrophage and one or more targeting moieties directed against a B cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties against a macrophage and one or more targeting moieties directed against a dendritic cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties against a macrophage and one or more targeting moieties directed against a NK cell.

In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against an NK cell and one or more targeting moieties directed against the same or another NK cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against an NK cell and one or more targeting moieties directed against a T cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties directed against an NK cell and one or more targeting moieties directed against a B cell. In one embodiment, the present chimeric protein comprises one or more targeting moieties against an NK cell and one or more targeting moieties directed against a macrophage. In one embodiment, the present chimeric protein comprises one or more targeting moieties against an NK cell and one or more targeting moieties directed against a dendritic cell.

In one embodiment, the present chimeric protein comprises a targeting moiety directed against a tumor cell and a second targeting moiety directed against the same or a different tumor cell. In such embodiments, the targeting moieties may bind to any of the tumor antigens described herein.

In various embodiments, the targeting moiety of the invention may comprise a sequence which has one or more amino acid mutations with respect to any one of the sequences disclosed herein. In various embodiments, the targeting moiety comprises a sequence that has about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 or more amino acid mutations with respect to any one of the amino acid sequences of targeting moieties disclosed herein. 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).

In various embodiments, the amino acid mutation may be in the CDRs of the targeting moiety (*e.g.*, the CDR1, CDR2 or CDR3 regions). In another embodiment, amino acid alteration may be in the framework regions (FRs) of the targeting moiety (*e.g.*, the FR1, FR2, FR3, or FR4 regions).

Modification of the amino acid sequences may be achieved using any known technique in the art *e.g.*, site-directed mutagenesis or PCR based mutagenesis. Such techniques are described, for example, in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., 1989 and Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., 1989.

Targeting Moiety Formats

In various embodiments, the targeting moiety of the present chimeric protein is a protein-based agent capable of specific binding, such as an antibody or derivatives thereof. In an embodiment, the targeting moiety comprises an antibody. In various embodiments, the antibody is a full-length multimeric protein that includes two heavy chains and two light chains. Each heavy chain includes one variable region (*e.g.*, V_H) and at least three constant regions (*e.g.*, CH_1, CH_2 and CH_3), and each light chain includes one variable region (V_L) and one constant region (C_L). The variable regions determine the specificity of the antibody. Each variable region comprises three hypervariable regions also known as complementarity determining regions (CDRs) flanked by four relatively conserved framework regions (FRs). The three CDRs, referred to as CDR1, CDR2, and CDR3, contribute to the antibody binding specificity. In some embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is a humanized antibody.

In some embodiments, the targeting moiety comprises antibody derivatives or formats. In some embodiments, the targeting moiety of the present chimeric protein is 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; a Microbody; a peptide 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; Affimers, a DuoBody, a Fv, a Fab, a Fab', a $F(ab')_2$, a peptide mimetic molecule, or a synthetic molecule, as described in US Patent Nos. or Patent Publication Nos. US 7,417,130, US 2004/132094, US 5,831,012, US 2004/023334, US 7,250,297, US 6,818,418, US 2004/209243, US 7,838,629, US 7,186,524, US 6,004,746, US 5,475,096, US 2004/146938, US 2004/157209, US 6,994,982, US 6,794,144, US 2010/239633, US 7,803,907, US 2010/119446, and/or US 7,166,697, the contents of which are hereby incorporated by reference in their entireties. *See* also, Storz MAbs. 2011 May-Jun; 3(3): 310–317.

In one embodiment, the targeting moiety comprises a single-domain antibody, such as VHH from, for example, an organism that produces VHH antibody such as a camelid, a shark, or a designed VHH. VHHs are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. VHH technology is based on fully functional antibodies from camelids that lack light chains. These heavy-chain antibodies contain a single variable domain (VHH) and two constant domains (CH2 and CH3). VHHs are commercially available under the trademark of NANOBODIES.

In some embodiments, the VHH is a humanized VHH or camelized VHH.

In various embodiments, the targeting moiety of the present chimeric protein is a protein-based agent capable of specific binding to a cell receptor, such as a natural ligand for the cell receptor. In various embodiments, the cell receptor is found on one or more immune cells, which can include, without limitation, 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, dendritic cells, or subsets thereof. In some embodiments, the cell receptor is found on megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils, or subsets thereof.

In some embodiments, the targeting moiety is a natural ligand such as a chemokine. Exemplary chemokines that may be included in the chimeric protein of the invention include, but are not limited to, CCL1, CCL2, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9, CCL10, CCL11, CCL12, CCL13, CCL14, CCL15, CCL16, CL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CLL25, CCL26, CCL27, CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL15, CXCL16, CXCL17, XCL1, XCL2, CX3CL1, HCC-4, and LDGF-PBP. In an illustrative embodiment, the targeting moiety may be XCL1 which is a chemokine that recognizes and binds to the dendritic cell receptor XCR1. In another illustrative embodiment, the targeting moiety is CCL1, which is a chemokine that recognizes and binds to CCR8. In another illustrative embodiment, the targeting moiety is CCL2, which is a chemokine that recognizes and binds

to CCR2 or CCR9. In another illustrative embodiment, the targeting moiety is CCL3, which is a chemokine that recognizes and binds to CCR1, CCR5, or CCR9. In another illustrative embodiment, the targeting moiety is CCL4, which is a chemokine that recognizes and binds to CCR1 or CCR5 or CCR9. In another illustrative embodiment, the targeting moiety is CCL5, which is a chemokine that recognizes and binds to CCR1 or CCR3 or CCR4 or CCR5. In another illustrative embodiment, the targeting moiety is CCL6, which is a chemokine that recognizes and binds to CCR1. In another illustrative embodiment, the targeting moiety is CCL7, which is a chemokine that recognizes and binds to CCR2 or CCR9. In another illustrative embodiment, the targeting moiety is CCL8, which is a chemokine that recognizes and binds to CCR1 or CCR2 or CCR2B or CCR5 or CCR9. In another illustrative embodiment, the targeting moiety is CCL9, which is a chemokine that recognizes and binds to CCR1. In another illustrative embodiment, the targeting moiety is CCL10, which is a chemokine that recognizes and binds to CCR1. In another illustrative embodiment, the targeting moiety is CCL11, which is a chemokine that recognizes and binds to CCR2 or CCR3 or CCR5 or CCR9. In another illustrative embodiment, the targeting moiety is CCL13, which is a chemokine that recognizes and binds to CCR2 or CCR3 or CCR5 or CCR9. In another illustrative embodiment, the targeting moiety is CCL14, which is a chemokine that recognizes and binds to CCR1 or CCR9. In another illustrative embodiment, the targeting moiety is CCL15, which is a chemokine that recognizes and binds to CCR1 or CCR3. In another illustrative embodiment, the targeting moiety is CCL16, which is a chemokine that recognizes and binds to CCR1, CCR2, CCR5, or CCR8. In another illustrative embodiment, the targeting moiety is CCL17, which is a chemokine that recognizes and binds to CCR4. In another illustrative embodiment, the targeting moiety is CCL19, which is a chemokine that recognizes and binds to CCR7. In another illustrative embodiment, the targeting moiety is CCL20, which is a chemokine that recognizes and binds to CCR6. In another illustrative embodiment, the targeting moiety is CCL21, which is a chemokine that recognizes and binds to CCR7. In another illustrative embodiment, the targeting moiety is CCL22, which is a chemokine that recognizes and binds to CCR4. In another illustrative embodiment, the targeting moiety is CCL23, which is a chemokine that recognizes and binds to CCR1. In another illustrative embodiment, the targeting moiety is CCL24, which is a chemokine that recognizes and binds to CCR3. In another illustrative embodiment, the targeting moiety is CCL25, which is a chemokine that recognizes and binds to CCR9. In another illustrative embodiment, the targeting moiety is CCL26, which is a chemokine that recognizes and binds to CCR3. In another illustrative embodiment, the targeting moiety is CCL27, which is a chemokine that recognizes and binds to CCR10. In another illustrative embodiment, the targeting moiety is CCL28, which is a chemokine that recognizes and binds to CCR3 or CCR10. In another illustrative embodiment, the targeting moiety is CXCL1, which is a chemokine that recognizes and binds to CXCR1 or CXCR2. In another illustrative embodiment, the targeting moiety is CXCL2, which is a chemokine that recognizes and binds to CXCR2. In another illustrative embodiment, the targeting moiety is CXCL3, which is a chemokine that recognizes and binds to CXCR2. In another illustrative embodiment, the targeting moiety is CXCL4, which is a chemokine that recognizes and binds to CXCR3B. In another illustrative embodiment, the targeting moiety is CXCL5, which is a chemokine that recognizes and binds to CXCR2. In another illustrative embodiment, the targeting moiety is CXCL6, which is a chemokine that recognizes and binds to CXCR1 or CXCR2. In another illustrative

embodiment, the targeting moiety is CXCL8, which is a chemokine that recognizes and binds to CXCR1 or CXCR2. In another illustrative embodiment, the targeting moiety is CXCL9, which is a chemokine that recognizes and binds to CXCR3. In another illustrative embodiment, the targeting moiety is CXCL10, which is a chemokine that recognizes and binds to CXCR3. In another illustrative embodiment, the targeting moiety is CXCL11, which is a chemokine that recognizes and binds to CXCR3 or CXCR7. In another illustrative embodiment, the targeting moiety is CXCL12, which is a chemokine that recognizes and binds to CXCR4 or CXCR7. In another illustrative embodiment, the targeting moiety is CXCL13, which is a chemokine that recognizes and binds to CXCR5. In another illustrative embodiment, the targeting moiety is CXCL16, which is a chemokine that recognizes and binds to CXCR6. In another illustrative embodiment, the targeting moiety is LDGF-PBP, which is a chemokine that recognizes and binds to CXCR2. In another illustrative embodiment, the targeting moiety is XCL2, which is a chemokine that recognizes and binds to XCR1. In another illustrative embodiment, the targeting moiety is CX3CL1, which is a chemokine that recognizes and binds to CX3CR1.

In various embodiments, the present chimeric protein comprises targeting moieties in various combinations. In an illustrative embodiment, the present chimeric protein may comprise two targeting moieties, wherein both targeting moieties are antibodies or derivatives thereof. In another illustrative embodiment, the present chimeric protein may comprise two targeting moieties, wherein both targeting moieties are natural ligands for cell receptors. In a further illustrative embodiment, the present chimeric protein may comprise two targeting moieties, wherein one of the targeting moieties is an antibody or derivative thereof, and the other targeting moiety is a natural ligand for a cell receptor.

In various embodiments, the recognition domain of the present chimeric protein functionally modulates (by way of non-limitation, partially or completely neutralizes) the target (*e.g.* antigen, receptor) of interest, *e.g.* substantially inhibiting, reducing, or neutralizing a biological effect that the antigen has. For example, various recognition domains may be directed against one or more tumor antigens that are actively suppressing, or have the capacity to suppress, the immune system of, for example, a patient bearing a tumor. For example, in some embodiments, the present chimeric protein functionally modulates immune inhibitory signals (*e.g.* checkpoint inhibitors), for example, one or more of TIM-3, BTLA, PD-1, CTLA-4, B7-H4, GITR, galectin-9, HVEM, PD-L1, PD-L2, B7-H3, CD244, CD160, TIGIT, SIRPα, ICOS, CD172a, and TMIGD2. For example, in some embodiments, the present chimeric protein is engineered to disrupt, block, reduce, and/or inhibit the transmission of an immune inhibitory signal, by way of non-limiting example, the binding of PD-1 with PD-L1 or PD-L2 and/or the binding of CTLA-4 with one or more of AP2M1, CD80, CD86, SHP-2, and PPP2R5A.

In various embodiments, the recognition domain of the present chimeric protein binds but does not functionally modulate the target (*e.g.* antigen, receptor) of interest, *e.g.* the recognition domain is, or is akin to, a binding antibody. For instance, in various embodiments, the recognition domain simply targets the antigen or receptor but does not substantially inhibit, reduce or functionally modulate a biological effect that the antigen or receptor has. For example, some of the smaller antibody formats described above (*e.g.* as compared to, for example, full antibodies) have the ability to target hard to access epitopes and provide a larger spectrum of specific binding

locales. In various embodiments, the recognition domain binds an epitope that is physically separate from an antigen or receptor site that is important for its biological activity (*e.g.* the antigen's active site).

Such non-neutralizing binding finds use in various embodiments of the present invention, including methods in which the present chimeric protein is used to directly or indirectly recruit active immune cells to a site of need via an effector antigen, such as any of those described herein. For example, in various embodiments, the present chimeric protein may be used to directly or indirectly recruit cytotoxic T cells via CD8 to a tumor cell in a method of reducing or eliminating a tumor (*e.g.* the chimeric protein may comprise an anti-CD8 recognition domain and a recognition domain directed against a tumor antigen). In such embodiments, it is desirable to directly or indirectly recruit CD8-expressing cytotoxic T cells but not to functionally modulate the CD8 activity. On the contrary, in these embodiments, CD8 signaling is an important piece of the tumor reducing or eliminating effect. By way of further example, in various methods of reducing or eliminating tumors, the present chimeric protein is used to directly or indirectly recruit dendritic cells (DCs) via CLEC9A (*e.g.* the chimeric protein may comprise an anti-CLEC9A recognition domain and a recognition domain directed against a tumor antigen). In such embodiments, it is desirable to directly or indirectly recruit CLEC9A-expressing DCs but not to functionally modulate the CLEC9A activity. On the contrary, in these embodiments, CLEC9A signaling is an important piece of the tumor reducing or eliminating effect.

In various embodiments, the recognition domain of the present chimeric protein binds to XCR1 *e.g.* on dendritic cells. For instance, the recognition domain, in some embodiments comprises all or part of XCL1 or a non-neutralizing anti-XCR1 agent.

In various embodiments, the recognition domain of the present chimeric protein binds to an immune modulatory antigen (*e.g.* immune stimulatory or immune inhibitory). In various embodiments, the immune modulatory antigen is one or more of 4-1BB, OX-40, HVEM, GITR, CD27, CD28, CD30, CD40, ICOS ligand; OX-40 ligand, LIGHT (CD258), GITR ligand, CD70, B7-1, B7-2, CD30 ligand, CD40 ligand, ICOS, ICOS ligand, CD137 ligand and TL1A. In various embodiments, such immune stimulatory antigens are expressed on a tumor cell. In various embodiments, the recognition domain of the present chimeric protein binds but does not functionally modulate such immune stimulatory antigens and therefore allows recruitment of cells expressing these antigens without the reduction or loss of their potential tumor reducing or eliminating capacity.

In various embodiments, the recognition domain of the present chimeric protein may be in the context of chimeric protein that comprises two recognition domains that have neutralizing activity, or comprises two recognition domains that have non-neutralizing (*e.g.* binding) activity, or comprises one recognition domain that has neutralizing activity and one recognition domain that has non-neutralizing (*e.g.* binding) activity.

Signaling Agents

In one aspect, the present invention provides a chimeric protein that includes a signaling agent (for instance, an immune-modulating agent). In various embodiments, the signaling agent is modified to have reduced affinity or

activity for one or more of its receptors, which allows for attenuation of activity (inclusive of agonism or antagonism) and/or prevents non-specific signaling or undesirable sequestration of the chimeric protein.

In various embodiments, the signaling agent is antagonistic in its wild type form and bears one or more mutations that attenuate its antagonistic activity. In various embodiments, the signaling agent is antagonistic due to one or more mutations, *e.g.* an agonistic signaling agent is converted to an antagonistic signaling agent and, such a converted signaling agent, optionally, also bears one or more mutations that attenuate its antagonistic activity (*e.g.* as described in WO 2015/007520, the entire contents of which are hereby incorporated by reference).

In various embodiments, the signaling agent is selected from modified versions of cytokines, growth factors, and hormones. Illustrative examples of such cytokines, growth factors, and hormones include, but are not limited to, lymphokines, monokines, traditional polypeptide hormones, such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and tumor necrosis factor-β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-α; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and –II ; osteo inductive factors; interferons such as, for example, interferon-α, interferon-β and interferon-γ (and interferon type I, II, and III), colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as, for example, IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, and IL-18; a tumor necrosis factor such as, for example, TNF-α or TNF-β; and other polypeptide factors including, for example, LIF and kit ligand (KL). As used herein, cytokines, growth factors, and hormones include proteins obtained from natural sources or produced from recombinant bacterial, eukaryotic or mammalian cell culture systems and biologically active equivalents of the native sequence cytokines.

In some embodiments, the signaling agent is a modified version of a growth factor selected from, but not limited to, transforming growth factors (TGFs) such as TGF-α and TGF-β, epidermal growth factor (EGF), insulin-like growth factor such as insulin-like growth factor-I and -II, fibroblast growth factor (FGF), heregulin, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF).

In an embodiment, the growth factor is a modified version of a fibroblast growth factor (FGF). Illustrative FGFs include, but are not limited to, FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF8, FGF9, FGF10, FGF11, FGF12, FGF13, FGF14, murine FGF15, FGF16, FGF17, FGF18, FGF19, FGF20, FGF21, FGF22, and FGF23.

In an embodiment, the growth factor is a modified version of a vascular endothelial growth factor (VEGF). Illustrative VEGFs include, but are not limited to, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PGF and isoforms thereof including the various isoforms of VEGF-A such as $VEGF_{121}$, $VEGF_{121}b$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165}b$, $VEGF_{189}$, and $VEGF_{206}$.

In an embodiment, the growth factor is a modified version of a transforming growth factor (TGF). Illustrative TGFs include, but are not limited to, TGF-α and TGF-β and subtypes thereof including the various subtypes of TGF-β including TGFβ1, TGFβ2, and TGFβ3.

In some embodiments, the signaling agent is a modified version of a hormone selected from, but not limited to, human chorionic gonadotropin, gonadotropin releasing hormone, an androgen, an estrogen, thyroid-stimulating hormone, follicle-stimulating hormone, luteinizing hormone, prolactin, growth hormone, adrenocorticotropic hormone, antidiuretic hormone, oxytocin, thyrotropin-releasing hormone, growth hormone releasing hormone, corticotropin-releasing hormone, somatostatin, dopamine, melatonin, thyroxine, calcitonin, parathyroid hormone, glucocorticoids, mineralocorticoids, adrenaline, noradrenaline, progesterone, insulin, glucagon, amylin, calcitriol, calciferol, atrial-natriuretic peptide, gastrin, secretin, cholecystokinin, neuropeptide Y, ghrelin, PYY3-36, insulin-like growth factor (IGF), leptin, thrombopoietin, erythropoietin (EPO), and angiotensinogen. In some embodiments, the signaling agent is EPO.

In some embodiments, the signaling agent is an immune-modulating agent, *e.g.* one or more of an interleukin, interferon, and tumor necrosis factor.

In some embodiments, the signaling agent is an interleukin or a modified interleukin, including for example IL-1; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; IL-8; IL-9; IL-10; IL-11; IL-12; IL-13; IL-14; IL-15; IL-16; IL-17; IL-18; IL-19; IL-20; IL-21; IL-22; IL-23; IL-24; IL-25; IL-26; IL-27; IL-28; IL-29; IL-30; IL-31; IL-32; IL-33; IL-35; IL-36 or a fragment, variant, analogue, or family-member thereof. Interleukins are a group of multi- functional cytokines synthesized by lymphocytes, monocytes, and macrophages. Known functions include stimulating proliferation of immune cells (*e.g.*, T helper cells, B cells, eosinophils, and lymphocytes), chemotaxis of neutrophils and T lymphocytes, and/or inhibition of interferons. Interleukin activity can be determined using assays known in the art: Matthews *et al.*, in *Lymphokines and Interferens: A Practical Approach*, Clemens *et al.*, eds, IRL Press, Washington, D.C. 1987, pp. 221-225; and Orencole & Dinarello (1989) Cytokine 1, 14-20.

In some embodiments, the signaling agent is an interferon or a modified version of an interferon such as interferon types I, II, and III. Illustrative interferons, including for example, interferon-α-1, 2, 4, 5, 6, 7, 8, 10, 13, 14, 16, 17, and 21, interferon-β and interferon-γ, interferon κ, interferon ε, interferon τ, and interferon ϖ.

In some embodiments, the signaling agent is a tumor necrosis factor (TNF) or a modified version of a tumor necrosis factor (TNF) or a protein in the TNF family, including but not limited to, TNF-α, TNF-β, LT-β, CD40L, CD27L, CD30L, FASL, 4-1BBL, OX40L, and TRAIL.

In various embodiments, the signaling agent is a modified (*e.g.* mutant) form of the signaling agent having one or more mutations. In various embodiments, the mutations allow for the modified signaling agent to have one or more of attenuated activity such as one or more of reduced binding affinity, reduced endogenous activity, and reduced specific bioactivity relative to unmodified or unmutated, *i.e.* the wild type form of the signaling agent (*e.g.* comparing the same signaling agent in a wild type form versus a modified (*e.g.* mutant) form). In some embodiments, the mutations which attenuate or reduce binding or affinity include those mutations which

substantially reduce or ablate binding or activity. In some embodiments, the mutations which attenuate or reduce binding or affinity are different than those mutations which substantially reduce or ablate binding or activity. Consequentially, in various embodiments, the mutations allow for the signaling agent to have improved safety, *e.g.* have reduced systemic toxicity, reduced side effects, and reduced off-target effects relative to unmutated, *i.e.* wild type, signaling agent (*e.g.* comparing the same signaling agent in a wild type form versus a modified (*e.g.* mutant) form).

In various embodiments, the signaling agent is modified to have one or more mutations that reduce its binding affinity or activity for one or more of its receptors. In some embodiments, the signaling agent is modified to have one or more mutations that substantially reduce or ablate binding affinity or activity for the receptors. In some embodiments, the activity provided by the wild type signaling agent is agonism at the receptor (*e.g.* activation of a cellular effect at a site of therapy). For example, the wild type signaling agent may activate its receptor. In such embodiments, the mutations result in the modified signaling agent to have reduced or ablated activating activity at the receptor. For example, the mutations may result in the modified signaling agent to deliver a reduced activating signal to a target cell or the activating signal could be ablated. In some embodiments, the activity provided by the wild type signaling agent is antagonism at the receptor (*e.g.* blocking or dampening of a cellular effect at a site of therapy). For example, the wild type signaling agent may antagonize or inhibit the receptor. In these embodiments, the mutations result in the modified signaling agent to have a reduced or ablated antagonizing activity at the receptor. For example, the mutations may result in the modified signaling agent to deliver a reduced inhibitory signal to a target cell or the inhibitory signal could be ablated. In various embodiments, the signaling agent is antagonistic due to one or more mutations, *e.g.* an agonistic signaling agent is converted to an antagonistic signaling agent (*e.g.* as described in WO 2015/007520, the entire contents of which are hereby incorporated by reference) and, such a converted signaling agent, optionally, also bears one or more mutations that reduce its binding affinity or activity for one or more of its receptors or that substantially reduce or ablate binding affinity or activity for one or more of its receptors.

In some embodiments, the reduced affinity or activity at the receptor is restorable by attachment with one or more of the targeting moieties. In other embodiments, the reduced affinity or activity at the receptor is not substantially restorable by the activity of one or more of the targeting moieties.

In various embodiments, the chimeric proteins of the present invention reduce off-target effects because their signaling agents have mutations that weaken or ablate binding affinity or activity at a receptor. In various embodiments, this reduction in side effects is observed relative with, for example, the wild type signaling agents. In various embodiments, the signaling agent is active on target cells because the targeting moiety(ies) compensates for the missing/insufficient binding (*e.g.*, without limitation and/or avidity) required for substantial activation. In various embodiments, the modified signaling agent is substantially inactive *en route* to the site of therapeutic activity and has its effect substantially on specifically targeted cell types which greatly reduces undesired side effects.

In some embodiments, the signaling agent may include one or more mutations that attenuate or reduce binding or affinity for one receptor (*i.e.*, a therapeutic receptor) and one or more mutations that substantially reduce or ablate binding or activity at a second receptor. In such embodiments, these mutations may be at the same or at different positions (*i.e.*, the same mutation or multiple mutations). In some embodiments, the mutation(s) that reduce binding and/or activity at one receptor is different than the mutation(s) that substantially reduce or ablate at another receptor. In some embodiments, the mutation(s) that reduce binding and/or activity at one receptor is the same as the mutation(s) that substantially reduce or ablate at another receptor. In some embodiments, the present chimeric proteins have a modified signaling agent that has both mutations that attenuate binding and/or activity at a therapeutic receptor and therefore allow for a more controlled, on-target therapeutic effect (*e.g.* relative wild type signaling agent) and mutations that substantially reduce or ablate binding and/or activity at another receptor and therefore reduce side effects (*e.g.* relative to wild type signaling agent).

In some embodiments, the substantial reduction or ablation of binding or activity is not substantially restorable with a targeting moiety. In some embodiments, the substantial reduction or ablation of binding or activity is restorable with a targeting moiety. In various embodiments, substantially reducing or ablating binding or activity at a second receptor also may prevent deleterious effects that are mediated by the other receptor. Alternatively, or in addition, substantially reducing or ablating binding or activity at the other receptor causes the therapeutic effect to improve as there is a reduced or eliminated sequestration of the therapeutic chimeric proteins away from the site of therapeutic action. For instance, in some embodiments, this obviates the need of high doses of the present chimeric proteins that compensate for loss at the other receptor. Such ability to reduce dose further provides a lower likelihood of side effects.

In various embodiments, the modified signaling agent comprises one or more mutations that cause the signaling agent to have reduced, substantially reduced, or ablated affinity, *e.g.* binding (*e.g.* K_D) and/or activation (for instance, when the modified signaling agent is an agonist of its receptor, measurable as, for example, K_A and/or EC_{50}) and/or inhibition (for instance, when the modified signaling agent is an antagonist of its receptor, measurable as, for example, K_I and/or IC_{50}), for one or more of its receptors. In various embodiments, the reduced affinity at the immumodulating agent's receptor allows for attenuation of activity (inclusive of agonism or antagonism). In such embodiments, the modified signaling agent has about 1%, or about 3%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 10%-20%, about 20%-40%, about 50%, about 40%-60%, about 60%-80%, about 80%-100% of the affinity for the receptor relative to the wild type signaling agent. In some embodiments, the binding affinity is at least about 2-fold lower, about 3-fold lower, about 4-fold lower, about 5-fold lower, about 6-fold lower, about 7-fold lower, about 8-fold lower, about 9-fold lower, at least about 10-fold lower, at least about 15-fold lower, at least about 20-fold lower, at least about 25-fold lower, at least about 30-fold lower, at least about 35-fold lower, at least about 40-fold lower, at least about 45-fold lower, at least about 50-fold lower, at least about 100-fold lower, at least about 150-fold lower,

or about 10-50-fold lower, about 50-100-fold lower, about 100-150-fold lower, about 150-200-fold lower, or more than 200-fold lower relative to the wild type signaling agent.

In embodiments wherein the chimeric protein has mutations that reduce binding at one receptor and substantially reduce or ablate binding at a second receptor, the attenuation or reduction in binding affinity of a modified signaling agent for one receptor is less than the substantial reduction or ablation in affinity for the other receptor. In some embodiments, the attenuation or reduction in binding affinity of a modified signaling agent for one receptor is less than the substantial reduction or ablation in affinity for the other receptor by about 1%, or about 3%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In various embodiments, substantial reduction or ablation refers to a greater reduction in binding affinity and/or activity than attenuation or reduction.

In various embodiments, the modified signaling agent comprises one or more mutations that reduce the endogenous activity of the signaling agent to about 75%, or about 70%, or about 60%, or about 50%, or about 40%, or about 30%, or about 25%, or about 20%, or about 10%, or about 5%, or about 3%, or about 1%, *e.g.*, relative to the wild type signaling agent

In various embodiments, the modified signaling agent comprises one or more mutations that cause the signaling agent to have reduced affinity and/or activity for a receptor of any one of the cytokines, growth factors, and hormones as described herein.

In some embodiments, the modified signaling agent comprises one or more mutations that cause the signaling agent to have reduced affinity for its receptor that is lower than the binding affinity of the targeting moiety(ies) for its(their) receptor(s). In some embodiments, this binding affinity differential is between signaling agent/receptor and targeting moiety/receptor on the same cell. In some embodiments, this binding affinity differential allows for the signaling agent, *e.g.* mutated signaling agent, to have localized, on-target effects and to minimize off-target effects that underlie side effects that are observed with wild type signaling agent. In some embodiments, this binding affinity is at least about 2-fold, or at least about 5-fold, or at least about 10-fold, or at least about 15-fold lower, or at least about 25-fold, or at least about 50-fold lower, or at least about 100-fold, or at least about 150-fold.

Receptor binding activity may be measured using methods known in the art. For example, affinity and/or binding activity may be assessed by Scatchard plot analysis and computer-fitting of binding data (*e.g.* Scatchard, 1949) or by reflectometric interference spectroscopy under flow through conditions, as described by Brecht *et al.* (1993), the entire contents of all of which are hereby incorporated by reference.

The amino acid sequences of the wild type signaling agents described herein are well known in the art. Accordingly, in various embodiments the modified signaling agent comprises an amino acid sequence that has 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 wild type amino acid sequences of the signaling agents described herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or 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 99% sequence identity).

In various embodiments the modified signaling agent comprises an amino acid sequence that has 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 any of amino acid sequences of the signaling agents disclosed herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or 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 99% sequence identity).

In various embodiments, the modified signaling agent comprises an amino acid sequence having one or more amino acid mutations. 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 as described elsewhere herein.

As described herein, the modified signaling agents bear mutations that affect affinity and/or activity at one or more receptors. In various embodiments, there is reduced affinity and/or activity at a therapeutic receptor, *e.g.* a receptor through which a desired therapeutic effect is mediated (*e.g.* agonism or antagonism). In various embodiments, the modified signaling agents bear mutations that substantially reduce or ablate affinity and/or activity at a receptor, *e.g.* a receptor through which a desired therapeutic effect is not mediated (*e.g.* as the result of promiscuity of binding). The receptors of any modified signaling agents, *e.g.* one of the cytokines, growth factors, and hormones as described herein, are known in the art.

Illustrative mutations which provide reduced affinity and/or activity (*e.g.* agonistic) at a receptor are found in WO 2013/107791 (*e.g.* with regard to interferons), WO 2015/007542 (*e.g.* with regard to interleukins), and WO 2015/007903 (*e.g.* with regard to TNF), the entire contents of each of which are hereby incorporated by reference. Illustrative mutations which provide reduced affinity and/or activity (*e.g.* antagonistic) at a therapeutic receptor are found in WO 2015/007520, the entire contents of which are hereby incorporated by reference.

In some embodiments, the modified signaling agent comprises one or more mutations that cause the signaling agent to have reduced affinity and/or activity for a type I cytokine receptor, a type II cytokine receptor, a chemokine receptor, a receptor in the Tumor Necrosis Factor Receptor (TNFR) superfamily, TGF-beta Receptors, a receptor in the immunoglobulin (Ig) superfamily, and/or a receptor in the tyrosine kinase superfamily.

In various embodiments, the receptor for the signaling agent is a Type I cytokine receptor. Type I cytokine receptors are known in the art and include, but are not limited to receptors for IL2 (beta-subunit), IL3, IL4, IL5, IL6, IL7, IL9, IL11, IL12, GM-CSF, G-CSF, LIF, CNTF, and also the receptors for Thrombopoietin (TPO), Prolactin, and Growth hormone. Illustrative type I cytokine receptors include, but are not limited to, GM-CSF receptor, G-CSF receptor, LIF receptor, CNTF receptor, TPO receptor, and type I IL receptors.

In various embodiments, the receptor for the signaling agent is a Type II cytokine receptor. Type II cytokine receptors are multimeric receptors composed of heterologous subunits, and are receptors mainly for interferons. This family of receptors includes, but is not limited to, receptors for interferon-α, interferon-β and interferon-γ, IL10, IL22, and tissue factor. Illustrative type II cytokine receptors include, but are not limited to, IFN-α receptor (*e.g.* IFNAR1 and IFNAR2), IFN- β receptor, IFN- γ receptor (*e.g.* IFNGR1 and IFNGR2), and type II IL receptors.

In various embodiments, the receptor for the signaling agent is a G protein-coupled receptor. Chemokine receptors are G protein-coupled receptors with seven transmembrane structure and coupled to G-protein for signal transduction. Chemokine receptors include, but are not limited to, CC chemokine receptors, CXC chemokine receptors, CX3C chemokine receptors, and XC chemokine receptor (XCR1). Exemplary chemokine receptors include, but are not limited to, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR3B, CXCR4, CXCR5, CSCR6, CXCR7, XCR1, and CX3CR1.

In various embodiments, the receptor for the signaling agent is a TNFR family member. Tumor necrosis factor receptor (TNFR) family members share a cysteine-rich domain (CRD) formed of three disulfide bonds

surrounding a core motif of CXXCXXC creating an elongated molecule. Exemplary tumor necrosis factor receptor family members include: CDI 20a (TNFRSFIA), CD 120b (TNFRSFIB), Lymphotoxin beta receptor (LTBR, TNFRSF3), CD 134 (TNFRSF4), CD40 (CD40, TNFRSF5), FAS (FAS, TNFRSF6), TNFRSF6B (TNFRSF6B), CD27 (CD27, TNFRSF7), CD30 (TNFRSF8), CD137 (TNFRSF9), TNFRSFIOA (TNFRSFIOA), TNFRSFIOB, (TNFRSFIOB), TNFRSFIOC (TNFRSFIOC), TNFRSFIOD (TNFRSFIOD), RANK (TNFRSFI IA), Osteoprotegerin (TNFRSFI IB), TNFRSF12A (TNFRSF12A), TNFRSF13B (TNFRSF13B), TNFRSF13C (TNFRSF13C), TNFRSF14 (TNFRSF14), Nerve growth factor receptor (NGFR, TNFRSF16), TNFRSF17 (TNFRSF17), TNFRSF18 (TNFRSF18), TNFRSF19 (TNFRSF19), TNFRSF21 (TNFRSF21), and TNFRSF25 (TNFRSF25).

In various embodiments, the receptor for the signaling agent is a TGF-beta receptor. TGF-beta receptors are single pass serine/threonine kinase receptors. TGF-beta receptors include, but are not limited to, TGFBR1, TGFBR2, and TGFBR3.

In various embodiments, the receptor for the signaling agent is an Ig superfamily receptor. Receptors in the immunoglobulin (Ig) superfamily share structural homology with immunoglobulins. Receptors in the Ig superfamily include, but are not limited to, interleukin-1 receptors, CSF-1R, PDGFR (*e.g.* PDGFRA and PDGFRB), and SCFR.

In various embodiments, the receptor for the signaling agent is a tyrosine kinase superfamily receptor. Receptors in the tyrosine kinase superfamily are well known in the art. There are about 58 known receptor tyrosine kinases (RTKs), grouped into 20 subfamilies. Receptors in the tyrosine kinase superfamily include, but are not limited to, FGF receptors and their various isoforms such as FGFR1, FGFR2, FGFR3, FGFR4, and FGFR5.

In an embodiment, the modified signaling agent is interferon α. In such embodiments, the modified IFN-α agent has reduced affinity and/or activity for the IFN-α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains. In some embodiments, the modified IFN-α agent has substantially reduced or ablated affinity and/or activity for the IFN-α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains.

Mutant forms of interferon α are known to the person skilled in the art. In an illustrative embodiment, the modified signaling agent is the allelic form IFN-α2a having the amino acid sequence of:

IFN-α2a (SEQ ID NO: 336):

CDLPQTHSLGSRRTLMLLAQMRKISLFSCLKDRHDFGFPQEEFGNQFQKAETIPVLH
EMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLMKED
SILAVRKYFQRITLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE

In an illustrative embodiment, the modified signaling agent is the allelic form IFN-α2b having the amino acid sequence of (which differs from IFN-α2a at amino acid position 23):

IFN-α2b (SEQ ID NO: 337):

CDLPQTHSLGSRRTLMLLAQMRRISLFSCLKDRHDFGFPQEEFGNQFQKAETIPVLH
EMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLMKED
SILAVRKYFQRITLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE

In some embodiments, said IFN-α2 mutant (IFN-α2a or IFN-α2b) is mutated at one or more amino acids at positions 144-154, such as amino acid positions 148, 149 and/or 153. In some embodiments, the IFN-α2 mutant comprises one or more mutations selected from L153A, R149A, and M148A. Such mutants are described, for example, in WO2013/107791 and Piehler *et al.*, (2000) J. Biol. Chem, 275:40425-33, the entire contents of all of which are hereby incorporated by reference.

In some embodiments, the IFN-α2 mutants have reduced affinity and/or activity for IFNAR1. In some embodiments, the IFN-α2 mutant comprises one or more mutations selected from F64A, N65A, T69A, L80A, Y85A, and Y89A, as described in WO2010/030671, the entire contents of which is hereby incorporated by reference.

In some embodiments, the IFN-α2 mutant comprises one or more mutations selected from K133A, R144A, R149A, and L153A as described in WO2008/124086, the entire contents of which is hereby incorporated by reference.

In some embodiments, the IFN-α2 mutant comprises one or more mutations selected from R120E and R120E/K121E, as described in WO2015/007520 and WO2010/030671, the entire contents of which are hereby incorporated by reference. In such embodiments, said IFN-α2 mutant antagonizes wild type IFN-α2 activity. In such embodiments, said mutant IFN-α2 has reduced affinity and/or activity for IFNAR1 while affinity and/or activity of IFNR2 is retained.

In some embodiments, the human IFN-α2 mutant comprises (1) one or more mutations selected from R120E and R120E/K121E, which, without wishing to be bound by theory, create an antagonistic effect and (2) one or more mutations selected from K133A, R144A, R149A, and L153A, which, without wishing to be bound by theory, allow for an attenuated effect at, for example, IFNAR2. In an embodiment, the human IFN-α2 mutant comprises R120E and L153A.

In some embodiments, the human IFN-α2 mutant comprises one or more mutations selected from, L15A, A19W, R22A, R23A, L26A, F27A, L30A, L30V, K31A, D32A, R33K, R33A, R33Q, H34A, D35A, Q40A, D114R, L117A, R120A, R125A , K134A, R144A, A145G, A145M, M148A, R149A, S152A, L153A, and N156A as disclosed in WO 2013/059885, the entire disclosures of which are hereby incorporated by reference. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or L30A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or R33A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or M148A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or L153A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations N65A, L80A, Y85A,

and/or Y89A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations N65A, L80A, Y85A, Y89A, and/or D114A as disclosed in WO 2013/059885.

In an embodiment, the modified signaling agent is interferon β. In such embodiments, the modified interferon β agent has reduced affinity and/or activity for the IFN-α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains. In some embodiments, the modified interferon β agent has substantially reduced or ablated affinity and/or activity for the IFN-α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains.

In an embodiment, the modified signaling agent is interferon γ. In such embodiments, the modified interferon γ agent has reduced affinity and/or activity for the interferon-gamma receptor (IFNGR), *i.e.*, IFNGR1 and IFNGR2 chains. In some embodiments, the modified interferon γ agent has substantially reduced or ablated affinity and/or activity for the interferon-gamma receptor (IFNGR), *i.e.*, IFNGR1 and/or IFNGR2 chains.

In some embodiments, the modified signaling agent is vascular endothelial growth factor (VEGF). VEGF is a potent growth factor that plays major roles in physiological but also pathological angiogenesis, regulates vascular permeability and can act as a growth factor on cells expressing VEGF receptors. Additional functions include, among others, stimulation of cell migration in macrophage lineage and endothelial cells. Several members of the VEGF family of growth factors exist, as well as at least three receptors (VEGFR-1, VEGFR -2, and VEGFR -3). Members of the VEGF family can bind and activate more than one VEGFR type. For example, VEGF-A binds VEGFR-1 and -2, while VEGF-C can bind VEGFR-2 and -3. VEGFR-1 and -2 activation regulates angiogenesis while VEGFR-3 activation is associated with lymphangiogenesis. The major pro-angiogenic signal is generated from activation of VEGFR-2. VEGFR-1 activation has been reported to be possibly associated with negative role in angiogenesis. It has also been reported that VEGFR-1 signaling is important for progression of tumors *in vivo* via bone marrow-derived VEGFR-1 positive cells (contributing to formation of premetastatic niche in the bone). Several therapies based on VEGF-A directed/neutralizing therapeutic antibodies have been developed, primarily for use in treatment of various human tumors relying on angiogenesis. These are not without side effects though. This may not be surprising considering that these operate as general, non-cell/tissue specific VEGF/VEGFR interaction inhibitors. Hence, it would be desirable to restrict VEGF (*e.g.* VEGF-A)/VEGFR-2 inhibition to specific target cells (*e.g.* tumor vasculature endothelial cells).

In some embodiments, the VEGF is VEGF-A, VEGF-B, VEFG-C, VEGF-D, or VEGF-E and isoforms thereof including the various isoforms of VEGF-A such as $VEGF_{121}$, $VEGF_{121}b$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165}b$, $VEGF_{189}$, and $VEGF_{206}$. In some embodiments, the modified signaling agent has reduced affinity and/or activity for VEGFR-1 (Flt-1) and/or VEGFR-2 (KDR/Flk-1). In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for VEGFR-1 (Flt-1) and/or VEGFR-2 (KDR/Flk-1). In an embodiment, the modified signaling agent has reduced affinity and/or activity for VEGFR-2 (KDR/Flk-1) and/or substantially reduced or ablated affinity and/or activity for VEGFR-1 (Flt-1). Such an embodiment finds use, for example, in wound healing methods or treatment of ischmia-related diseases (without wishing to be bound by theory, mediated by VEGFR-2's effects on endothelial cell function and angiogenesis). In various embodiments,

binding to VEGFR-1 (Flt-1), which is linked to cancers and pro-inflammatory activities, is avoided. In various embodiments, VEGFR-1 (Flt-1) acts a decoy receptor and therefore substantially reduces or ablates affinity at this receptor avoids sequestration of the therapeutic agent. In an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for VEGFR-1 (Flt-1) and/or substantially reduced or ablated affinity and/or activity for VEGFR-2 (KDR/Flk-1). In some embodiments, the VEGF is VEGF-C or VEGF-D. In such embodiments, the modified signaling agent has reduced affinity and/or activity for VEGFR-3. Alternatively, the modified signaling agent has substantially reduced or ablated affinity and/or activity for VEGFR-3.

Proangiogenic therapies are also important in various diseases (*e.g.* ischemic heart disease, bleeding *etc.*), and include VEGF-based therapeutics. Activation of VEGFR-2 is proangiogenic (acting on endothelial cells). Activation of VEFGR-1 can cause stimulation of migration of inflammatory cells (including, for example, macrophages) and lead to inflammation associated hypervascular permeability. Activation of VEFGR-1 can also promote bone marrow associated tumor niche formation. Thus, VEGF based therapeutic selective for VEGFR-2 activation would be desirable in this case. In addition, cell specific targeting, *e.g.* to endothelial cells, would be desirable.

In some embodiments, the modified signaling agent has reduced affinity and/or activity (*e.g.* antagonistic) for VEGFR-2 and/or has substantially reduced or ablated affinity and/or activity for VEGFR-1. When targeted to tumor vasculature endothelial cells via a targeting moiety that binds to a tumor endothelial cell marker (*e.g.* PSMA and others), such construct inhibits VEGFR-2 activation specifically on such marker-positive cells, while not activating VEGFR-1 *en route* and on target cells (if activity ablated), thus eliminating induction of inflammatory responses, for example. This would provide a more selective and safe anti-angiogenic therapy for many tumor types as compared to VEGF-A neutralizing therapies.

In some embodiments, the modified signaling agent has reduced affinity and/or activity (*e.g.* agonistic) for VEGFR-2 and/or has substantially reduced or ablated affinity and/or activity for VEGFR-1. Through targeting to vascular endothelial cells, such construct, in some embodiments, promotes angiogenesis without causing VEGFR-1 associated induction of inflammatory responses. Hence, such a construct would have targeted proangiogenic effects with substantially reduced risk of side effects caused by systemic activation of VEGFR-2 as well as VEGR-1.

In an illustrative embodiment, the modified signaling agent is $VEGF_{165}$, which has the amino acid sequence:

VEGF 165 (wild type) (SEQ ID NO: 338)

APMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSC
VPLMRCGGCCNDEGLECVPTEESNITMQIMRIKPHQGQHIGEMSFLQHNK
CECRPKKDRARQENPCGPCSERRKHLFVQDPQTCKCSCKNTDSRCKAR
QLELNERTCRCDKPRR

In another illustrative embodiment, the modified signaling agent is $VEGF_{165b}$, which has the amino acid sequence:

VEGF 165b (wild type) (SEQ ID NO: 339)

APMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSC
VPLMRCGGCCNDEGLECVPTEESNITMQIMRIKPHQGQHIGEMSFLQHNK
CECRPKKDRARQENPCGPCSERRKHLFVQDPQTCKCSCKNTDSRCKAR
QLELNERTCRSLTRKD

In these embodiments, the modified signaling agent has a mutation at amino acid I83 (*e.g.*, a substitution mutation at I83, *e.g.*, I83K, I83R, or I83H). Without wishing to be bound by theory, it is believed that such mutations may result in reduced receptor binding affinity. See, for example, U.S. Patent No. 9,078,860, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is TNF-α. TNF is a pleiotropic cytokine with many diverse functions, including regulation of cell growth, differentiation, apoptosis, tumorigenesis, viral replication, autoimmunity, immune cell functions and trafficking, inflammation, and septic shock. It binds to two distinct membrane receptors on target cells: TNFR1 (p55) and TNFR2 (p75). TNFR1 exhibits a very broad expression pattern whereas TNFR2 is expressed preferentially on certain populations of lymphocytes, Tregs, endothelial cells, certain neurons, microglia, cardiac myocytes and mesenchymal stem cells. Very distinct biological pathways are activated in response to receptor activation, although there is also some overlap. As a general rule, without wishing to be bound by theory, TNFR1 signaling is associated with induction of apoptosis (cell death) and TNFR2 signaling is associated with activation of cell survival signals (*e.g.* activation of NFkB pathway). Administration of TNF is systemically toxic, and this is largely due to TNFR1 engagement. However, it should be noted that activation of TNFR2 is also associated with a broad range of activities and, as with TNFR1, in the context of developing TNF based therapeutics, control over TNF targeting and activity is important.

In some embodiments, the modified signaling agent has reduced affinity and/or activity for TNFR1 and/or TNFR2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for TNFR1 and/or TNFR2. TNFR1 is expressed in most tissues, and is involved in cell death signaling while, by contrast, TNFR2 is involved in cell survival signaling. Accordingly, in embodiments directed to methods of treating cancer, the modified signaling agent has reduced affinity and/or activity for TNFR1 and/or substantially reduced or ablated affinity and/or activity for TNFR2. In these embodiments, the chimeric proteins may be targeted to a cell for which apoptosis is desired, *e.g.* a tumor cell or a tumor vasculature endothelial cell. In embodiments directed to methods of promoting cell survival, for example, in neurogenesis for the treatment of neurodegenerative disorders, the modified signaling agent has reduced affinity and/or activity for TNFR2 and/or substantially reduced or ablated affinity and/or activity for TNFR1. Stated another way, the present chimeric proteins, in some embodiments, comprise modified TNF-α agent that allows of favoring either death or survival signals.

In some embodiments, the chimeric protein has a modified TNF having reduced affinity and/or activity for TNFR1 and/or substantially reduced or ablated affinity and/or activity for TNFR2. Such a chimera, in some embodiments, is a more potent inducer of apoptosis as compared to a wild type TNF and/or a chimera bearing only mutation(s) causing reduced affinity and/or activity for TNFR1. Such a chimera, in some embodiments, finds use in inducing tumor cell death or a tumor vasculature endothelial cell death (*e.g.* in the treatment of cancers). Also, in some embodiments, these chimeras avoid or reduce activation of T_{reg} cells via TNFR2, for example, thus further supporting TNFR1-mediated antitumor activity *in vivo*.

In some embodiments, the chimeric protein has a modified TNF having reduced affinity and/or activity for TNFR2 and/or substantially reduced or ablated affinity and/or activity for TNFR1. Such a chimera, in some embodiments, is a more potent activator of cell survival in some cell types, which may be a specific therapeutic objective in various disease settings, including without limitation, stimulation of neurogenesis. In addition, such a TNFR2-favoring chimeras also are useful in the treatment of autoimmune diseases (*e.g.* Crohn's, diabetes, MS, colitis *etc.* and many others described herein). In some embodiments, the chimera is targeted to auto-reactive T cells. In some embodiments, the chimera promotes T_{reg} cell activation and indirect suppression of cytotoxic T cells.

In some embodiments, the chimera causes the death of auto-reactive T cells, *e.g.* by activation of TNFR2 and/or avoidance of TNFR1 (*e.g.* a modified TNF having reduced affinity and/or activity for TNFR2 and/or substantially reduced or ablated affinity and/or activity for TNFR1). Without wishing to be bound by theory these auto-reactive T cells, have their apoptosis/survival signals altered *e.g.* by NFkB pathway activity/signaling alterations. In some embodiments, the chimera causes the death of autoreactive T cells having lesions or modifications in the NFkB pathway, which underlie an imbalance of their cell death (apoptosis)/survival signaling properties and, optionally, altered susceptibility to certain death-inducing signals (*e.g.*, TNFR2 activation).

In some embodiments, a TNFR2 based chimera has additional therapeutic applications in diseases, including various autoimmune diseases, heart disease, de-myelinating and neurodegenerative disorders, and infectious disease, among others.

In an embodiment, the wild type TNF-α has the amino acid sequence of:

TNF-α (SEQ ID NO: 340)

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLV
VPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQ
RETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYF
GIIAL

In such embodiments, the modified TNF-α agent has mutations at one or more amino acid positions 29, 31, 32, 84, 85, 86, 87, 88, 89, 145, 146 and 147 which produces a modified TNF-α with reduced receptor binding affinity. *See*, for example, U.S. Patent No. 7,993,636, the entire contents of which are hereby incorporated by reference.

In some embodiments, the modified human TNF-α moiety has mutations at one or more amino acid positions R32, N34, Q67, H73, L75, T77, S86, Y87, V91 , I97, T105, P106, A109, P113, Y115, E127, N137, D143, and A145, as described, for example, in WO/2015/007903, the entire contents of which is hereby incorporated by reference (numbering according to the human TNF sequence, Genbank accession number BAG70306, version BAG70306.1 GI: 197692685). In some embodiments, the modified human TNF-α moiety has substitution mutations selected from R32G, N34G, Q67G, H73G, L75G, L75A, L75S, T77A, S86G, Y87Q, Y87L, Y87A, Y87F, V91G, V91A, I97A, I97Q, I97S, T105G, P106G, A109Y, P113G, Y115G, Y115A, E127G, N137G, D143N, A145G and A145T. In an embodiment, the human TNF-α moiety has a mutation selected from Y87Q, Y87L, Y87A, and Y87F. In another embodiment, the human TNF-α moiety has a mutation selected from I97A, I97Q, and I97S. In a further embodiment, the human TNF-α moiety has a mutation selected from Y115A and Y115G.

In some embodiments, the modified TNF-α agent has one or more mutations selected from N39Y, S147Y, and Y87H, as described in WO2008/124086, the entire contents of which is hereby incorporated by reference.

In some embodiments, the modified human TNF-α moiety has mutations that provide receptor selectivity as described in PCT/IB2016/001668, the entire contents of which are hereby incorporated by reference. In some embodiments, the mutations to TNF are TNF-R1 selective. In some embodiments, the mutations to TNF which are TNF-R1 selective are at one or more of positions R32, S86, and E146. In some embodiments, the mutations to TNF which are TNF-R1 selective are one or more of R32W, S86T, and E146K. In some embodiments, the mutations to TNF which are TNF-R1 selective are one or more of R32W, R32W/S86T, R32W/E146K and E146K. In some embodiments, the mutations to TNF are TNF-R2 selective. In some embodiments, the mutations to TNF which are TNF-R2 selective are at one or more of positions A145, E146, and S147. In some embodiments, the mutations to TNF which are TNF-R2 selective are one or more of A145T, A145R, E146D, and S147D. In some embodiments, the mutations to TNF which are TNF-R2 selective are one or more of A145R, A145T/S147D, and A145T/E146D/S147D.

In an embodiment, the modified signaling agent is TNF-β. TNF-β can form a homotrimer or a heterotrimer with LT-β (LT-α1β2). In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for TNFR1 and/or TNFR2 and/or herpes virus entry mediator (HEVM) and/or LT-βR.

In an embodiment, the wild type TNF-β has the amino acid sequence of:

TNF-beta (SEQ ID NO: 341)

LPGVGLTPSAAQTARQHPKMHLAHSNLKPAAHLIGDPSKQNSLLWRANTD
RAFLQDGFSLSNNSLLVPTSGIYFVYSQVVFSGKAYSPKATSSPLYLAHEV
QLFSSQYPFHVPLLSSQKMVYPGLQEPWLHSMYHGAAFQLTQGDQLSTH
TDGIPHLVLSPSTVFFGAFAL

In such embodiments, the modified TNF-β agent may comprise mutations at one or more amino acids at positions 106-113, which produce a modified TNF-β with reduced receptor binding affinity to TNFR2. In an

embodiment, the modified signaling agent has one or more substitution mutations at amino acid positions 106-113. In illustrative embodiments, the substitution mutations are selected from Q107E, Q107D, S106E, S106D, Q107R, Q107N, Q107E/S106E, Q107E/S106D, Q107D/S106E, and Q107D/S106D. In another embodiment, the modified signaling agent has an insertion of about 1 to about 3 amino acids at positions 106-113.

In some embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which can be a single chain trimeric version as described in WO 2015/007903, the entire contents of which are incorporated by reference.

In some embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which has reduced affinity and/or activity, *i.e.* antagonistic activity (*e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) at TNFR1. In these embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which also, optionally, has substantially reduced or ablated affinity and/or activity for TNFR2. In some embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which has reduced affinity and/or activity, *i.e.* antagonistic activity (*e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) at TNFR2. In these embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which also, optionally, has substantially reduced or ablated affinity and/or activity for TNFR1. The constructs of such embodiments find use in, for example, methods of dampening TNF response in a cell specific manner. In some embodiments, the antagonistic TNF family member (*e.g.* TNF-alpha, TNF-beta) is a single chain trimeric version as described in WO 2015/007903.

In an embodiment, the modified signaling agent is TRAIL. In some embodiments, the modified TRAIL agent has reduced affinity and/or activity for DR4 (TRAIL-RI) and/or DR5 (TRAIL-RII) and/or DcR1 and/or DcR2. In some embodiments, the modified TRAIL agent has substantially reduced or ablated affinity and/or activity for DR4 (TRAIL-RI) and/or DR5 (TRAIL-RII) and/or DcR1 and/or DcR2.

In an embodiment, the wild type TRAIL has the amino acid sequence of:

TRAIL (SEQ ID NO: 342)

MAMMEVQGGPSLGQTCVLIVIFTVLLQSLCVAVTYVYFTNELKQMQDKYSK
SGIACFLKEDDSYWDPNDEESMNSPCWQVKWQLRQLVRKMILRTSEETIS
TVQEKQQNISPLVRERGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKIN
SWESSRSGHSFLSNLHLRNGELVIHEKGFYYIYSQTYFRFQEEIKENTKND
KQMVQYIYKYTSYPDPILLMKSARNSCWSKDAEYGLYSIYQGGIFELKEND
RIFVSVTNEHLIDMDHEASFFGAFLVG

In such embodiments, the modified TRAIL agent may comprise a mutation at amino acid positions T127-R132, E144-R149, E155-H161, Y189-Y209, T214-1220,K224-A226, W231, E236-L239, E249-K251, T261-H264 and

H270-E271 (Numbering based on the human sequence, Genbank accession number NP _003801, version 10 NP _003801.1, GI: 4507593; see above).

In an embodiment, the modified signaling agent is TGFα. In such embodiments, the modified TGFα agent has reduced affinity and/or activity for the epidermal growth factor receptor (EGFR). In some embodiments, the modified TGFα agent has substantially reduced or ablated affinity and/or activity for the epidermal growth factor receptor (EGFR).

In an embodiment, the modified signaling agent is TGFβ. In such embodiments, the modified signaling agent has reduced affinity and/or activity for TGFBR1 and/or TGFBR2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for TGFBR1 and/or TGFBR2. In some embodiments, the modified signaling agent optionally has reduced or substantially reduced or ablated affinity and/or activity for TGFBR3 which, without wishing to be bound by theory, may act as a reservoir of ligand for TGF-beta receptors. In some embodiments, the TGFβ may favor TGFBR1 over TGFBR2 or TGFBR2 over TGFBR1. Similarly, LAP, without wishing to be bound by theory, may act as a reservoir of ligand for TGF-beta receptors. In some embodiments, the modified signaling agent has reduced affinity and/or activity for TGFBR1 and/or TGFBR2 and/or substantially reduced or ablated affinity and/or activity for Latency Associated Peptide (LAP). In some embodiments, such chimeras find use in Camurati-Engelmann disease, or other diseases associated with inappropriate TGFβ signaling.

In some embodiments, the modified agent is a TGF family member (*e.g.* TGFα, TGFβ) which has reduced affinity and/or activity, *i.e.* antagonistic activity (*e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) at one or more of TGFBR1, TGFBR2, TGFBR3. In these embodiments, the modified agent is a TGF family member (*e.g.* TGFα, TGFβ) which also, optionally, has substantially reduced or ablated affinity and/or activity at one or more of TGFBR1, TGFBR2, TGFBR3.

In some embodiments, the modified agent is a TGF family member (*e.g.* TGFα, TGFβ) which has reduced affinity and/or activity, *i.e.* antagonistic activity (*e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) at TGFBR1 and/or TGFBR2. In these embodiments, the modified agent is a TGF family member (*e.g.* TGFα, TGFβ) which also, optionally, has substantially reduced or ablated affinity and/or activity at TGFBR3.

In an embodiment, the modified signaling agent is an interleukin. In an embodiment, the modified signaling agent is IL-1. In an embodiment, the modified signaling agent is IL-1α or IL-1β. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-1R1 and/or IL-1RAcP. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-1R1 and/or IL-1RAcP. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-1R2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-1R2.

For instance, in some embodiments, the present modified IL-1 agents avoid interaction at IL-1R2 and therefore substantially reduce its function as a decoy and/or sink for therapeutic agents.

In an embodiment, the wild type IL-1β has the amino acid sequence of:

IL-1 beta (mature form, wild type) (SEQ ID NO: 343)

APVRSLNCTLRDSQQKSLVMSGPYELKALHLQGQDMEQQVVFSMSFVQG
EESNDKIPVALGLKEKNLYLSCVLKDDKPTLQLESVDPKNYPKKKMEKRFV
FNKIEINNKLEFESAQFPNWYISTSQAENMPVFLGGTKGGQDITDFTMQFV
SS

IL1 is a proinflammatory cytokine and an important immune system regulator. It is a potent activator of CD4 T cell responses, increases proportion of Th17 cells and expansion of IFNγ and IL-4 producing cells. IL-1 is also a potent regulator of $CD8^+$ T cells, enhancing antigen-specific $CD8^+$ T cell expansion, differentiation, migration to periphery and memory. IL-1 receptors comprise IL-1R1 and IL-1R2. Binding to and signaling through the IL-1R1 constitutes the mechanism whereby IL-1 mediates many of its biological (and pathological) activities. IL1-R2 can function as a decoy receptor, thereby reducing IL-1 availability for interaction and signaling through the IL-1R1.

In some embodiments, the modified IL-1 has reduced affinity and/or activity (*e.g.* agonistic activity) for IL-1R1. In some embodiments, the modified IL-1 has substantially reduced or ablated affinity and/or activity for IL-1R2. In such embodiments, there is restorable IL-1/ IL-1R1 signaling and prevention of loss of therapeutic chimeras at IL-R2 and therefore a reduction in dose of IL-1 that is required (*e.g.* relative to wild type or a chimera bearing only an attenuation mutation for IL-R1). Such constructs find use in, for example, methods of treating cancer, including, for example, stimulating the immune system to mount an anti-cancer response.

In some embodiments, the modified IL-1 has reduced affinity and/or activity (*e.g.* antagonistic activity, *e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) for IL-1R1. In some embodiments, the modified IL-1 has substantially reduced or ablated affinity and/or activity for IL-1R2. In such embodiments, there is the IL-1/ IL-1R1 signaling is not restorable and prevention of loss of therapeutic chimeras at IL-R2 and therefore a reduction in dose of IL-1 that is required (*e.g.* relative to wild type or a chimera bearing only an attenuation mutation for IL-R1). Such constructs find use in, for example, methods of treating autoimmune diseases, including, for example, suppressing the immune system.

In such embodiments, the modified signaling agent has a deletion of amino acids 52-54 which produces a modified human IL-1β with reduced binding affinity for type I IL-1R and reduced biological activity. See, for example, WO 1994/000491, the entire contents of which are hereby incorporated by reference. In some embodiments, the modified human IL-1β has one or more substitution mutations selected from A117G/P118G, R120X, L122A, T125G/L126G, R127G, Q130X, Q131G, K132A, S137G/Q138Y, L145G, H146X, L145A/L147A, Q148X, Q148G/Q150G, Q150G/D151A, M152G, F162A, F162A/Q164E, F166A, Q164E/E167K, N169G/D170G,

I172A, V174A, K208E, K209X, K209A/K210A, K219X, E221X, E221 S/N224A, N224S/K225S, E244K, N245Q (where X can be any change in amino acid, *e.g.*, a non-conservative change), which exhibit reduced binding to IL-1R, as described, for example, in WO2015/007542 and WO/2015/007536, the entire contents of which is hereby incorporated by reference (numbering base on the human IL-1 β sequence, Genbank accession number NP_000567, version NP-000567.1 , GI: 10835145). In some embodiments, the modified human IL-1β may have one or more mutations selected from R120A, R120G, Q130A, Q130W, H146A, H146G, H146E, H146N, H146R, Q148E, Q148G, Q148L, K209A, K209D, K219S, K219Q, E221S and E221K. In an embodiment, the modified human IL-1β comprises the mutations Q131G and Q148G. In an embodiment, the modified human IL-1β comprises the mutations Q148G and K208E. In an embodiment, the modified human IL-1β comprises the mutations R120G and Q131G. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146A. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146N. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146R. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146E. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146G. In an embodiment, the modified human IL-1β comprises the mutations R120G and K208E. In an embodiment, the modified human IL-1β comprises the mutations R120G, F162A, and Q164E.

In an embodiment, the modified signaling agent is IL-2. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-2Rα and/or IL-2Rβ and/or IL-2Rγ. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-2Rβ and/or IL-2Rγ. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-2Rα. Such embodiments may be relevant for treatment of cancer, for instance when the modified IL-2 is agonistic at IL-2Rβ and/or IL-2Rγ. For instance, the present constructs may favor attenuated activation of $CD8^+$ T cells (which can provide an anti-tumor effect), which have IL2 receptors β and γ and disfavor T_{regs} (which can provide an immune suppressive, pro-tumor effect), which have IL2 receptors α, β, and γ. Further, in some embodiments, the preferences for IL-2Rβ and/or IL-2Rγ over IL-2Rα avoid IL-2 side effects such as pulmonary edema. Also, IL-2-based chimeras are useful for the treatment of autoimmune diseases, for instance when the modified IL-2 is antagonistic (*e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) at IL-2Rβ and/or IL-2Rγ. For instance, the present constructs may favor attenuated suppression of $CD8^+$ T cells (and therefore dampen the immune response), which have IL2 receptors β and γ and disfavor T_{regs} which have IL2 receptors α, β, and γ. Alternatively, in some embodiments, the chimeras bearing IL-2 favor the activation of T_{regs}, and therefore immune suppression, and activation of disfavor of $CD8^+$ T cells. For instance, these constructs find use in the treatment of diseases or diseases that would benefit from immune suppression, *e.g.* autoimmune disorders.

In some embodiments, the chimeric protein has targeting moieties as described herein directed to $CD8^+$ T cells as well as a modified IL-2 agent having reduced affinity and/or activity for IL-2Rβ and/or IL-2Rγ and/or substantially reduced or ablated affinity and/or activity for IL-2Rα. In some embodiments, these constructs

provide targeted $CD8^+$ T cell activity and are generally inactive (or have substantially reduced activity) towards T_{reg} cells. In some embodiments, such constructs have enhanced immune stimulatory effect compared to wild type IL-2 (*e.g.*, without wishing to be bound by theory, by not stimulating Tregs), whilst eliminating or reducing the systemic toxicity associated with IL-2.

In an embodiment, the wild type IL-2 has the amino acid sequence of:

IL-2 (mature form, wild type) (SEQ ID NO: 344)

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATE
LKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMC
EYADETATIVEFLNRWITFCQSIISTLT

In such embodiments, the modified IL-2 agent has one or more mutations at amino acids L72 (L72G, L72A, L72S, L72T, L72Q, L72E, L72N, L72D, L72R, or L72K), F42 (F42A, F42G, F42S, F42T, F42Q, F42E, F42N, F42D, F42R, or F42K) and Y45 (Y45A, Y45G, Y45S, Y45T, Y45Q, Y45E, Y45N, Y45D, Y45R or Y45K). Without wishing to be bound by theory, it is believed that these modified IL-2 agents have reduced affinity for the high-affinity IL-2 receptor and preserves affinity to the intermediate-affinity IL-2 receptor, as compared to the wild-type IL-2. See, for example, US Patent Publication No. 2012/0244112, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-3. In some embodiments, the modified signaling agent has reduced affinity and/or activity for the IL-3 receptor, which is a heterodimer with a unique alpha chain paired with the common beta (beta c or CD131) subunit. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the IL-3 receptor, which is a heterodimer with a unique alpha chain paired with the common beta (beta c or CD131) subunit.

In an embodiment, the modified signaling agent is IL-4. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for type 1 and/or type 2 IL-4 receptors. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for type 1 and/or type 2 IL-4 receptors. Type 1 IL-4 receptors are composed of the IL-4Rα subunit with a common γ chain and specifically bind IL-4. Type 2 IL-4 receptors include an IL-4Rα subunit bound to a different subunit known as IL-13Rα1. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity the type 2 IL-4 receptors.

In an embodiment, the wild type IL-4 has the amino acid sequence of:

IL-4 (mature form, wild type) (SEQ ID NO: 345)

HKCDITLQEIIKTLNSLTEQKTLCTELTVTDIFAASKNTTEKETFCRAATVLRQ
FYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGLNSCPVKEA
NQSTLENFLERLKTIMREKYSKCSS

In such embodiments, the modified IL-4 agent has one or more mutations at amino acids R121 (R121A, R121D, R121E, R121F, R121H, R121I, R121K, R121N, R121P, R121T, R121W), E122 (E122F), Y124 (Y124A, Y124Q, Y124R, Y124S, Y124T) and S125 (S125A). Without wishing to be bound by theory, it is believed that these modified IL-4 agents maintain the activity mediated by the type I receptor, but significantly reduces the biological activity mediated by the other receptors. See, for example, US Patent No. 6,433,157, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-6. IL-6 signals through a cell-surface type I cytokine receptor complex including the ligand-binding IL-6R chain (CD126), and the signal-transducing component gp130. IL-6 may also bind to a soluble form of IL-6R (sIL-6R), which is the extracellular portion of IL-6R. The sIL-6R/IL-6 complex may be involved in neurites outgrowth and survival of neurons and, hence, may be important in nerve regeneration through remyelination. Accordingly, in some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-6R/gp130 and/or sIL-6R. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-6R/gp130 and/or sIL-6R.

In an embodiment, the wild type IL-6 has the amino acid sequence of IL-6 (mature form, wild type) (SEQ ID NO: 346):

APVPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCE
SSKEALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQN
RFESSEEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLTTKLQAQN
QWLQDMTTHLILRSFKEFLQSSLRALRQM

In such embodiments, the modified signaling agent has one or more mutations at amino acids 58, 160, 163, 171 or 177. Without wishing to be bound by theory, it is believed that these modified IL-6 agents exhibit reduced binding affinity to IL-6Ralpha and reduced biological activity. See, for example, WO 97/10338, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-10. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-10 receptor-1 and IL-10 receptor-2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-10 receptor-1 and IL-10 receptor-2

In an embodiment, the modified signaling agent is IL-11. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-11Rα and/or IL-11Rβ and/or gp130. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-11Rα and/or IL-11Rβ and/or gp130.

In an embodiment, the modified signaling agent is IL-12. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-12Rβ1 and/or IL-12Rβ2. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-12Rβ1 and/or IL-12Rβ2.

In an embodiment, the modified signaling agent is IL-13. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for the IL-4 receptor (IL-4Rα) and IL-13Rα1. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-4 receptor (IL-4Rα) or IL-13Rα1.

In an embodiment, the wild type IL-13 has the amino acid sequence of IL-13 (mature form, wild type) (SEQ ID NO: 347):

SPGPVPPSTALRELIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALES
LINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLH
LKKLFREGRFN

In such embodiments, the modified IL-13 agent has one or more mutations at amino acids 13, 16, 17, 66, 69, 99, 102, 104, 105, 106, 107, 108, 109, 112, 113 and 114. Without wishing to be bound by theory, it is believed that these modified IL-13 agents exhibit reduced biological activity. See, for example, WO 2002/018422, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-18. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-18Rα and/or IL-18Rβ. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-18Rα and/or IL-18Rβ. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-18Rα type II, which is an isoform of IL-18Rα that lacks the TIR domain required for signaling.

In an embodiment, the wild type IL-18 has the amino acid sequence of IL-18 (wild type) (SEQ ID NO: 348):

MAAEPVEDNCINFVAMKFIDNTLYFIAEDDENLESDYFGKLESKLSVIRNLN
DQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISMYKDSQPRGMAVTISVKC
EKISTLSCENKIISFKEMNPPDNIKDTKSDIIFFQRSVPGHDNKMQFESSSYE
GYFLACEKERDLFKLILKKEDELGDRSIMFTVQNEDL

In such embodiments, the modified IL-18 agent may comprise one or more mutations in amino acids or amino acid regions selected from Y37-K44, R49-Q54, D59-R63, E67-C74, R80, M87-A97, N 127-K129, Q139-M149, K165-K171, R183 and Q190-N191, as described in WO/2015/007542, the entire contents of which are hereby incorporated by reference (numbering based on the human IL-18 sequence, Genbank accession number AAV38697, version AAV38697.1, GI: 54696650).

In an embodiment, the modified signaling agent is IL-33. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for the ST-2 receptor and IL-1RAcP. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the ST-2 receptor and IL-1RAcP.

In an embodiment, the wild type IL-33 has the amino acid sequence of:

MKPKMKYSTNKISTAKWKNTASKALCFKLGKSQQKAKEVCPMYFMKLRSG
LMIKKEACYFRRETTKRPSLKTGRKHKRHLVLAACQQQSTVECFAFGISGV

QKYTRALHDSSITGISPITEYLASLSTYNDQSITFALEDESYEIYVEDLKKDEK
KDKVLLSYYESQHPSNESGDGVDGKMLMVTLSPTKDFWLHANNKEHSVE
LHKCEKPLPDQAFFVLHNMHSNCVSFECKTDPGVFIGVKDNHLALIKVDSS
ENLCTENILFKLSET (SEQ ID NO: 349)

In such embodiments, the modified IL-33 agent may comprise one or more mutations in amino acids or amino acid regions selected from I113-Y122, S127-E139, E144-D157, Y163-M183, E200, Q215, L220-C227 and T260-E269, as described in WO/2015/007542, the entire contents of which are hereby incorporated by reference (numbering based on the human sequence, Genbank accession number NP_254274, version NP_254274.1, GI:15559209).

In an embodiment, the modified signaling agent is epidermal growth factor (EGF). EGF is a member of a family of potent growth factors. Members include EGF, HB-EGF, and others such as TGFalpha, amphiregulin, neuregulins, epiregulin, betacellulin. EGF family receptors include EGFR (ErbB1), ErbB2, ErbB3 and ErbB4. These may function as homodimeric and /or heterodimeric receptor subtypes. The different EGF family members exhibit differential selectivity for the various receptor subtypes. For example, EGF associates with ErbB1/ErbB1, ErbB1/ErbB2, ErbB4/ErbB2 and some other heterodimeric subtypes. HB-EGF has a similar pattern, although it also associates with ErbB4/4. Modulation of EGF (EGF-like) growth factor signaling, positively or negatively, is of considerable therapeutic interest. For example, inhibition of EGFRs signaling is of interest in the treatment of various cancers where EGFR signaling constitutes a major growth promoting signal. Alternatively, stimulation of EGFRs signaling is of therapeutic interest in, for example, promoting wound healing (acute and chronic), oral mucositis (a major side-effect of various cancer therapies, including, without limitation radiation therapy).

In some embodiments, the modified signaling agent has reduced affinity and/or activity for ErbB1, ErbB2, ErbB3, and/or ErbB4. Such embodiments find use, for example, in methods of treating wounds. In some embodiments, the modified signaling agent binds to one or more ErbB1, ErbB2, ErbB3, and ErbB4 and antagonizes the activity of the receptor. In such embodiments, the modified signaling agent has reduced affinity and/or activity for ErbB1, ErbB2, ErbB3, and/or ErbB4 which allows for the activity of the receptor to be antagonized in an attenuated fashion. Such embodiments find use in, for example, treatments of cancer. In an embodiment, the modified signaling agent has reduced affinity and/or activity for ErbB1. ErbB1 is the therapeutic target of kinase inhibitors - most have side effects because they are not very selective (*e.g.*, gefitinib, erlotinib, afatinib, brigatinib and icotinib). In some embodiments, attenuated antagonistic ErbB1 signaling is more on-target and has less side effects than other agents targeting receptors for EGF.

In some embodiments, the modified signaling agent has reduced affinity and/or activity (*e.g.* antagonistic *e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) for ErbB1 and/or substantially reduced or ablated affinity and/or activity for ErbB4 or other subtypes it may interact with. Through specific targeting via the targeting moiety, cell-selective suppression (antagonism *e.g.* natural antagonistic activity or

antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) of ErbB1/ErbB1 receptor activation would be achieved – while not engaging other receptor subtypes potentially associated with inhibition-associated side effects. Hence, in contrast to EGFR kinase inhibitors, which inhibit EGFR activity in all cell types in the body, such a construct would provide a cell-selective (*e.g.*, tumor cell with activated EGFR signaling due to amplification of receptor, overexpression *etc.*) anti-EGFR (ErbB1) drug effect with reduced side effects.

In some embodiments, the modified signaling agent has reduced affinity and/or activity (*e.g.* agonistic) for ErbB4 and/or other subtypes it may interact with. Through targeting to specific target cells through the targeting moiety, a selective activation of ErbB1 signaling is achieved (*e.g.* epithelial cells). Such a construct finds use, in some embodiments, in the treatment of wounds (promoting would healing) with reduced side effects, especially for treatment of chronic conditions and application other than topical application of a therapeutic (*e.g.* systemic wound healing).

In an embodiment, the modified signaling agent is insulin or insulin analogs. In some embodiments, the modified insulin or insulin analog has reduced affinity and/or activity for the insulin receptor and/or IGF1 or IGF2 receptor. In some embodiments, the modified insulin or insulin analog has substantially reduced or ablated affinity and/or activity for the insulin receptor and/or IGF1 or IGF2 receptor. Attenuated response at the insulin receptor allows for the control of diabetes, obesity, metabolic disorders and the like while directing away from IGF1 or IGF2 receptor avoids pro-cancer effects.

In an embodiment, the modified signaling agent is insulin-like growth factor-I or insulin-like growth factor-II (IGF-1 or IGF-2). In an embodiment, the modified signaling agent is IGF-1. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for the insulin receptor and/or IGF1 receptor. In an embodiment, the modified signaling agent may bind to the IGF1 receptor and antagonize the activity of the receptor. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IGF1 receptor which allows for the activity of the receptor to be antagonized in an attenuated fashion. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the insulin receptor and/or IGF1 receptor. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IGF2 receptor which allows for the activity of the receptor to be antagonized in an attenuated fashion. In an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the insulin receptor and accordingly does not interfere with insulin signaling. In various embodiments, this applies to cancer treatment. In various embodiments, the present agents may prevent IR isoform A from causing resistance to cancer treatments.

In an embodiment, the modified signaling agent is EPO. In various embodiments, the modified EPO agent has reduced affinity and/or activity for the EPO receptor (EPOR) receptor and/or the ephrin receptor (EphR) relative to wild type EPO or other EPO based agents described herein. In some embodiments, the modified EPO agent has substantially reduced or ablated affinity and/or activity for the EPO receptor (EPOR) receptor and/or the Eph

receptor (EphR). Illustrative EPO receptors include, but are not limited to, an EPOR homodimer or an EPOR/CD131 heterodimer. Also included as an EPO receptor is beta-common receptor (βcR). Illustrative Eph receptors include, but are not limited to, EPHA1, EPHA2, EPHA3, EPHA4, EPHA5, EPHA6, EPHA7, EPHA8, EPHA9, EPHA10, EPHB1, EPHB2, EPHB3, EPHB4, EPHB5, and EPHB6. In some embodiments, the modified EPO protein comprises one or more mutations that cause the EPO protein to have reduced affinity for receptors that comprise one or more different EPO receptors or Eph receptors (*e.g.* heterodimer, heterotrimers, *etc.*, including by way of non-limitation: EPOR-EPHB4, EPOR- βcR-EPOR). Also provided are the receptors of EP Patent Publication No. 2492355 the entire contents of which are hereby incorporated by reference, including by way of non-limitation, NEPORs.

In an embodiment, the human EPO has the amino acid sequence of (the signal peptide is underlined):

MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLE
AKEAENITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVE
VWQGLALLSEAVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLT
TLLRALGAQKEAISPPDAASAAPLRTITADTFRKLFRVYSNFLRGK
LKLYTGEACRTGDR (SEQ ID NO: 350)

In an embodiment, the human EPO protein is the mature form of EPO (with the signal peptide being cleaved off) which is a glycoprotein of 166 amino acid residues having the sequence of:

APPRLICDSRVLERYLLEAKEAENITTGCAEHCSLNENITVPDTKV
NFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQALLVNSSQPWE
PLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPLRTITA
DTFRKLFRVYSNFLRGKLKLYTGEACRTGDR (SEQ ID NO: 351)

The structure of the human EPO protein is predicted to comprise four-helix bundles including helices A, B, C, and D. In various embodiments, the modified EPO protein comprises one or more mutations located in four regions of the EPO protein which are important for bioactivity, *i.e.*, amino acid residues 10-20, 44-51, 96-108, and 142-156. In some embodiments, the one or more mutations are located at residues 11-15, 44-51, 100-108, and 147-151. These residues are localized to helix A (Val11, Arg14, and Tyr15), helix C (Ser100, Arg103, Ser104, and Leu108), helix D (Asn147, Arg150, Gly151, and Leu155), and the A/B connecting loop (residues 42-51). In some embodiments, the modified EPO protein comprises mutations in residues between amino acids 41-52 and amino acids 147, 150, 151, and 155. Without wishing to be bound by theory, it is believed that mutations of these residues have substantial effects on both receptor binding and in vitro biological activity. In some embodiments, the modified EPO protein comprises mutations at residues 11, 14, 15, 100, 103, 104, and 108. Without wishing to be bound by theory, it is believed that mutations of these residues have modest effects on receptor binding activity and much greater effects on in vitro biological activity. Illustrative substitutions include, but are not limited to, one or more of Val11Ser, Arg14Ala, Arg14Gln, Tyr15Ile, Pro42Asn, Thr44Ile, Lys45Asp, Val46Ala, Tyr51Phe,

Ser100Glu, Ser100Thr, Arg103Ala, Ser104Ile, Ser104Ala, Leu108Lys, Asn147Lys, Arg150Ala, Gly151Ala, and Leu155Ala.

In some embodiments, the modified EPO protein comprises mutations that effect bioactivity and not binding, *e.g.* those listed in Eliot, *et al.* Mapping of the Active Site of Recombinant Human Erythropoietin January 15, 1997; *Blood*: 89 (2), the entire contents of which are hereby incorporated by reference.

In some embodiments, the modified EPO protein comprises one or more mutations involving surface residues of the EPO protein which are involved in receptor contact. Without wishing to be bound by theory, it is believed that mutations of these surface residues are less likely to affect protein folding thereby retaining some biological activity. Illustrative surface residues that may be mutated include, but are not limited to, residues 147 and 150. In illustrative embodiments, the mutations are substitutions including, one or more of N147A, N147K, R150A and R150E.

In some embodiments, the modified EPO protein comprises one or more mutations at residues N59, E62, L67, and L70, and one or more mutations that affect disulfide bond formation. Without wishing to be bound by theory, it is believed that these mutations affect folding and/or are predicted be in buried positions and thus affects biological activity indirectly.

In an embodiment, the modified EPO protein comprises a K20E substitution which significantly reduces receptor binding. *See* Elliott, *et al.,* (1997) *Blood,* 89:493-502, the entire contents of which are hereby incorporated by reference.

Additional EPO mutations that may be incorporated into the chimeric EPO protein of the invention are disclosed in, for example, Elliott, *et al.,* (1997) *Blood,* 89:493-502, the entire contents of which are hereby incorporated by reference and Taylor *et al*., (2010) *PEDS*, 23(4): 251-260, the entire contents of which are hereby incorporated by reference.

In some embodiments, the chimeric protein of the invention comprises a modified IL-1 (*e.g.*, IL-1α and/or IL-1β) as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells such as T helper cells (*e.g.* for co-stimulation and activation) and/or B cells (*e.g.* for maturation, proliferation), and/or NK cells (*e.g.* for activation) and/or macrophages (*e.g.* for activation).

In some embodiments, the chimeric protein of the invention comprises a modified IL-2 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for growth and differentiation) and/or B cells (*e.g.* for growth) and/or NK cells (*e.g.* for growth) and/or macrophages.

In some embodiments, the chimeric protein of the invention comprises a modified IL-3 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) hematopoietic stem cells (*e.g.*, for growth, differentiation and proliferation) and/or myeloid progenitor cells (*e.g.* for differentiation and proliferation) and/or mast cells (*e.g.* for growth).

In some embodiments, the chimeric protein of the invention comprises a modified IL-4 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for growth, proliferation, and survival) and/or B cells (*e.g.*, for activation, growth, differentiation, and proliferation) and/or mast cells (*e.g.* for growth) and/or macrophages (*e.g.*, for inhibition of activation).

In some embodiments, the chimeric protein of the invention comprises a modified IL-5 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) eosinophils (*e.g.* for growth and differentiation) and/or B cells (*e.g.* for differentiation and production).

In some embodiments, the chimeric protein of the invention comprises a modified IL-6 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) B cells (*e.g.*, for growth and differentiation) and/or plasma cells (*e.g.* for antibody secretion) and/or hematopoietic stem cells (*e.g.* for differentiation) and/or T cells (*e.g.* for growth and differentiation).

In some embodiments, the chimeric protein of the invention comprises a modified IL-7 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for growth and differentiation and survival) and/or B cells (*e.g.* for growth and differentiation and survival) and/or NK cells (*e.g.* for growth and differentiation and survival).

In some embodiments, the chimeric protein of the invention comprises a modified IL-8 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) neutrophils and/or basophils and/or B cells and/or T cells and/or NK cells.

In some embodiments, the chimeric protein of the invention comprises a modified IL-9 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells and/or B cells (*e.g.* activation) and/or mast cells (*e.g.* stimulation).

In some embodiments, the chimeric protein of the invention comprises a modified IL-10 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) macrophages (*e.g.* for inhibition) and/or B cells (*e.g.* for activation) and/or mast cells (*e.g.* for co-stimulation of growth) and/or Th_1 cells (*e.g.* for inhibition) and/or Th_2 cells (*e.g.* for stimulation).

In some embodiments, the chimeric protein of the invention comprises a modified IL-12 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for differentiation) and/or NK cells (*e.g.* for activation).

In some embodiments, the chimeric protein of the invention comprises a modified IL-13 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.*, Th_1 and Th_2 cells; for example, for inhibition of Th_1 cells) and/or B cells (*e.g.* for growth and differentiation) and/or macrophages (*e.g.*, for inhibition).

In some embodiments, the chimeric protein of the invention comprises a modified IL-14 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) B cells (*e.g.*, for growth and proliferation).

In some embodiments, the chimeric protein of the invention comprises a modified IL-15 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for growth) and/or B cells and or NK cells (*e.g.* for growth).

In some embodiments, the chimeric protein of the invention comprises a modified IL-16 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.*, CD4+ T cells; for example, for survival).

In some embodiments, the chimeric protein of the invention comprises a modified IL-18 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) Th_1 cells (*e.g.*, for induction) and/or NK cells (*e.g.*, for induction of IFNγ production).

In some embodiments, the chimeric protein of the invention comprises a modified IL-21 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for activation and proliferation and/or differentiation) and/or B cells (*e.g.* for proliferation and differentiation) and/or NK cells and/or dendritic cells.

In some embodiments, the chimeric protein of the invention comprises a modified IL-27 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells and/or B cells.

In some embodiments, the chimeric protein of the invention comprises a modified IL-32 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) monocytes and/or macrophages.

In some embodiments, the chimeric protein of the invention comprises a modified IL-33 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.*, T helper cells).

In some embodiments, the chimeric protein of the invention comprises a modified IL-35 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.*, T helper cells).

In some embodiments, the chimeric protein of the invention comprises a modified IL-36 as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells and/or dendritic cells.

In some embodiments, the chimeric protein of the invention comprises a modified TNF-α as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) macrophages (*e.g.*, for activation).

In some embodiments, the chimeric protein of the invention comprises a modified TNF-β as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for killing) and/or B cells (*e.g.*, for inhibition) and/or macrophages (*e.g.*, for activation) and/or neutrophils (*e.g.* for activation).

In some embodiments, the chimeric protein of the invention comprises a modified CD40L as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) B cells (*e.g.* for activation).

In some embodiments, the chimeric protein of the invention comprises a modified CD27L as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for proliferation).

In some embodiments, the chimeric protein of the invention comprises a modified CD30L as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for proliferation) and/or B cells (*e.g.* for proliferation).

In some embodiments, the chimeric protein of the invention comprises a modified 4-1BBL as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for co-stimulation) and/or B cells (*e.g.* for co-stimulation).

In some embodiments, the chimeric protein of the invention comprises a modified TGF-β as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) B cells (*e.g.* for inhibition of growth) and/or macrophages (*e.g.* for inhibition of activation) and/or neutrophils (*e.g.* for activation).

In some embodiments, the chimeric protein of the invention comprises a modified IFN-γ as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for inhibition of Th_2 growth) and/or B cells (*e.g.* for differentiation) and/or NK cells (*e.g.* for activation) and/or macrophages (*e.g.* for activation).

In some embodiments, the chimeric protein of the invention comprises a modified GM-CSF as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) T cells (*e.g.* for inhibition of growth) and/or B cells (*e.g.* for differentiation) and/or macrophages (*e.g.* for activation and differentiation) and hematopoietic stem cells (*e.g.* for differentiation) and/or myeloid progenitor cells (*e.g.* for growth and differentiation).

In some embodiments, the chimeric protein of the invention comprises a modified G-CSF as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) neutrophils (*e.g.* for development and differentiation).

In some embodiments, the chimeric protein of the invention comprises a modified EPO as the signaling agent. In such embodiments, the chimeric protein has effects on (and one targeting moiety targets) hematopoietic

progenitor and/or precursor cells and/or erythroid progenitor and/or precursor cells (*e.g.*, BFU-E (burst forming unit-erythroid) and/or CFU-E (colony-forming units-erythroid)).

By way of non-limiting example, in one embodiment, the present chimeric protein has (i) a targeting moiety directed against a T cell, for example, mediated by targeting to one of CD8, CD4, and CD3 and (ii) a targeting moiety is directed against a tumor cell (*e.g.,* mediated by targeting to, without limitation, a checkpoint inhibitor, *e.g.,* without limitation, PD-L1 or PD-L2), along with any of the modified (*e.g.* mutant) signaling agents described herein, for example, modified (*e.g.* mutant) signaling agent that may alter the activity of a T cell, for example, interferon alpha, including without limitation, one or more of following mutations F64A, N65A, T69A, L80A, Y85A, Y89A, R120E, R120E/K121E, K133A, R144A, M148A, R149A, and L153A. In an embodiment, the present chimeric protein has a targeting moiety directed against CD8 on T cells, a second targeting moiety directed against a checkpoint inhibitor, *e.g.,* without limitation, PD-L1 or PD-L2, on tumor cells, and a modified (*e.g.* mutant) interferon agent as described herein.

By way of non-limiting example, in one embodiment, the present chimeric protein has (i) a targeting moiety directed against a B cell, for example, mediated by targeting to CD20 and (ii) a targeting moiety is directed against a tumor cell (*e.g.,* mediated by targeting to, without limitation, a checkpoint inhibitor, *e.g.,* without limitation, PD-L1 or PD-L2), along with any of the modified (*e.g.* mutant) signaling agents described herein, for example, modified (*e.g.* mutant) signaling agent that may alter the activity of a B cell, for example, interferon alpha, including without limitation, one or more of the following mutations F64A, N65A, T69A, L80A, Y85A, Y89A, R120E, R120E/K121E, K133A, R144A, M148A, R149A, and L153A.

By way of non-limiting example, in one embodiment, the present chimeric protein has (i) a targeting moiety directed against a dendritic cell, for example, mediated by targeting to one of CLEC9A, XCR1, and RANK and (ii) a targeting moiety is directed against a tumor cell (*e.g.,* mediated by targeting to, without limitation, a checkpoint inhibitor, *e.g.,* without limitation, PD-L1 or PD-L2), along with any of the modified (*e.g.* mutant) signaling agents described herein, for example, modified (*e.g.* mutant) signaling agent that may alter the activity of a dendritic cell, for example, interferon alpha, including without limitation, one or more of the following mutations F64A, N65A, T69A, L80A, Y85A, Y89A, R120E, R120E/K121E, K133A, R144A, M148A, R149A, and L153A. In an embodiment, the present chimeric protein has a targeting moiety directed against CLEC9A on dendritic cells, a second targeting moiety directed against a checkpoint inhibitor, *e.g.,* without limitation, PD-L1 or PD-L2, on tumor cells, and a modified (*e.g.* mutant) interferon agent as described herein.

By way of non-limiting example, in one embodiment, the present chimeric protein has (i) a targeting moiety directed against a macrophage, for example, mediated by targeting to SIRP1a and (ii) a targeting moiety is directed against a tumor cell (*e.g.,* mediated by targeting to, without limitation, a checkpoint inhibitor, *e.g.,* without limitation, PD-L1 or PD-L2), along with any of the modified (*e.g.* mutant) signaling agents described herein, for example, modified (*e.g.* mutant) signaling agent that may alter the activity of a macrophage, for example,

interferon alpha, including without limitation, one or more of the following mutations F64A, N65A, T69A, L80A, Y85A, Y89A, R120E, R120E/K121E, K133A, R144A, M148A, R149A, and L153A.

By way of non-limiting example, in one embodiment, the present chimeric protein has (i) a targeting moiety directed against a NK cell, for example, mediated by targeting to TIGIT or KIR1 and (ii) a targeting moiety is directed against a tumor cell (*e.g.,* mediated by targeting to, without limitation, a checkpoint inhibitor, *e.g.,* without limitation, PD-L1 or PD-L2), along with any of the modified (*e.g.* mutant) signaling agents described herein, for example, modified (*e.g.* mutant) signaling agent that may alter the activity of a NK cell, for example, interferon alpha, including without limitation, one or more of the following mutations F64A, N65A, T69A, L80A, Y85A, Y89A, R120E, R120E/K121E, K133A, R144A, M148A, R149A, and L153A.

In various embodiments, the signaling agent is a toxin or toxic enzyme. In some embodiments, the toxin or toxic enzyme is derived from plants and bacteria. Illustrative toxins or toxic enzymes include, but are not limited to, the diphtheria toxin, Pseudomonas toxin, anthrax toxin, ribosome-inactivating proteins (RIPs) such as ricin and saporin, modeccin, abrin, gelonin, and poke weed antiviral protein. Additional toxins include those disclosed in Mathew et al., (2009) Cancer Sci 100(8): 1359-65, the entire disclosures are hereby incorporated by reference. In such embodiments, the chimeric proteins of the invention may be utilized to induce cell death in cell-type specific manner. In such embodiments, the toxin may be modified, *e.g.* mutated, to reduce affinity and/or activity of the toxin for an attenuated effect, as described with other signaling agents herein.

Linkers

In some embodiments, the present chimeric protein optionally comprises one or more linkers. In some embodiments, the present chimeric protein comprises a linker connecting the targeting moiety and the signaling agent. In some embodiments, the present chimeric protein comprises a linker within the signaling agent (*e.g.* in the case of single chain TNF, which can comprise two linkers to yield a trimer).

In some embodiments vectors encoding the present chimeric proteins linked as a single nucleotide sequence to any of the linkers described herein are provided and may be used to prepare such chimeric proteins.

In some embodiments, the linker length allows for efficient binding of a targeting moiety and the signaling agent to their receptors. For instance, in some embodiments, the linker length allows for efficient binding of one of the targeting moieties and the signaling agent to receptors on the same cell as well as the efficient binding of the other targeting moiety to another cell. Illustrative pairs of cells are provided elsewhere herein.

In some embodiments the linker length is at least equal to the minimum distance between the binding sites of one of the targeting moieties and the signaling agent to receptors on the same cell. In some embodiments the linker length is at least twice, or three times, or four times, or five times, or ten times, or twenty times, or 25 times, or 50 times, or one hundred times, or more the minimum distance between the binding sites of one of the targeting moieties and the signaling agent to receptors on the same cell.

As described herein, the linker length allows for efficient binding of one of the targeting moieties and the signaling agent to receptors on the same cell, the binding being sequential, *e.g.* targeting moiety/receptor binding preceding signaling agent/receptor binding.

In some embodiments, there are two linkers in a single chimera, each connecting the signaling agent to a targeting moiety. In various embodiments, the linkers have lengths that allow for the formation of a site that has a disease cell and an effector cell without steric hindrance that would prevent modulation of the either cell.

The invention contemplates the use of a variety of linker sequences. 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 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 chimeric protein.

In some embodiments, the linker is a polypeptide. In some embodiments, the linker is less than about 100 amino acids long. For example, the linker 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 some embodiments, the linker is a polypeptide. In some embodiments, the linker is greater than about 100 amino acids long. For example, the linker may be greater 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 some embodiments, the linker is flexible. In another embodiment, the linker is rigid.

In some embodiments, a linker connects the two targeting moieties to each other and this linker has a short length and a linker connects a targeting moiety and a signaling agent this linker is longer than the linker connecting the two targeting moieties. For example, the difference in amino acid length between the linker connecting the two targeting moieties and the linker connecting a targeting moiety and a signaling agent may be 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.

In various embodiments, the linker 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). For example, in some embodiments, the linker is $(Gly_4Ser)_n$, where n is from about 1 to about 8, *e.g.* 1, 2, 3, 4, 5, 6, 7, or 8. In an embodiment, the linker sequence is GGSGGSGGGGSGGGGS (SEQ ID NO: 352). Additional illustrative linkers include, but are not limited to, linkers having the sequence LE, GGGGS (SEQ ID NO: 353), $(GGGGS)_n$ (n=1-4) (SEQ ID NO: 354), $(Gly)_8$ (SEQ ID NO: 355), $(Gly)_6$ (SEQ ID NO: 356), $(EAAAK)_n$ (n=1-3) (SEQ ID NO: 357), $A(EAAAK)_nA$ (n = 2-5) (SEQ ID NO: 358), AEAAAKEAAAKA (SEQ ID NO: 359), $A(EAAAK)_4ALEA(EAAAK)_4A$ (SEQ ID NO: 360), PAPAP (SEQ ID NO: 361), KESGSVSSEQLAQFRSLD (SEQ ID NO: 362), EGKSSGSGSESKST (SEQ ID NO: 363), GSAGSAAGSGEF (SEQ ID NO: 364), and $(XP)_n$, with X designating any amino acid, *e.g.*, Ala, Lys, or Glu. In various embodiments, the linker is GGS.

In some embodiments, the linker 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)). In various embodiments, the linker 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.

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 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 of the present invention comprises one or more glycosylation sites. In various embodiments, the linker is a hinge-CH2-CH3 domain of a human IgG4 antibody.

If desired, the present chimeric protein can be linked to an antibody Fc region, comprising one or both of C_H2 and C_H3 domains, and optionally a hinge region. For example, vectors encoding the present chimeric proteins linked as a single nucleotide sequence to an Fc region can be used to prepare such polypeptides.

In some embodiments, the linker is a synthetic linker such as PEG.

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 chimeric protein. In another example, the linker may function to target the chimeric protein to a particular cell type or location.

Production of Chimeric Proteins

Methods for producing the chimeric proteins of the invention are described herein. For example, DNA sequences encoding the chimeric proteins of the invention (*e.g.*, DNA sequences encoding the modified signaling agent and the targeting moiety and the linker) can be chemically synthesized using methods known in the art. Synthetic DNA sequences can be ligated to other appropriate nucleotide sequences, including, *e.g.*, expression control sequences, to produce gene expression constructs encoding the desired chimeric proteins. Accordingly, in various embodiments, the present invention provides for isolated nucleic acids comprising a nucleotide sequence encoding the chimeric protein of the invention.

Nucleic acids encoding the chimeric protein of the invention can be incorporated (ligated) into expression vectors, which can be introduced into host cells through transfection, transformation, or transduction techniques. For example, nucleic acids encoding the chimeric protein of the invention can be introduced into host cells by retroviral transduction. Illustrative host cells are *E.coli* cells, Chinese hamster ovary (CHO) cells, human embryonic kidney 293 (HEK 293) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (*e.g.*, Hep G2), and myeloma cells. Transformed host cells can be grown under conditions that permit the host cells to express the genes that encode the chimeric protein of the invention. Accordingly, in various embodiments, the present invention provides expression vectors comprising

nucleic acids that encode the chimeric protein of the invention. In various embodiments, the present invention additional provides host cells comprising such expression vectors.

Specific expression and purification conditions will vary depending upon the expression system employed. For example, if a gene is to be expressed in *E. coli*, it is first cloned into an expression vector by positioning the engineered gene downstream from a suitable bacterial promoter, *e.g.*, Trp or Tac, and a prokaryotic signal sequence. In another example, if the engineered gene is to be expressed in eukaryotic host cells, *e.g.*, CHO cells, it is first inserted into an expression vector containing for example, a suitable eukaryotic promoter, a secretion signal, enhancers, and various introns. The gene construct can be introduced into the host cells using transfection, transformation, or transduction techniques.

The chimeric protein of the invention can be produced by growing a host cell transfected with an expression vector encoding the chimeric protein under conditions that permit expression of the protein. Following expression, the protein can be harvested and purified using techniques well known in the art, *e.g.*, affinity tags such as glutathione-S-transferase (GST) and histidine tags or by chromatography.

Accordingly, in various embodiments, the present invention provides for a nucleic acid encoding a chimeric protein of the present invention. In various embodiments, the present invention provides for a host cell comprising a nucleic acid encoding a chimeric protein of the present invention.

Pharmaceutically Acceptable Salts and Excipients

The chimeric proteins 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.

Pharmaceutically acceptable salts include, by way of non-limiting example, sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, camphorsulfonate, pamoate, phenylacetate, trifluoroacetate, acrylate, chlorobenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, methylbenzoate, o-acetoxybenzoate, naphthalene-2-benzoate, isobutyrate, phenylbutyrate, α-hydroxybutyrate, butyne-1,4-dicarboxylate, hexyne-1,4-dicarboxylate, caprate, caprylate, cinnamate, glycollate, heptanoate, hippurate, malate, hydroxymaleate, malonate, mandelate, mesylate, nicotinate, phthalate, teraphthalate, propiolate, propionate, phenylpropionate, sebacate, suberate, p-bromobenzenesulfonate, chlorobenzenesulfonate, ethylsulfonate, 2-hydroxyethylsulfonate,

methylsulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, naphthalene-1,5-sulfonate, xylenesulfonate, and tartarate salts.

The term "pharmaceutically acceptable salt" also refers to a salt of the compositions of the present invention having an acidic functional group, such as a carboxylic acid functional group, and a base. Suitable bases include, but are not limited to, hydroxides of alkali metals such as sodium, potassium, and lithium; hydroxides of alkaline earth metal such as calcium and magnesium; hydroxides of other metals, such as aluminum and zinc; ammonia, and organic amines, such as unsubstituted or hydroxy-substituted mono-, di-, or tri-alkylamines, dicyclohexylamine; tributyl amine; pyridine; N-methyl, N-ethylamine; diethylamine; triethylamine; mono-, bis-, or tris-(2-OH-lower alkylamines), such as mono-; bis-, or tris-(2-hydroxyethyl)amine, 2-hydroxy-tert-butylamine, or tris-(hydroxymethyl)methylamine, N,N-di-lower alkyl-N-(hydroxyl-lower alkyl)-amines, such as N,N-dimethyl-N-(2-hydroxyethyl)amine or tri-(2-hydroxyethyl)amine; N-methyl-D-glucamine; and amino acids such as arginine, lysine, and the like.

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

Pharmaceutical Compositions and Formulations

In various embodiments, the present invention pertains to pharmaceutical compositions comprising the chimeric proteins described herein and a pharmaceutically acceptable carrier or excipient. Any pharmaceutical compositions 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.

In various embodiments, 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. 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.

The present invention includes the described pharmaceutical compositions (and/or additional therapeutic agents) in various formulations. Any inventive pharmaceutical composition (and/or additional therapeutic agents) described herein can take the form of solutions, suspensions, emulsion, drops, tablets, pills, pellets, capsules,

capsules containing liquids, gelatin capsules, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, lyophilized powder, frozen suspension, dessicated powder, or any other form suitable for use. In one embodiment, the composition is in the form of a capsule. In another embodiment, the composition is in the form of a tablet. In yet another embodiment, the pharmaceutical composition is formulated in the form of a soft-gel capsule. In a further embodiment, the pharmaceutical composition is formulated in the form of a gelatin capsule. In yet another embodiment, the pharmaceutical composition is formulated as a liquid.

Where necessary, the inventive pharmaceutical compositions (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.

The formulations comprising the inventive pharmaceutical compositions (and/or additional agents) of the present invention 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 various embodiments, any pharmaceutical compositions (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: oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically. Administration can be local or systemic. In some embodiments, the administering is effected orally. In another embodiment, the administration is by parenteral injection. The mode of administration can be left to the discretion of the practitioner, and depends in-part upon the site of the medical condition. In most instances, administration results in the release of any agent described herein into the bloodstream.

In one embodiment, the chimeric protein described herein is formulated in accordance with routine procedures as a composition adapted for oral administration. Compositions for oral delivery can be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, emulsions, capsules, syrups, or elixirs, for example. Orally administered compositions can comprise one or more agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents, to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions can be coated to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving any chimeric proteins described herein are also suitable for orally

administered compositions. In these latter platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the spiked profiles of immediate release formulations. A time-delay material such as glycerol monostearate or glycerol stearate can also be useful. Oral compositions can include standard excipients such as mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, and magnesium carbonate. In one embodiment, the excipients are of pharmaceutical grade. Suspensions, in addition to the active compounds, may contain suspending agents such as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, tragacanth, *etc.*, and mixtures thereof.

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. Formulation components suitable for parenteral administration include a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose.

For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). The carrier should be stable under the conditions of manufacture and storage, and should be preserved against microorganisms. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol), and suitable mixtures thereof.

The compositions provided herein, alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Any inventive pharmaceutical compositions (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, hydropropyl cellulose, hydropropylmethyl cellulose, polyvinylpyrrolidone, 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. Suitable controlled- or sustained-release formulations known to those skilled in the art, including those described herein, can be readily selected for use with the active ingredients of the agents described herein. The invention thus provides single unit dosage forms suitable for oral administration such as, but not limited to, tablets, capsules, gelcaps, and caplets that are adapted for controlled- or sustained-release.

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, 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 (s*ee, 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.

Pharmaceutical formulations preferably are sterile. Sterilization can be accomplished, for example, by filtration through sterile filtration membranes. Where the composition is lyophilized, filter sterilization can be conducted prior to or following lyophilization and reconstitution.

Administration and Dosage

It will be appreciated that the actual dose of the chimeric protein to be administered according to the present invention will vary according to the particular dosage form, and the mode of administration. Many factors that may modify the action of the chimeric protein (*e.g.*, body weight, gender, diet, time of administration, route of administration, rate of excretion, condition of the subject, drug combinations, genetic disposition and reaction sensitivities) can be taken into account by those skilled in the art. Administration can be carried out continuously or in one or more discrete doses within the maximum tolerated dose. Optimal administration rates for a given set of conditions can be ascertained by those skilled in the art using conventional dosage administration tests.

In some embodiments, a suitable dosage of the chimeric protein is in a range of about 0.01 mg/kg to about 10 g/kg of body weight of the subject, about 0.01 mg/kg to about 1 g/kg of body weight of the subject, about 0.01 mg/kg to about 100 mg/kg of body weight of the subject, 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, about 100 mg/kg body weight, about 1 g/kg of body weight, about 10 g/kg of body weight, inclusive of all values and ranges therebetween.

Individual doses of the chimeric protein can be administered in unit dosage forms (*e.g.*, tablets or capsules) containing, for example, from about 0.01 mg to about 100 g, from about 0.01 mg to about 75 g, from about 0.01 mg to about 50 g, from about 0.01 mg to about 25 g, about 0.01 mg to about 10 g, about 0.01 mg to about 7.5 g, about 0.01 mg to about 5 g, about 0.01 mg to about 2.5 g, about 0.01 mg to about 1 g, about 0.01 mg to about 100 mg, from about 0.1 mg to about 100 mg, from about 0.1 mg to about 90 mg, from about 0.1 mg to about 80 mg, from about 0.1 mg to about 70 mg, from about 0.1 mg to about 60 mg, from about 0.1 mg to about 50 mg, from about 0.1 mg to about 40 mg active ingredient, from about 0.1 mg to about 30 mg, from about 0.1 mg to about 20 mg, from about 0.1 mg to about 10 mg, from about 0.1 mg to about 5 mg, from about 0.1 mg to about 3 mg, from about 0.1 mg to about 1 mg per unit dosage form, or from about 5 mg to about 80 mg per unit dosage form. For example, a unit dosage form can be about 0.01 mg, about 0.02 mg, about 0.03 mg, about 0.04 mg, about 0.05 mg, about 0.06 mg, about 0.07 mg, about 0.08 mg, about 0.09 mg, about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg about 10 mg, about 15 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg, about 200 mg, about 500 mg, about 1 g, about 2.5 g, about 5 g, about 10 g, about 25 g, about 50 g, about 75 g, about 100 g, inclusive of all values and ranges therebetween.

In one embodiment, the chimeric protein is administered at an amount of from about 0.01 mg to about 100 g daily, from about 0.01 mg to about 75 g daily, from about 0.01 mg to about 50 g daily, from about 0.01 mg to about 25 g daily, from about 0.01 mg to about 10 g daily, from about 0.01 mg to about 7.5 g daily, from about 0.01 mg to about 5 g daily, from about 0.01 mg to about 2.5 g daily, from about 0.01 mg to about 1 g daily, from about 0.01 mg to about 100 mg daily, from about 0.1 mg to about 100 mg daily, from about 0.1 mg to about 95 mg daily, from about 0.1 mg to about 90 mg daily, from about 0.1 mg to about 85 mg daily, from about 0.1 mg to about 80 mg daily, from about 0.1 mg to about 75 mg daily, from about 0.1 mg to about 70 mg daily, from about 0.1 mg to about 65 mg daily, from about 0.1 mg to about 60 mg daily, from about 0.1 mg to about 55 mg daily, from about 0.1 mg to about 50 mg daily, from about 0.1 mg to about 45 mg daily, from about 0.1 mg to about 40 mg daily, from about 0.1 mg to about 35 mg daily, from about 0.1 mg to about 30 mg daily, from about 0.1 mg to about 25 mg daily, from about 0.1 mg to about 20 mg daily, from about 0.1 mg to about 15 mg daily, from about 0.1 mg to about 10 mg daily, from about 0.1 mg to about 5 mg daily, from about 0.1 mg to about 3 mg daily, from about 0.1 mg to about 1 mg daily, or from about 5 mg to about 80 mg daily. In various embodiments, the chimeric protein is administered at a daily dose of about 0.01 mg, about 0.02 mg, about 0.03 mg, about 0.04 mg, about 0.05 mg, about 0.06 mg, about 0.07 mg, about 0.08 mg, about 0.09 mg, about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg about 10 mg, about

15 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg, about 200 mg, about 500 mg, about 1 g, about 2.5 g, about 5 g, about 7.5 g, about 10 g, about 25 g, about 50 g, about 75 g, about 100 g, inclusive of all values and ranges therebetween.

In accordance with certain embodiments of the invention, the pharmaceutical composition comprising the chimeric protein may be administered, for example, more than once daily (*e.g.*, about two times, about three times, about four times, about five times, about six times, about seven times, about eight times, about nine times, or about ten times daily), about once per day, about every other day, about every third day, about once a week, about once every two weeks, about once every month, about once every two months, about once every three months, about once every six months, or about once every year.

Combination Therapy and Additional Therapeutic Agents

In various embodiments, the pharmaceutical composition of the present invention is co-administered in conjunction with additional therapeutic agent(s). Co-administration can be simultaneous or sequential.

In one embodiment, the additional therapeutic agent and the chimeric protein of the present invention are administered to a subject simultaneously. The term "simultaneously" as used herein, means that the additional therapeutic agent and the chimeric protein are administered with a time separation of no more than about 60 minutes, such as no more than about 30 minutes, no more than about 20 minutes, no more than about 10 minutes, no more than about 5 minutes, or no more than about 1 minute. Administration of the additional therapeutic agent and the chimeric protein can be by simultaneous administration of a single formulation (*e.g.*, a formulation comprising the additional therapeutic agent and the chimeric protein) or of separate formulations (*e.g.*, a first formulation including the additional therapeutic agent and a second formulation including the chimeric protein).

Co-administration does not require the therapeutic agents to be administered simultaneously, if the timing of their administration is such that the pharmacological activities of the additional therapeutic agent and the chimeric protein overlap in time, thereby exerting a combined therapeutic effect. For example, the additional therapeutic agent and the chimeric protein can be administered sequentially. The term "sequentially" as used herein means that the additional therapeutic agent and the chimeric protein are administered with a time separation of more than about 60 minutes. For example, the time between the sequential administration of the additional therapeutic agent and the chimeric protein can be more than about 60 minutes, more than about 2 hours, more than about 5 hours, more than about 10 hours, more than about 1 day, more than about 2 days, more than about 3 days, more than about 1 week apart, more than about 2 weeks apart, or more than about one month apart. The optimal administration times will depend on the rates of metabolism, excretion, and/or the pharmacodynamic activity of the additional therapeutic agent and the chimeric protein being administered. Either the additional therapeutic agent or the chimeric protein cell may be administered first.

Co-administration also does not require the therapeutic agents to be administered to the subject by the same route of administration. Rather, each therapeutic agent can be administered by any appropriate route, for example, parenterally or non-parenterally.

In some embodiments, the chimeric protein described herein acts synergistically when co-administered with another therapeutic agent. In such embodiments, the chimeric protein and the additional therapeutic agent may be administered at doses that are lower than the doses employed when the agents are used in the context of monotherapy.

In some embodiments, the present invention pertains to chemotherapeutic agents as additional therapeutic agents. For example, without limitation, such combination of the present chimeric proteins and chemotherapeutic agent find use in the treatment of cancers, as described elsewhere herein. 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 some embodiments, the present bi-specific chimeras are combined with FMS-like tyrosine kinase 3 ligand (FLT3L), *e.g.* anti-human Clec9a VHH/anti-human PDL1 VHH/human IFN -R149A bi-specific chimera can be combined with FLT3L.

In some embodiments, inclusive of, without limitation, infectious disease applications, the present invention pertains to anti-infectives as additional therapeutic 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 autoimmmune applications, the additional therapeutic 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 invention 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 invention, 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). Additional anti-inflammatory agents are described, for example, in U.S. Patent No. 4,537,776, the entire contents of which is incorporated by reference herein.

In some embodiments, the present invention pertains to various agents used for treating obesity as additional therapeutic agents. Illustrative agents used for treating obesity include, but are not limited to, orlistat (*e.g.* ALL1, XENICAL), loracaserin (*e.g.* BELVIQ), phentermine-topiramate (*e.g.* QSYMIA), sibutramme (*e.g.* REDUCTIL or MERJDIA), rimonabant (ACOMPLLA), exenatide (*e.g.* BYETTA), pramlintide (*e.g.* SYMLIN) phentermine, benzphetamine, diethylpropion, phendimetrazme, bupropion, and metformin. Agents that interfere with the body's ability to absorb specific nutrients in food are among the additional agents, *e.g.* orlistat (*e.g.* ALU, XENICAL), glucomannan, and guar gum. Agents that suppress apetite are also among the additional agents, *e.g.* catecholamines and their derivatives (such as phenteimine and other amphetamine-based drugs), various antidepressants and mood stabilizers (*e.g.* bupropion and topiramate), anorectics (*e.g.* dexedrine, digoxin). Agents that increase the body's metabolism are also among the additional agents.

In some embodiments, additional therapeutic agents may be selected from among appetite suppressants, neurotransmitter reuptake inhibitors, dopaminergic agonists, serotonergic agonists, modulators of GABAergic signaling, anticonvulsants, antidepressants, monoamine oxidase inhibitors, substance P (NK1) receptor

antagonists, melanocortin receptor agonists and antagonists, lipase inhibitors, inhibitors of fat absorption, regulators of energy intake or metabolism, cannabinoid receptor modulators, agents for treating addiction, agents for treating metabolic syndrome, peroxisome proliferator-activated receptor (PPAR) modulators; dipcptidyl peptidase 4 (DPP- 4) antagonists, agents for treating cardiovascular disease, agents for treating elevated triglyceride levels, agents for treating low HDL, agents for treating hypercholesterolemia, and agents for treating hypertension. Some agents for cardiovascular disease include statins (*e.g.* lovastatin, atorvastatin, fluvastatin, rosuvastatin, simvastatin and pravastatin) and omega-3 agents (*e.g.* LOVAZA, EPANQVA, VASCEPA, esterified omega-3's in general, fish oils, krill oils, algal oils). In some embodiments, additional agents may be selected from among amphetamines, benzodiazepines, suifonyl ureas, meglitinides, thiazolidinediones, biguanides, beta-blockers, XCE inhibitors, diuretics, nitrates, calcium channel blockers, phenlermine, sibutramine, iorcaserin, cetilistat, rimonabant, taranabant, topiramate, gabapentin, valproate, vigabatrin, bupropion, tiagabine, sertraline, fluoxetine, trazodone, zonisamide, methylphenidate, varenicline, naltrexone, diethylpropion, phendimetrazine, rcpaglini.de, nateglinide, glimepiride, metformin, pioglitazone, rosiglilazone, and sitagliptin.

In some embodiments, the present invention pertains to an agent used for treating diabetes as additional therapeutic agents. Illustrative anti-diabetic agents include, but are not limited to, sulfonylurea (*e.g.*. DYMELOR (acetohexamide), DIABINESE (chlorpropamide), ORINASE (tolbutamide), and TOLINASE (tolazamide), GLUCOTROL (glipizide), GLUCOTROL XL (extended release), DIABETA (glyburide), MICRONASE (glyburide), GLYNASE PRESTAB (glyburide), and AMARYL (glimepiride)); a Biguanide (*e.g.* metformin (GLUCOPHAGE, GLUCOPHAGE XR, RIOMET, FORTAMET, and GLUMETZA)); a thiazolidinedione (*e.g.* ACTOS (pioglitazone) and AVANDIA (rosiglitazone); an alpha-glucosidase inhibitor (*e.g.*, PRECOSE (acarbose) and GLYSET (miglitol); a Meglitinide (*e.g.*, PRANDIN (repaglinide) and STARLIX (nateglinide)); a Dipeptidyl peptidase IV (DPP-IV) inhibitor (*e.g.*, JANUVIA (sitagliptin), NESINA (alogliptin), ONGLYZA (saxagliptin), and TRADJENTA (linagliptin)); Sodium-glucose co-transporter 2 (SGLT2) inhibitor (*e.g.* INVOKANA (canaglifozin)); and a combination pill (*e.g.* GLUCOVANCE, which combines glyburide (a sulfonylurea) and metformin, METAGLIP, which combines glipizide (a sulfonylurea) and metformin, and AVANDAMET, which uses both metformin and rosiglitazone (AVANDIA) in one pill, KAZANO (alogliptin and metformin), OSENI (alogliptin plus pioglitazone), METFORMIN oral, ACTOS oral, BYETTA subcutaneous, JANUVIA oral, WELCHOL oral, JANUMET oral, glipizide oral, glimepiride oral, GLUCOPHAGE oral, LANTUS subcutaneous, glyburide oral, ONGLYZA oral, AMARYl oral, LANTUS SOLOSTAR subcutaneous, BYDUREON subcutaneous, LEVEMIR FLEXPEN subcutaneous, ACTOPLUS MET oral, GLUMETZA oral, TRADJENTA oral, bromocriptine oral, KOMBIGLYZE XR oral, INVOKANA oral, PRANDIN oral, LEVEMIR subcutaneous, PARLODEL oral, pioglitazone oral, NOVOLOG subcutaneous, NOVOLOG FLEXPEN subcutaneous, VICTOZA 2-PAK subcutaneous, HUMALOG subcutaneous, STARLIX oral, FORTAMET oral, GLUCOVANCE oral, GLUCOPHAGE XR oral, NOVOLOG Mix 70-30 FLEXPEN subcutaneous, GLYBURIDE-METFORMIN oral, acarbose oral, SYMLINPEN 60 subcutaneous, GLUCOTROl XL oral, NOVOLIN R inj, GLUCOTROL oral, DUETACT oral, sitagliptin oral, SYMLINPEN 120 subcutaneous, HUMALOG KWIKPEN subcutaneous, JANUMET XR oral, GLIPIZIDE-METFORMIN oral, CYCLOSET oral,

HUMALOG MIX 75-25 subcutaneous, nateglinide oral, HUMALOG Mix 75-25 KWIKPEN subcutaneous, HUMULIN 70/30 subcutaneous, PRECOSE oral, APIDRA subcutaneous, Humulin R inj, Jentadueto oral, Victoza 3-Pak subcutaneous, Novolin 70/30 subcutaneous, NOVOLIN N subcutaneous, insulin detemir subcutaneous, glyburide micronized oral, GLYNASE oral, HUMULIN N subcutaneous, insulin glargine subcutaneous, RIOMET oral, pioglitazone-metformin oral, APIDRA SOLOSTAR subcutaneous, insulin lispro subcutaneous, GLYSET oral, HUMULIN 70/30 Pen subcutaneous, colesevelam oral, sitagliptin-metformin oral, DIABETA oral, insulin regular human inj, HUMULIN N Pen subcutaneous, exenatide subcutaneous, HUMALOG Mix 50-50 KWIKPEN subcutaneous, liraglutide subcutaneous, KAZANO oral, repaglinide oral, chlorpropamide oral, insulin aspart subcutaneous, NOVOLOG Mix 70-30 subcutaneous, HUMALOG Mix 50-50 subcutaneous, saxagliptin oral, ACTOPLUS Met XR oral, miglitol oral, NPH insulin human recomb subcutaneous, insulin NPH and regular human subcutaneous, tolazamide oral, mifepristone oral, insulin aspart protam-insulin aspart subcutaneous, repaglinide-metformin oral, saxagliptin-metformin oral, linagliptin-metformin oral, NESINA oral, OSENI oral, tolbutamide oral, insulin lispro protamine and lispro subcutaneous, pramlintide subcutaneous, insulin glulisine subcutaneous, pioglitazone-glimepiride oral, PRANDIMET oral, NOVOLOG PenFill subcutaneous, linagliptin oral, exenatide microspheres subcutaneous, KORLYM oral, alogliptin oral, alogliptin-pioglitazone oral, alogliptin-metformin oral, canagliflozin oral, Lispro (HUMALOG); Aspart (NOVOLOG); Glulisine (APIDRA); Regular (NOVOLIN R or HUMULIN R); NPH (NOVOLIN N or HUMULIN N); Glargine (LANTUS); Detemir (LEVEMIR); HUMULIN or NOVOLIN 70/30; and NOVOLOG Mix 70/30 HUMALOG Mix 75/25 or 50/50.

In some embodiments, the present invention relates to combination therapy with a blood transfusion. For instance, the present compositions may supplement a blood transfusion. In some embodiments, the present invention relates to combination therapy with iron supplements.

In some embodiments, the present invention relates to combination therapy with one or more EPO-based agents. For example, the present compositions may be used as an adjuvant to other EPO-based agents. In some embodiments, the present compositions are used as a maintenance therapy to other EPO-based agents. Other EPO-based agents include the following: epoetin alfa, including without limitation, DARBEPOETIN (ARANESP), EPOCEPT (LUPIN PHARMA), NANOKINE (NANOGEN PHARMACEUTICAL), EPOFIT (INTAS PHARMA), EPOGEN (AMGEN), EPOGIN, EPREX, (JANSSEN-CILAG), BINOCRIT (SANDOZ), PROCRIT; epoetin beta, including without limitation, NEORECORMON (HOFFMANN–LA ROCHE), RECORMON, Methoxy polyethylene glycol-epoetin beta (MIRCERA, ROCHE); epoetin delta, including without limitation, DYNEPO (erythropoiesis stimulating protein, SHIRE PLC); epoetin omega, including without limitation, EPOMAX; epoetin zeta, including without limitation, SILAPO (STADA) and RETACRIT (HOSPIRA) and other EPOs, including without limitation, EPOCEPT (LUPIN PHARMACEUTICALS), EPOTRUST (PANACEA BIOTEC LTD), ERYPRO SAFE (BIOCON LTD.), REPOITIN (SERUM INSTITUTE OF INDIA LIMITED), VINTOR (EMCURE PHARMACEUTICALS), EPOFIT (INTAS PHARMA), ERYKINE (INTAS BIOPHARMACEUTICA), WEPOX (WOCKHARDT BIOTECH), ESPOGEN (LG LIFE SCIENCES), RELIPOIETIN (RELIANCE LIFE SCIENCES),

SHANPOIETIN (SHANTHA BIOTECHNICS LTD), ZYROP (CADILA HEALTHCARE LTD.), EPIAO (RHUEPO) (SHENYANG SUNSHINE PHARMACEUTICAL CO. LTD), CINNAPOIETIN (CINNAGEN).

In some embodiments, the present invention relates to combination therapy with one or more immune-modulating agents, for example, without limitation, agents that modulate immune checkpoint. In various embodiments, the immune-modulating agent targets one or more of PD-1, PD-L1, and PD-L2. In various embodiments, the immune-modulating agent is PD-1 inhibitor. In various embodiments, the immune-modulating agent is an antibody specific for one or more of PD-1, PD-L1, and PD-L2. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, 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), MPDL328OA (ROCHE). In some embodiments, the immune-modulating agent targets one or more of CD137 or CD137L. In various embodiments, the immune-modulating agent is an antibody specific for one or more of CD137 or CD137L. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, urelumab (also known as BMS-663513 and anti-4-1BB antibody). In some embodiments, the present chimeric protein is combined with urelumab (optionally with one or more of nivolumab, lirilumab, and urelumab) for the treatment of solid tumors and/or B-cell non-Hodgkins lymphoma and/or head and neck cancer and/or multiple myeloma. In some embodiments, the immune-modulating agent is an agent that targets one or more of CTLA-4, AP2M1, CD80, CD86, SHP-2, and PPP2R5A. In various embodiments, the immune-modulating agent is an antibody specific for one or more of CTLA-4, AP2M1, CD80, CD86, SHP-2, and PPP2R5A. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, ipilimumab (MDX-010, MDX-101, Yervoy, BMS) and/or tremelimumab (Pfizer). In some embodiments, the present chimeric protein is combined with ipilimumab (optionally with bavituximab) for the treatment of one or more of melanoma, prostate cancer, and lung cancer. In various embodiments, the immune-modulating agent targets CD20. In various embodiments, the immune-modulating agent is an antibody specific CD20. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, Ofatumumab (GENMAB), obinutuzumab (GAZYVA), AME-133v (APPLIED MOLECULAR EVOLUTION), Ocrelizumab (GENENTECH), TRU-015 (TRUBION/EMERGENT), veltuzumab (IMMU-106).

In some embodiments, the present invention relates to combination therapy with one or more chimeric agents described in WO 2013/10779, WO 2015/007536, WO 2015/007520, WO 2015/007542, and WO 2015/007903, the entire contents of which are hereby incorporated by reference in their entireties.

In some embodiments, the chimeric protein 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 tunicamycin, *etc.*

In still other embodiments, the chimeric protein 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 chimeric protein 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.

Illustrative cytotoxic agents include, but are not limited to, methotrexate, aminopterin, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine; alkylating agents such as mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU), mitomycin C, lomustine (CCNU), 1-methylnitrosourea, cyclothosphamide, mechlorethamine, busulfan, dibromomannitol, streptozotocin, mitomycin C, cis-dichlorodiamine platinum (II) (DDP) cisplatin and carboplatin (paraplatin); anthracyclines include daunorubicin (formerly daunomycin), doxorubicin (adriamycin), detorubicin, carminomycin, idarubicin, epirubicin, mitoxantrone and bisantrene; antibiotics include dactinomycin (actinomycin D), bleomycin, calicheamicin, mithramycin, and anthramycin (AMC); and antimytotic agents such as the vinca alkaloids, vincristine and vinblastine. Other cytotoxic agents include paclitaxel (taxol), ricin, pseudomonas exotoxin, gemcitabine, cytochalasin B, gramicidin D, ethidium bromide, emetine, etoposide, tenoposide, colchicin, dihydroxy anthracin dione, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, procarbazine, hydroxyurea, asparaginase, corticosteroids, mytotane (O,P'-(DDD)), interferons, and mixtures of these cytotoxic agents.

Further cytotoxic agents include, but are not limited to, chemotherapeutic agents such as carboplatin, cisplatin, paclitaxel, gemcitabine, calicheamicin, doxorubicin, 5-fluorouracil, mitomycin C, actinomycin D, cyclophosphamide, vincristine, bleomycin, VEGF antagonists, EGFR antagonists, platins, taxols, irinotecan, 5-fluorouracil, gemcytabine, leucovorine, steroids, cyclophosphamide, melphalan, vinca alkaloids (*e.g.*, vinblastine, vincristine, vindesine and vinorelbine), mustines, tyrosine kinase inhibitors, radiotherapy, sex hormone antagonists, selective androgen receptor modulators, selective estrogen receptor modulators, PDGF antagonists, TNF antagonists, IL-1 antagonists, interleukins (*e.g.* IL-12 or IL-2), IL-12R antagonists, Toxin conjugated monoclonal antibodies, tumor antigen specific monoclonal antibodies, Erbitux, Avastin, Pertuzumab, anti-CD20 antibodies, Rituxan, ocrelizumab, ofatumumab, DXL625, HERCEPTIN®, or any combination thereof. Toxic enzymes from plants and bacteria such as ricin, diphtheria toxin and Pseudomonas toxin may be conjugated to the therapeutic agents (*e.g.* antibodies) to generate cell-type-specific-killing reagents (Youle, *et al.*, Proc. Nat'l Acad. Sci. USA 77:5483 (1980); Gilliland, *et al.*, Proc. Nat'l Acad. Sci. USA 77:4539 (1980); Krolick, *et al.*, Proc. Nat'l Acad. Sci. USA 77:5419 (1980)).

Other cytotoxic agents include cytotoxic ribonucleases as described by Goldenberg in U.S. Pat. No. 6,653,104. Embodiments of the invention also relate to radioimmunoconjugates where a radionuclide that emits alpha or beta particles is stably coupled to the chimeric protein, with or without the use of a complex-forming agent. Such radionuclides include beta-emitters such as Phosphorus-32, Scandium-47, Copper-67, Gallium-67, Yttrium-88, Yttrium-90, Iodine-125, Iodine-131, Samarium-153, Lutetium-177, Rhenium-186 or Rhenium-188, and alpha-emitters such as Astatine-211, Lead-212, Bismuth-212, Bismuth-213 or Actinium-225.

Illustrative detectable moieties further include, but are not limited to, horseradish peroxidase, acetylcholinesterase, alkaline phosphatase, beta-galactosidase and luciferase. Further illustrative fluorescent materials include, but are not limited to, rhodamine, fluorescein, fluorescein isothiocyanate, umbelliferone, dichlorotriazinylamine, phycoerythrin and dansyl chloride. Further illustrative chemiluminescent moieties include, but are not limited to, luminol. Further illustrative bioluminescent materials include, but are not limited to, luciferin and aequorin. Further illustrative radioactive materials include, but are not limited to, Iodine-125, Carbon-14, Sulfur-35, Tritium and Phosphorus-32.

Methods of Treatment

Methods and compositions described herein have application to treating various diseases and disorders, including, but not limited to cancer, infections, immune disorders, anemia, autoimmune diseases, cardiovascular diseases, wound healing, ischemia-related diseases, neurodegenerative diseases, metabolic diseases and many other diseases and disorders.

Further, any of the present agents may be for use in the treating, or the manufacture of a medicament for treating, various diseases and disorders, including, but not limited to cancer, infections, immune disorders, inflammatory diseases or conditions, and autoimmune diseases.

In some embodiments, the present invention relates to the treatment of, or a patient having one or more of cancer, heart failure, autoimmune disease, sickle cell disease, thalassemia, blood loss, transfusion reaction, diabetes, vitamin B12 deficiency, collagen vascular disease, Shwachman syndrome, thrombocytopenic purpura, Celiac disease, endocrine deficiency state such as hypothyroidism or Addison's disease, autoimmune disease such as Crohn's Disease, systemic lupus erythematosis, rheumatoid arthritis or juvenile rheumatoid arthritis, ulcerative colitis immune disorders such as eosinophilic fasciitis, hypoimmunoglobulinemia, or thymoma/thymic carcinoma, graft versus host disease, preleukemia, Nonhematologic syndrome (*e.g.* Down's, Dubowwitz, Seckel), Felty syndrome, hemolytic uremic syndrome, myelodysplasic syndrome, nocturnal paroxysmal hemoglobinuria, osteomyelofibrosis, pancytopenia, pure red-cell aplasia, Schoenlein-Henoch purpura, malaria, protein starvation, menorrhagia, systemic sclerosis, liver cirrhosis, hypometabolic states, and congestive heart failure.

In some embodiments, the present invention relates to the treatment of, or a patient having cancer. As used herein, cancer refers to any uncontrolled growth of cells that may interfere with the normal functioning of the bodily organs and systems, and includes both primary and metastatic tumors. Primary tumors or cancers that

migrate from their original location and seed vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs. A metastasis is a cancer cell or group of cancer cells, distinct from the primary tumor location, resulting from the dissemination of cancer cells from the primary tumor to other parts of the body. Metastases may eventually result in death of a subject. For example, cancers can include benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases.

Illustrative cancers that may be treated include, but are not limited to, carcinomas, *e.g.* various subtypes, including, for example, adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma), sarcomas (including, for example, bone and soft tissue), leukemias (including, for example, acute myeloid, acute lymphoblastic, chronic myeloid, chronic lymphocytic, and hairy cell), lymphomas and myelomas (including, for example, Hodgkin and non-Hodgkin lymphomas, light chain, non-secretory, MGUS, and plasmacytomas), and central nervous system cancers (including, for example, brain (*e.g.* gliomas (*e.g.* astrocytoma, oligodendroglioma, and ependymoma), meningioma, pituitary adenoma, and neuromas, and spinal cord tumors (*e.g.* meningiomas and neurofibroma).

Illustrative cancers that may be treated include, but are not limited to, 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 present invention relates to the treatment of, or a patient having a microbial infection and/or chronic infection. Illustrative infections include, but are not limited to, HIV/AIDS, tuberculosis, osteomyelitis, hepatitis B, hepatitis C, Epstein-Barr virus or parvovirus, T cell leukemia virus, bacterial overgrowth syndrome, fungal or parasitic infections.

In various embodiments, the present compositions are used to treat or prevent one or more inflammatory diseases or conditions, such as inflammation, acute inflammation, chronic inflammation, respiratory disease, atherosclerosis, restenosis, asthma, allergic rhinitis, atopic dermatitis, septic shock, rheumatoid arthritis, inflammatory bowel disease, inflammatory pelvic disease, pain, ocular inflammatory disease, celiac disease, Leigh Syndrome, Glycerol Kinase Deficiency, Familial eosinophilia (FE), autosomal recessive spastic ataxia, laryngeal inflammatory disease; Tuberculosis, Chronic cholecystitis, Bronchiectasis, Silicosis and other pneumoconioses.

In various embodiments, the present compositions are used to treat or prevent one or more autoimmune diseases or conditions, such as 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 compositions are used to treat, control or prevent cardiovascular disease, such as a disease or condition affecting the heart and vasculature, including but not limited to, coronary heart disease (CHD), cerebrovascular disease (CVD), aortic stenosis, peripheral vascular disease, atherosclerosis, arteriosclerosis, myocardial infarction (heart attack), cerebrovascular diseases (stroke), transient ischaemic attacks (TIA), angina (stable and unstable), atrial fibrillation, arrhythmia, vavular disease, and/or congestive heart failure.

In various embodiments, the present compositions are used to treat or prevent one or more metabolic-related disorders. In various embodiments, the present invention is useful for the treatment, controlling or prevention of diabetes, including Type 1 and Type 2 diabetes and diabetes associated with obesity. The compositions and methods of the present invention are useful for the treatment or prevention of diabetes-related disorders, including without limitation diabetic nephropathy, hyperglycemia, impaired glucose tolerance, insulin resistance, obesity, lipid disorders, dyslipidemia, hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, low HDL levels, high LDL levels, atherosclerosis and its sequelae, vascular restenosis, irritable bowel syndrome, inflamatory bowel disease, including Crohn's disease and ulcerative colitis, other inflammatory conditions, pancreatitis, abdominal obesity, neurodegenerative disease, retinopathy, neoplastic conditions, adipose cell tumors, adipose cell carcinomas, such as liposarcoma, prostate cancer and other cancers, including gastric, breast, bladder and colon cancers, angiogenesis, Alzheimer's disease, psoriasis, high blood pressure, Metabolic Syndrome (*e.g.* a person has three or more of the following disorders: abdominal obesity, hypertriglyceridemia, low HDL cholesterol, high blood pressure, and high fasting plasma glucose), ovarian hyperandrogenism (polycystic ovary syndrome), and other disorders where insulin resistance is a component, such as sleep apnea. The compositions and methods of the present invention are useful for the treatment, control, or prevention of obesity, including

genetic or environmental, and obesity-related disorders. The obesity-related disorders herein are associated with, caused by, or result from obesity. Examples of obesity-related disorders include obesity, diabetes, overeating, binge eating, and bulimia, hypertension, elevated plasma insulin concentrations and insulin resistance, dyslipidemia, hyperlipidemia, endometrial, breast, prostate, kidney and colon cancer, osteoarthritis, obstructive sleep apnea, gallstones, heart disease, abnormal heart rhythms and arrythmias, myocardial infarction, congestive heart failure, coronary heart disease, sudden death, stroke, polycystic ovary disease, craniopharyngioma, Prader-Willi Syndrome, Frohlich's syndrome, GH-deficient subjects, normal variant short stature, Turner's syndrome, and other pathological conditions showing reduced metabolic activity or a decrease in resting energy expenditure as a percentage of total fat-free mass, e.g, children with acute lymphoblastic leukemia. Further examples of obesity-related disorders are Metabolic Syndrome, insulin resistance syndrome, reproductive hormone abnormalities, sexual and reproductive dysfunction, such as impaired fertility, infertility, hypogonadism in males and hirsutism in females, fetal defects associated with maternal obesity, gastrointestinal motility disorders, such as obesity-related gastro-esophageal reflux, respiratory disorders, such as obesity-hypoventilation syndrome (Pickwickian syndrome), breathlessness, cardiovascular disorders, inflammation, such as systemic inflammation of the vasculature, arteriosclerosis, hypercholesterolemia, lower back pain, gallbladder disease, hyperuricemia, gout, and kidney cancer, and increased anesthetic risk. The compositions and methods of the present invention are also useful to treat Alzheimer's disease.

In various embodiments, the present compositions are used to treat or prevent one or more respiratory diseases, such as asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, allergic rhinitis, sinusitis, pulmonary vasoconstriction, inflammation, allergies, impeded respiration, respiratory distress syndrome, cystic fibrosis, pulmonary hypertension, pulmonary vasoconstriction, emphysema, Hantavirus pulmonary syndrome (HPS), Loeffler's syndrome, Goodpasture's syndrome, Pleurisy, pneumonitis, pulmonary edema, pulmonary fibrosis, Sarcoidosis, complications associated with respiratory syncitial virus infection, and other respiratory diseases.

In some embodiments, the present invention is used to treat or prevent one or more neurodegenerative disease. Illustrative neurodegenerative disease include, but are not limited to, multiple sclerosis (including without limitation, benign multiple sclerosis; relapsing-remitting multiple sclerosis (RRMS); secondary progressive multiple sclerosis (SPMS); progressive relapsing multiple sclerosis (PRMS); and primary progressive multiple sclerosis (PPMS)), Alzheimer's. disease (including, without limitation, Early-onset Alzheimer's, Late-onset Alzheimer’s, and Familial Alzheimer’s disease (FAD), Parkinson’s disease and parkinsonism (including, without limitation, Idiopathic Parkinson's disease, Vascular parkinsonism, Drug-induced parkinsonism, Dementia with Lewy bodies, Inherited Parkinson's, Juvenile Parkinson's), Huntington's disease, Amyotrophic lateral sclerosis (ALS, including, without limitation, Sporadic ALS, Familial ALS, Western Pacific ALS, Juvenile ALS, Hiramaya Disease).

In various embodiments, the present chimeric proteins find use in treating wounds, *e.g.*, a non-healing wound, an ulcer, a burn, or frostbite, a chronic or acute wound, open or closed wound, internal or external wound (illustrative

external wounds are penetrating and non-penetrating wound. In various embodiments, the present chimeric proteins find use in treating ischemia, by way of non-limiting example, ischemia associated with acute coronary syndrome, acute lung injury (ALI), acute myocardial infarction (AMI), acute respiratory distress syndrome (ARDS), arterial occlusive disease, arteriosclerosis, articular cartilage defect, aseptic systemic inflammation, atherosclerotic cardiovascular disease, autoimmune disease, bone fracture, bone fracture, brain edema, brain hypoperfusion, Buerger's disease, burns, cancer, cardiovascular disease, cartilage damage, cerebral infarct, cerebral ischemia, cerebral stroke, cerebrovascular disease, chemotherapy-induced neuropathy, chronic infection, chronic mesenteric ischemia, claudication, congestive heart failure, connective tissue damage, contusion, coronary artery disease (CAD), critical limb ischemia (CLI), Crohn's disease, deep vein thrombosis, deep wound, delayed ulcer healing, delayed wound-healing, diabetes (type I and type II), diabetic neuropathy, diabetes induced ischemia, disseminated intravascular coagulation (DIC), embolic brain ischemia, frostbite, graft-versus-host disease, hereditary hemorrhagic telengiectasiaischemic vascular disease, hyperoxic injury, hypoxia, inflammation, inflammatory bowel disease, inflammatory disease, injured tendons, intermittent claudication, intestinal ischemia, ischemia, ischemic brain disease, ischemic heart disease, ischemic peripheral vascular disease, ischemic placenta, ischemic renal disease, ischemic vascular disease, ischemic-reperfusion injury, laceration, left main coronary artery disease, limb ischemia, lower extremity ischemia, myocardial infarction, myocardial ischemia, organ ischemia, osteoarthritis, osteoporosis, osteosarcoma, Parkinson's disease, peripheral arterial disease (PAD), peripheral artery disease, peripheral ischemia, peripheral neuropathy, peripheral vascular disease, pre-cancer, pulmonary edema, pulmonary embolism, remodeling disorder, renal ischemia, retinal ischemia, retinopathy, sepsis, skin ulcers, solid organ transplantation, spinal cord injury, stroke, subchondral-bone cyst, thrombosis, thrombotic brain ischemia, tissue ischemia, transient ischemic attack (TIA), traumatic brain injury, ulcerative colitis, vascular disease of the kidney, vascular inflammatory conditions, von Hippel-Lindau syndrome, or wounds to tissues or organs

In various embodiments, the present invention relates to the treatment of one or more of anemia, including anemia resulting from chronic kidney disease (*e.g.* from dialysis) and/or an anti-cancer agent (*e.g.* chemotherapy and/or HIV treatment (*e.g.* Zidovudine (INN) or azidothymidine (AZT)), inflammatory bowel disease (*e.g.* Crohn's disease and ulcer colitis), anemia linked to inflammatory conditions (*e.g.* arthritis, lupus, IBD), anemia linked to diabetes, schizophrenia, cerebral malaria, as aplastic anemia, and myelodysplasia from the treatment of cancer (*e.g.* chemotherapy and/or radiation), and various myelodysplastic syndrome diseases (*e.g.* sickle cell anemia, hemoglobin SC disease, hemoglobin C disease, alpha- and beta-thalassemias, neonatal anemia after premature birth, and comparable conditions).

In some embodiments, the present invention relates to the treatment of, or a patient having anemia, *i.e.* a condition in which the number of red blood cells and/or the amount of hemoglobin found in the red blood cells is below normal. In various embodiments, the anemia may be acute or chronic. For example, the present anemias include but are not limited to iron deficiency anemia, renal anemia, anemia of chronic diseases/inflammation, pernicious anemia such as macrocytic achylic anemia, juvenile pernicious anemia and congenital pernicious

anemia, cancer-related anemia, anti-cancer-related anemia (*e.g.* chemotherapy-related anemia, radiotherapy-related anemia), pure red cell aplasia, refractory anemia with excess of blasts, aplastic anemia, X-lined siderobalstic anemia, hemolytic anemia, sickle cell anemia, anemia caused by impaired production of ESA, myelodysplasia syndromes, hypochromic anemia, microcytic anemia, sideroblastic anemia, autoimmune hemolytic anemia, Cooley's anemia, Mediterranean anemia, Diamond Blackfan anemia, Fanconi's anemia and drug-induced immune hemolytic anemia. Anemia may cause serious symptoms, including hypoxia, chronic fatigue, lack of concentration, pale skin, low blood pressure, dizziness and heart failure.

In some embodiments, the present invention relates to the treatment of anemia resulting from chronic renal failure. In some embodiments, the present invention relates to the treatment of anemia resulting from the use of one or more renal replacement therapies, inclusive of dialysis, hemodialysis, peritoneal dialysis, hemofiltration, hemodiafiltration, and renal transplantation.

In some embodiments, the present invention relates to the treatment of anemia in patients with chronic kidney disease who are not on dialysis. For instance, the present invention relates to patients in stage 1 CKD, or stage 2 CKD, or stage 3 CKD, or stage 4 CKD, or stage 5 CKD. In some embodiments, the present patient is stage 4 CKD or stage 5 CKD. In some embodiments, the present patient has undergone a kidney transplant. In some embodiments, the present invention relates to the treatment of anemia is a patient having an acute kidney injury (AKI).

In some embodiments, the anemia is induced by chemotherapy. For instance, the chemotherapy may be any myelosuppressive chemotherapy. In some embodiment, the chemotherapy is one or more of Revlimid, Thalomid, dexamethasone, Adriamycin and Doxil. In some embodiments, the chemotherapy is one or more platinum-based drugs including cisplatin (*e.g.* PLATINOL) and carboplatin (*e.g.* PARAPLATIN). In some embodiments, the chemotherapy is any one of the chemotherapeutic agents described herein. In some embodiments, the chemotherapy is any agent described in Groopman *et al.* J Natl Cancer Inst (1999) 91 (19): 1616-1634, the contents of which are hereby incorporated by reference in their entireties. In some embodiments, the present compositions and methods are used in the treatment of chemotherapy-related anemia in later stage cancer patients (*e.g.* a stage IV, or stage III, or stage II cancer). In some embodiments, the present compositions and methods are used in the treatment of chemotherapy-related anemia in cancer patients receiving dose-dense chemotherapy or other aggressive chemotherapy regimens.

In some embodiments, the present invention relates to the treatment of anemia in a patient having one or more blood-based cancers, such as leukemia, lymphoma, and multiple myeloma. Such cancers may affect the bone marrow directly. Further, the present invention relates to metastatic cancer that has spread to the bone or bone marrow. In some embodiments, the present invention relates to the treatment of anemia in a patient undergoing radiation therapy. Such radiation therapy may damage the bone marrow, lowering its ability to make red blood cells. In further embodiments, the present invention relates to the treatment of anemia in a patient having a reduction or deficiency of one or more of iron, vitamin B12, and folic acid. In further embodiments, the present

invention relates to the treatment of anemia in a patient having excessive bleeding including without limitation, after surgery or from a tumor that is causing internal bleeding. In further embodiments, the present invention relates to the treatment of anemia in a patient having anemia of chronic disease.

In some embodiments, the present methods and compositions stimulate red blood cell production. In some embodiments, the present methods and compositions stimulate division and differentiation of committed erythroid progenitors in the bone marrow.

Certain embodiments of the present invention are particularly useful for treating chemotherapy-induced anemia in cancer patients. In some embodiments, the present methods and compositions allows for continued administration of the chimeric protein after a cancer patient's chemotherapy is finished. In some embodiments, the present methods and compositions allows for treatment of a cancer patient without dose reduction relative to a non-cancer patient. In some embodiments, the present methods and compositions allows for treatment of a cancer patient receiving chemotherapy and considered curable. In various embodiments, the cancer patient has one or more of a history of blood clots, recent surgery, prolonged periods of bed rest or limited activity, and treatment with a chemotherapeutic agent.

Kits

The invention also provides kits for the administration of any agent described herein (*e.g.* the chimeric protein with or without various additional therapeutic agents). The kit is an assemblage of materials or components, including at least one of the inventive pharmaceutical compositions described herein. Thus, in some embodiments, the kit contains at least one of the pharmaceutical compositions described herein.

The exact nature of the components configured in the kit depends on its intended purpose. In one embodiment, the kit is configured for the purpose of treating human subjects.

Instructions for use may be included in the kit. Instructions for use typically include a tangible expression describing the technique to be employed in using the components of the kit to effect a desired outcome, such as to treat anemia. Optionally, the kit also contains other useful components, such as, diluents, buffers, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or measuring tools, bandaging materials or other useful paraphernalia as will be readily recognized by those of skill in the art.

The materials and components assembled in the kit can be provided to the practitioner stored in any convenience and suitable ways that preserve their operability and utility. For example, the components can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging materials. In various embodiments, the packaging material is constructed by well-known methods, preferably to provide a sterile, contaminant-free environment. The packaging material may have an external label which indicates the contents and/or purpose of the kit and/or its components.

Definitions

As used herein, "a," "an," or "the" can mean one or more than one.

Further, the term "about" when used in connection with a referenced numeric indication means the referenced numeric indication plus or minus up to 10% of that referenced numeric indication. For example, the language "about 50" covers the range of 45 to 55.

An "effective amount," when used in connection with medical uses is an amount that is effective for providing a measurable treatment, prevention, or reduction in the rate of pathogenesis of a disease of interest.

As used herein, something is "decreased" if a read-out of activity and/or effect is reduced by a significant amount, such as by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or more, up to and including at least about 100%, in the presence of an agent or stimulus relative to the absence of such modulation. As will be understood by one of ordinary skill in the art, in some embodiments, activity is decreased and some downstream read-outs will decrease but others can increase.

Conversely, activity is "increased" if a read-out of activity and/or effect is increased by a significant amount, for example by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or more, up to and including at least about 100% or more, at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 50-fold, at least about 100-fold, in the presence of an agent or stimulus, relative to the absence of such agent or stimulus.

As referred to herein, all compositional percentages are by weight of the total composition, unless otherwise specified. As used herein, the word "include," and its variants, is intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the compositions and methods of this technology. Similarly, the terms "can" and "may" and their variants are intended to be non-limiting, such that recitation that an embodiment can or may comprise certain elements or features does not exclude other embodiments of the present technology that do not contain those elements or features.

Although the open-ended term "comprising," as a synonym of terms such as including, containing, or having, is used herein to describe and claim the invention, the present invention, or embodiments thereof, may alternatively be described using alternative terms such as "consisting of" or "consisting essentially of."

As used herein, the words "preferred" and "preferably" refer to embodiments of the technology that afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the technology.

The amount of compositions described herein needed for achieving a therapeutic effect may be determined empirically in accordance with conventional procedures for the particular purpose. Generally, for administering therapeutic agents for therapeutic purposes, the therapeutic agents are given at a pharmacologically effective dose. A "pharmacologically effective amount," "pharmacologically effective dose," "therapeutically effective amount," or "effective amount" refers to an amount sufficient to produce the desired physiological effect or amount capable of achieving the desired result, particularly for treating the disorder or disease. An effective amount as used herein would include an amount sufficient to, for example, delay the development of a symptom of the disorder or disease, alter the course of a symptom of the disorder or disease (*e.g.*, slow the progression of a symptom of the disease), reduce or eliminate one or more symptoms or manifestations of the disorder or disease, and reverse a symptom of a disorder or disease. Therapeutic benefit also includes halting or slowing the progression of the underlying disease or disorder, regardless of whether improvement is realized.

Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to about 50% of the population) and the ED50 (the dose therapeutically effective in about 50% of the population). The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. In some embodiments, compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from in vitro assays, including, for example, cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 as determined in cell culture, or in an appropriate animal model. Levels of the described compositions in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment.

In certain embodiments, the effect will result in a quantifiable change of at least about 10%, at least about 20%, at least about 30%, at least about 50%, at least about 70%, or at least about 90%. In some embodiments, the effect will result in a quantifiable change of about 10%, about 20%, about 30%, about 50%, about 70%, or even about 90% or more. Therapeutic benefit also includes halting or slowing the progression of the underlying disease or disorder, regardless of whether improvement is realized.

As used herein, "methods of treatment" are equally applicable to use of a composition for treating the diseases or disorders described herein and/or compositions for use and/or uses in the manufacture of a medicaments for treating the diseases or disorders described herein.This invention is further illustrated by the following non-limiting examples.

EXAMPLES

The term "AcTaferon" is occasionally used herein to reference an interferon-based chimera.

In the following examples, unless noted, mutations to IFN are relative to human IFN-α2 - SEQ ID NO: 336.

The Q124R mutant is representative of an attenuated human IFN alpha 2 mutant that can be assayed *in vivo* in a murine model. Specifically, Q124R is a human IFN mutation that is suitable for use in the mouse (*i.e.* it is a human mutant IFN that functions in mouse). *See Nat. Comm.* 2014;5:3016. doi: 10.1038/ncomms4016, the entire contents of which are hereby incorporated by reference.

The R33A/E120R mutant is representative of human IFN alpha 2 mutant that is non-functional (and is used as a control)

Anti-human PD-1 VHH used in these Examples is SEQ ID NO: 132.

Anti-human PD-L1 VHH used in these Examples is SEQ ID NO: 267.

Anti-human CLEC9A VHH used in these Examples is R2CHCL24 (SEQ ID NO: 87)

Anti-human CD3 scFV used in these Examples is SEQ ID NO: 365.

Anti-human CD20 scFV used in these Examples is SEQ ID NO: 366.

Anti-human CD8 VHH used in these Examples is R2HCD26 (SEQ ID NO:21).

Anti-BclI10 VHH is used in these Examples as a control (targeting an irrelevant antigen, *i.e.* "untargeted").

Example 1: Characterization of Bispecific Chimeras Using an In Vivo B16 Melanoma Model.

Various bispecific chimeric constructs were engineered with anti- mouse PD-L1, anti- mouse CD20, anti- mouse Clec9A, and/or anti- mouse CD8 targeting moieties and a modified interferon signaling domain (*i.e.*, the mutant human interferon (IFN) α2-Q124R or R149A). A list of the chimeric constructs is provided below. The *in vivo* activities of the chimera were characterized using an *in vivo* B16 melanoma model.

- PBS
- BCII.10-Q124R (10120)
- PD-L1-Q124R (12246)
- CLEC9A-Q124R (10666)
- CLEC9A-Q124R-PD-L1 (11645)
- PD-L1-Q124R-CLEC9A (12938)
- CLEC9A-PD-L1-Q124R (13271)
- PD-L1-CLEC9A-Q124R (13272)

- CLEC9A-R149A (10685)
- CLEC9A-R149A-PD-L1 (13305)
- PD-L1-R149A-CLEC9A
- CLEC9A-PD-L1-R149A (13331)
- PD-L1-CLEC9A-R149A (13332)

- CD8-Q124R (11487)
- CD8-Q124R-PD-L1 (12277)
- PD-L1-Q124R-CD8 (12939)
- CD8-PD-L1-Q124R (13309)
- PD-L1-CD8-Q124R (13310)

- CD8-R149A (13339)
- CD8-R149A-PD-L1 (13306)
- PD-L1-R149A-CD8
- CD8-PD-L1-R149A (13337)
- PD-L1-CD8-R149A (13338)

- CLEC9A-Q124R-CD8 (12942)
- CD8-Q124R-CLEC9A (12941)
- CLEC9A-CD8-Q124R (13276)
- CD8-CLEC9A-Q124R (13308)

- CLEC9A-R149A-CD8
- CD8-R149A-CLEC9A
- CLEC9A-CD8-R149A
- CD8-CLEC9A-R149A

FIG. 1 shows the anti-tumor activities of an anti-CD20 VHH-human IFNalpha (Q124R) mutant-anti-PD-L1 VHH bispecific construct using a B16 tumor model. In the experiment, C57BL/6 mice were inoculated subcutaneously (50 µl) with $6x10^5$ B16mCD20cl1 melanoma tumor cells (a mouse melanoma cell line that stably expresses mouse CD20). Perilesional treatment with 30 µg of various constructs (100 µl) was started when tumors reached a size of ± 10 mm^2 as measured by caliper. The constructs tested included a monospecific anti-PD-L1 VHH, a monospecific anti-PD-L1 VHH-human IFNalpha, Q124R mutant construct, a monospecific anti-CD20 VHH-human IFNalpha, Q124R mutant construct, and the bispecific anti-CD20 VHH-human IFNalpha, Q124R mutant-anti-PD-L1 VHH construct. **FIG. 1**, panel A, shows that anti-PD-L1 had no effect in the B16 tumor model while the monospecific PD-L1-Q124R construct showed some anti-tumor activity and reduced tumor size. Panel B shows that a bispecific chimera that targets both CD20 and PD-L1 ("mCD20-Q124R-PD-L1") was superior to a monospecific chimera that only targets CD20 ("mCD20-Q124R") with respect to anti-tumor activity (panel B). In both the bispecific and monospecific constructs, a modified interferon signaling domain was present (Q124R).

FIG. 2 shows the anti-tumor activities of a bispecific chimera ("Clec9A-Q124R-PD-L1") using the B16 tumor model described above. As shown in **FIG. 2**, the bispecific chimera Clec9A-Q124R-PD-L1 was superior to a monospecific anti-Clec9a chimera ("Clec9A-Q124R") and PD-L1 ("PD-L1-Q124R") chimera. All of the chimeras were fused to modified interferon: Q124R.

Both mCD20-Q124R-PD-L1 and Clec9A-Q124R-PD-L1 bi-specific constructs were shown to be safe (**FIG. 3** and **FIG. 4**). In each figure, panels A-G show: change in body weight of the mice in the tumor studies described

above (panel A), white blood cell counts ("wbc") and lymphocytes count ("ly") (panel B), neutrophil count ("ne") and monocyte count ("mo") (panel C); red blood cell count ("rbc") and, for FIG. 4, hemoglobin ("hb") (panel D); hemocrit ("hct"), mean corpuscular volume ("mcv"), mean corpuscular hemoglobin ("mch"), mean corpuscular hemoglobin concentration ("mchc") (panel E); pitted red blood cells ("pit") (panel F); and mean platelet volume ("mpv") (panel G).

FIG. 5 shows a B16 tumor model study in which a bi-specific (anti-Clec9A and anti-PD-L1) fusion to modified human IFN alpha (Q124R) provided better anti-tumor activity as compared to the co-administration of a fusion of anti-Clec9A to modified human IFN alpha (Q124R) and a fusion of anti-PD-L1 to modified human IFN alpha (Q124R) or the co-administration of a fusion of anti-Clec9A to modified human IFN alpha (Q124R) and an anti-PD-L1 VHH.

The anti-tumor activities of a bispecific chimeric ("CD8-Q124R-PD-L1") was also analyzed using the B16 melanoma model. As shown in **FIG. 6**, the bi-specific (anti-CD8 and anti-PD-L1) fusion to modified human IFN alpha (Q124R) provided better anti-tumor activity as compared to a fusion of anti-CD8 to modified human IFN alpha (Q124R).

A Batf3 knockout (Batf3-/-) mouse model was utilized to study the role of cross-presenting dendritic cells in tumorigenesis and the anti-tumor activities of the bispecific constructs. The Batf3 -/- C57BL6 mice lack the Batf3 gene and thus do not have CD8α+ cross-presenting dendritic cells. In the experiment, Batf3 -/- mice were inoculated subcutaneously with B16mCD20cl1 melanoma tumor cells. When tumors reached a certain size, the mice were then treated with a bi-specific (anti-Clec9A and anti-PD-L1) fusion to modified human IFN alpha (Q124R). As shown in **FIG. 7**, the Batf3 -/- mice were more susceptible to tumor formation and formed larger tumors than wild type C57BL6 mice. Administration of the bispecific (anti-Clec9A and anti-PD-L1) fusion to modified human IFN alpha (Q124R) reduced tumor size in both Batf3 -/- and wild type C57BL6 mice. Further, without wishing to be bound by theory, comparing the effectiveness of the bispecific (anti-Clec9A and anti-PD-L1) fusion to modified human IFN alpha (Q124R) in the two genetic backgrounds underscores the importance of dendritic cells for Clec9A targeting.

Example 2: Characterization of Bispecific Chimeras Using an In Vivo 4T1 Mammary Tumor Model.

The antitumor activities of the various mono-specific and bi-specific chimeric constructs were also tested using a 4T1 mammary tumor model. In the experiment, mice were inoculated with 4T1 mammary tumor cells. Once tumors reached a certain size, the mice were treated with a monospecific anti-PD-L1 VHH or a monospecific anti-PD-L1 VHH-human IFNalpha, Q124R mutant construct. As shown in **FIG. 8**, panel A, anti-PD-L1 has no effect in the 4T1 mammary tumor model while the monospecific PD-L1-Q124R construct had anti-tumor activity.

FIG. 8, panel B shows a 4T1 mammary tumor model study in which a bi-specific (anti-Clec9A and anti-PD-L1) fusion to modified human IFN alpha (Q124R) provided better anti-tumor activity as compared to the co-administration of a fusion of anti-Clec9A to modified human IFN alpha (Q124R) and a fusion of anti-PD-L1 to

modified human IFN alpha (Q124R) or the co-administration of a fusion of anti-Clec9A to modified human IFN alpha (Q124R) and an anti-PD-L1 VHH.

FIG. 8, panel C shows a 4T1 mammary tumor model study in which a bi-specific (anti-CD8 and anti-PD-L1) fusion to modified human IFN alpha (Q124R) provided better anti-tumor activity as compared to the co-administration of a fusion of anti-CD8 to modified human IFN alpha (Q124R) and a fusion of anti-PD-L1 to modified human IFN alpha (Q124R) or the co-administration of a fusion of anti-CD8 to modified human IFN alpha (Q124R) and an anti-PD-L1 VHH.

Example 3: Combination Therapy Using Bispecific Chimera and Doxorubicin in the B16 Melanoma and 4T1 Mammary Tumor Models

The anti-tumor effects of a combination therapy using doxorubicin and bispecific constructs were tested. In one set of experiments, mice were transplanted with B16 cells (a mouse melanoma cell line that stably expresses mouse CD20) to induce tumors. In a second set of experiments, the mice were transplanted with 4T1 mammary tumor cells. The mice were subsequently treated with a bi-specific (anti- mouse Clec9A and anti- mouse PD-L1) fusion to modified human IFN alpha (Q124R) with or without doxorubicin. In a third set of experiment, the mice were transplanted with 4T1 mammary tumor cells and subsequently treated with a bi-specific (anti-CD8 and anti-PD-L1) fusion to modified human IFN alpha (Q124R) with or without doxorubicin. As shown in **FIG. 9**, panels A-C, in all three tumor models, combining the bispecific chimera with doxorubicin significantly reduced tumor size compared with treatment using the bispecific chimera alone or doxorubicin alone. Notably as shown in **FIG. 9**, panel B, in the 4T1 mammary tumor model, the use of the bi-specific (anti-Clec9A and anti-PD-L1) fusion to modified human IFN alpha (Q124R) with doxorubicin resulted in a curative effect in 5 out of 6 mice (*i.e.*, the mice were completely tumor free). Similarly, as shown in **FIG. 9**, panel C, in the 4T1 mammary tumor model, the use of the bi-specific (anti-CD8 and anti-PD-L1) fusion to modified human IFN alpha (Q124R) with doxorubicin also resulted in a curative effect in 3 out of 6 mice (*i.e.*, the mice were completely tumor free). Without wishing to be bound by theory, it is believed that the combination of the bispecific agent and doxorubicin resulted in a synergistic effect (*i.e.*, complete eradiation of tumors) compared to use of the agents individually.

The 4T1 tumor model was utilized conduct a tumor re-challenge experiment, in which mice that remained tumor free (*i.e.*, the 5 mice that were tumor free as demonstrated in **FIG. 9**, panel B) were re-challenged with additional 4T1 tumor cells. Specifically, in the experiment, mice were initially inoculated with 4T1 mammary tumor cells and treated with a combination of doxorubicin and a fusion of anti-Clec9A to modified human IFN alpha (Q124R) or a bi-specific (anti-Clec9A and anti-PD-L1) fusion to modified human IFN alpha (Q124R) as described previously (for example, in **FIG. 9**). Mice that remained tumor free were then re-challenged with 10^5 4T1 tumor cells and monitored for tumor growth without any additional treatment.

Without wishing to be bound by theory, **FIG. 10** shows that the present bi-specific (anti-Clec9A and anti-PD-L1) fusion to modified human IFN alpha (Q124R) provided an anti-tumor memory effect.

Example 4: Efficacy of Bispecific Chimera in a Mouse Model of Multiple Sclerosis

The efficacy of the bispecific constructs in treating multiple sclerosis was tested using an established mouse experimental autoimmune encephalomyelitis (EAE) model. Specifically, the mice were subcutaneously administered peptides corresponding to the immunodominant epitopes of MOG (MOG_{35-55}) suspended in complete Freund's adjuvant (CFA) derived from killed Mycobacterium tuberculosis bacilli (Mtb). Pertussis toxin was also administered to the mice by intraperitoneal injections on the day of and two days later. The mice were then treated from days 7-25 with either 100 IU or 1000 IU of a monospecific fusion of anti- mouse Clec9A to modified human IFN alpha Q124R ("Clec9A-Q124R") or a monospecific fusion of anti- mouse mCD20 to modified human IFN alpha Q124R ("mCD20-Q124R") chimera or a combination of both mono-specific chimeras. Alternatively, the mice were treated with either 100 IU or 1000 IU of a Clec9A-Q124R-mCD20 (*i.e.* bi1) or Clec-9A-mCD20-Q124R (*i.e.* bi2) bispecific chimera, which differ in the configuration of the targeting moieties (*i.e.*, the anti-Clec9A and anti-mCD20 VHHs) and the signaling agent (*i.e.*, IFNalpha, Q124R mutant). The mice were phenotypically scored on a daily basis as depicted in **FIG. 11**, panel A.

As shown in **FIG. 11**, panel B, at the 100 IU dosage, the Clec-9A-mCD20-Q124R bispecific chimera significantly improved clinical scores compared to treatment with either of the monospecific chimeras or a combination of the monospecific chimeras. The Clec-9A-mCD20-Q124R bispecific chimera also exhibited superior activities compared to the Clec9A-Q124R-mCD20 bispecific chimera. Similarly, at the 1000 IU dosage level, the Clec-9A-mCD20-Q124R bispecific chimera significantly improved clinical scores compared to treatment with either of the monospecific chimeras or a combination of the monospecific chimeras (**FIG. 11**, panel C). At this dosage level, the Clec9A-Q124R-mCD20 bispecific chimera also exhibited superior activities compared to treatment with either of the monospecific chimeras or a combination of the monospecific chimeras (**FIG. 11**, panel C). Additionally, it was shown that the Clec-9A-mCD20-Q124R bispecific chimera was safe to use at the 1000 IU dosage level. As shown in **FIG. 11**, panel D, at this dosage, the bispecific chimera did not induce lymphopenia in treated mice.

In summary, as further shown in **FIG. 11**, panel E, the Clec9A-Q124R-mCD20 bispecific chimera provided superior protection against multiple sclerosis in the EAE model at a dosage of 1000 IU. In comparison, the Clec-9A-mCD20-Q124R bispecific chimera provided superior protection against multiple sclerosis in the EAE model at both the 100 IU and 1000 IU dosage levels and induced no lymphopenia in treated mice (**FIG. 11**, panel F). Notably, in mice treated with 100 IU of the Clec-9A-mCD20-Q124R bispecific chimera (*i.e.*, bi2), 1 out 5 mice did not show any disease symptoms.

Experiments were also carried out to assess the efficacy of the Clec-9A-mCD20-Q124R bispecific chimera at the 5000 IU dosage level. In these experiments, a control Clec-9A-mCD20-R149A bispecific chimera was also used. In these experiments, the bispecific chimeras were administered starting at day 7 or day 12 and continued until day 25.

As shown in **FIG. 12**, panel A, the Clec-9A-mCD20-Q124R bispecific chimera significantly improved clinical scores compared to controls when administered at either day 7 or day 12 at a dosage level of 5000 IU. More specifically, when the bispecific chimera was administered before disease onset, *i.e.*, at day 7, disease onset

was delayed or stopped altogether. Importantly, when the bispecific chimera was administered after disease onset, *i.e.* at day 12, disease progression was slowed. Without wishing to be bound by theory, administration of the bispecific chimera can be utilized for both prevention and treatment (*e.g.*, slowing progression) of multiple sclerosis. The bispecific chimera also appeared to be safe to use at this dosage level as the treated mice did not show significant loss in body weight up to day 20 of treatment (**FIG. 12**, panel B). As shown in **FIG. 12**, panel C, the Clec-9A-mCD20-Q124R bispecific chimera also significantly delayed disease onset and incidence compared to controls.

Example 5. Tumor-Antigen Specific CD8+ T cell Proliferation and Activation with Bi-Specific Chimeras

FIG. 13 shows an evaluation of tumor-antigen specific $CD8^+$ T cell proliferation and activation both in tumor-draining lymph nodes as well as in spleen, two prime organs in the induction of anti-tumor immunity.

Proliferation of tumor-antigen specific T cells

To evaluate proliferation of tumor-antigen specific T cells, T cell receptor transgenic $CD8^+$ T cells (OT-I cells) were utilized which specifically recognized the model antigen ovalbumin (OVA) present on B16-OVA tumor cells. $CD8^+$ T cells were isolated from the spleens of C57BL/6 OT-I mice using the CD8α T cell isolation kit (Miltenyi Biotec) and labeled with 5 µM carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher). One million of CFSE-labeled CD8 purified OT-I cells were adoptively transferred to C57BL/6 mice inoculated 9 days earlier with $6x10^5$ B16-OVA melanoma cells expressing ovalbumin. One day after OT-I transfer, mice were treated perilesional with PBS or with 30 µg bispecific chimera of anti-Clec9A VHH/ anti-PD-L1 VHH /human IFN Q124R. Some mice were additionally injected perilesionaly with doxorubicin (3 mg/kg, Sigma). Five days post adoptive OT-I transfer, tumor-draining lymph nodes and spleen were isolated and OT-I CD8 T cell proliferation was assessed by flow cytometry (**FIG 13**, panels A and C). Samples were acquired on an Attune Nxt Acoustic Focusing Cytometer (Life Technologies) and analyzed using FlowJo software.

Activation of tumor-antigen specific T cells

To analyze the phenotype of the proliferated OT-I CD8 T cells, single cell suspension of tumor-draining lymph nodes and spleens were stained for different T cell activation markers. Therefore, Fc receptors were blocked using CD16/CD32 antibody (eBioscience) prior to staining with CD8-APC (clone 53-6.7, BD Pharmingen), CD44-PercP-Cy5.5 (clone IMF7, Biolegend) and CD62L-APC-Cy7 (clone MEL-14, Biolegend). Gating strategy was first performed on $CD8^+$ cells of which the CFSE diluting (proliferating) T cells were selected. Naive T cells were based on $CD44^{low}$ $CD62L^{high}$ phenotype, effector T cells on $CD44^{high}$ $CD62L^{low}$ phenotype and memory T cells based on $CD44^{high}$ $CD62L^{high}$ phenotype (**FIG 13**, panels B and D). Samples were acquired on an Attune Nxt Acoustic Focusing Cytometer (Life Technologies) and analyzed using FlowJo software.

It is well established that $CD8^+$ cytotoxic T lymphocytes play a crucial role in eradicating tumor cells. The data of this Example shows, *inter alia*, that the bispecific chimera of anti-Clec9A VHH/ anti-PD-L1 VHH /human IFN

Q124R induced tumor-antigen specific $CD8^+$ T cell proliferation and activation both in tumor-draining lymph nodes (**FIG 13**, panels A and B) as well as in spleen (**FIG 13**, panels C and D).

Example 6. Sirp1α and DNAM Bi-Specific Chimeras

The efficiency of mouse PD-L1 targeting by various bi-specific mouse chimeras was assessed. Specifically, FACS analysis was carried out to quantify STAT1 phosphorylation in the mouse PD-L1 positive B16 cell line.

B16 cells were stimulated with various bispecific chimeras for 15 minutes at 37°C in DMEM medium supplemented with 10% FBS. After stimulation, cells were fixed by adding 1 volume Fix Buffer I (BD Biosciences) for 10 minutes at 37°C, and permeabilized by resuspension in 2 volumes Perm III Buffer I (BD Biosciences) for 30 minutes on ice. Samples were stained with an anti-STAT1 pY701 antibody (BD Biosciences) for 20 minutes at 4°C and analyzed with a FACSCalibur (BD Biosciences) and the CellQuest Pro Version 4.0.2 software (BD Biosciences).

As shown in **FIG. 14**, B16 cells were stimulated with 100 ng/ml of anti-mouse Sirp1α VHH/ anti- mouse PD-L1 VHH /human IFN Q124R bi-specific chimera, anti- mouse DNAM VHH/ anti-mouse PD-L1 VHH /human IFN Q124R bi-specific chimera of BcII10 VHH-human Q124R IFN or left unstimulated for 15 minutes at 37°C. After fixation and permeabilization, cells were stained for phospho STAT1 and analyzed in FACS. Data clearly illustrate that PD-L1 targeting significantly increased STAT1 phosphorylation by the bispecific chimeras when compared to the untargeted (BcII10 VHH) chimera.

Example 7. Human CD8 Targeting of Mono-Specific and Bi-Specific Chimeras

The efficiency of human CD8 targeting of mono-specific human chimeras was examined by quantification of STAT1 phosphorylation in CD8-positive and CD8-negative peripheral blood mononuclear cells (PBMCs) by FACS.

A chimera of anti-human CD8 VHH/human IFN R149A (*i.e.,* pmTW-SIgK-hCD8_R2HCD26 (SEQ ID NO:21)-$(GGS)_{20}$-hIFNa2_R149A-GGS-$(His)_9$ construct) and anti-human CD8 VHH/human IFN R33A/E120A (*i.e.*, pmTW-SIgK-hCD8_R2HCD26 (SEQ ID NO:21)-$(GGS)_{20}$-hIFNa2_R33A/E120A-GGS-$(His)_9$ construct) were produced in Hek293F cells. Cells were grown to a density of $0.6x10^6$ cells per ml in Freestyle medium and transfected with 25K PEI (polyethylenimine) according to standard protocols. Three days after transfection, fresh medium was added to the cultures and cells were grown for two or three additional days. Medium was harvested, cells removed by centrifugation and filter-sterilized. Recombinant proteins were purified using Ni Excel resin (GE Healthcare) according to the manufacturer's instructions and imidazole removed from the samples with PD10 columns (GE Healthcare).

PBMCs from buffy coats of healthy donors were isolated using density gradient centrifugation with Ficoll-Paque (GE Healthcare). Cells were washed twice with FACS buffer (2% FBS, 1 mM EDTA in PBS) and stained with anti-human CD8 APC (clone RPE-T8; BD Pharmingen) for 20 minutes at 4°C. After two washes, cells were stimulated with a serial dilution of CD8-targeting chimeras for 15 minutes at 37°C. After fixation (10 minutes,

37°C, Fix Buffer I; BD Biosciences) and permeabilisation (30 minutes, on ice, Perm III Buffer I; BD Biosciences) and washing, cells were stained with anti-STAT1 pY701 Ab (BD Biosciences). Samples were acquired with a FACSCalibur (BD Biosciences), with the CellQuest Pro Version 4.0.2 software (BD Biosciences).

Isolated PBMCs were stimulated with a serial dilution of CD8-targeting chimeras (anti-human CD8 VHH/human IFN R149A, anti-human CD8 VHH/human IFN R33A/E120A, or anti-BcII10 VHH/human IFN R149A) and stained for CD8 (APC) and pSTAT1 (PE). Data clearly showed that the biological activity of anti-human CD8 VHH/human IFN R149A and anti-BcII10 VHH- human IFN R149A were comparable in CD8-negative cells (**FIG. 15,** panel C), but CD8 targeting resulted in a clear and pronounced increase (at least 500 fold) in STAT1 phosphorylation by anti-human CD8 VHH/human IFN R149A (**FIG. 15,** panels A and B). The combined IFN mutations in the anti-human CD8 VHH/human IFN R33A/E120A chimera completely blocked STAT1 phosphorylation and CD8 targeting did not rescue biological activity. This was in contrast to the IFN R149A mutation which maintained STAT1 phosphorylation.

The efficiency of human CD8 targeting of bi-specific human chimeras (anti-human CD8 VHH/anti-human PD-L1 VHH/human IFN R149A) was also examined by quantification of STAT1 phosphorylation in CD8-positive and CD8-negative peripheral blood mononuclear cells (PBMCs) in FACS.

Anti-human CD8 VHH/anti-human PD-L1 VHH/human IFN R149A bi-specific chimera (i.e., pmTW-SIgK-hCD8_R2HCD26-$(GGS)_{10}$-hPD-L1 VHH-$(GGS)_{20}$-hIFNa2_R149A-GGS-$(His)_9$ construct) and anti-human CD8 VHH/anti-human PD-L1 VHH/human IFN R33A/E120A bi-specific chimera (i.e., pmTW-SIgK-hCD8_R2HCD26-$(GGS)_{10}$-hPD-L1 VHH-$(GGS)_{20}$-hIFNa2_R33A/E120A-GGS-$(His)_9$ construct) were produced in Hek293F cells. Cells were grown to a density of $0.6x10^6$ cells per ml in Freestyle medium and transfected with 25K PEI (polyethylenimine) according to standard protocols. Three days after transfection, fresh medium was added to the cultures and cells were grown for two or three additional days. Medium was harvested, cells removed by centrifugation and filter-sterilized. Recombinant proteins were purified using Ni Excel resin (GE Healthcare) according to the manufacturer's instructions and imidazole from the samples removed with PD10 columns (GE Healthcare).

PBMCs from buffy coats of healthy donors were isolated using density gradient centrifugation with Ficoll-Paque (GE Healthcare). Cells were washed twice with FACS buffer (2% FBS, 1 mM EDTA in PBS) and stained with anti-human CD8 APC (clone RPE-T8; BD Pharmingen) for 20 minutes at 4°C. After two washes, cells were stimulated with a serial dilution of CD8/PD-L1 bi-specific chimeras for 15 minutes at 37°C. After fixation (10 minutes, 37°C, Fix Buffer I; BD Biosciences) and permeabilisation (30 minutes, on ice, Perm III Buffer I; BD Biosciences) and washing, cells were stained with anti-STAT1 pY701 Ab (BD Biosciences). Samples were acquired with a FACSCalibur (BD Biosciences), with the CellQuest Pro Version 4.0.2 software (BD Biosciences).

Isolated PBMCs were stimulated with a serial dilution of bi-specific chimeras (anti-human CD8 VHH/anti-human PD-L1 VHH/human IFN R149A bi-specific chimera, anti-human CD8 VHH/anti-human PD-L1 VHH/human IFN R33A/E120E bi-specific chimera, or anti-human BcII10 VHH/human IFN R149A) and stained for CD8 (APC) and

pSTAT1 (PE). Anti-human CD8 VHH/anti-human PD-L1 VHH/human IFN R149A bi-specific chimera was approximately 100-fold more effective in STAT1 phosphorylation than anti-human BcII10 VHH/human IFN R149A in CD8-negative cells (panels D and F), pointing to a PD-L1 targeting effect on PD-L1 expressing cells. The effect of CD8 targeting was clearly illustrated by the difference in pSTAT1 levels in CD8 positive cells (**FIG. 15,** panel E) versus CD8 negative cells (**FIG. 15,** panel F) upon stimulation by the anti-human CD8 VHH/anti-human PD-L1 VHH/human IFN R149A bi-specific chimera. The combined human IFN R33A/E120A mutation completely blocked STAT1 phosphorylation and CD8 and/or PD-L1 targeting did not rescue biological activity. This was in contrast to the R149A mutation which maintained STAT1 phosphorylation.

Example 8. Human PD-L1 Targeting of Mono- and Bi-Specific Chimera

The efficiency of human PD-L1 targeting of (bi-specific) human chimeras was examined by quantification of STAT1 phosphorylation in the human PD-L1 positive MDA-MB-321 cell-line by FACS analysis. Chimeras studied were anti-human PD-L1 VHH/human IFN R149A; anti-human PD-L1 VHH/human IFN R33A/E120R; anti-human Clec9A VHH/anti-human PD-L1 VHH/human IFN R149A; anti-human Clec9A VHH/ anti-human PD-L1 VHH/human IFN R33A/E120R; and anti-human Bcl10 VHH/human IFN R149A.

MDA-MB-321 cells were stimulated with human chimeras as indicated for 15 minutes at 37°C in DMEM medium supplemented with 10% FBS. After stimulation, cells were fixed by adding 1 volume Fix Buffer I (BD Biosciences) for 10 minutes at 37°C, and permeabilized by resuspension in 2 volumes Perm III Buffer I (BD Biosciences) for 30 minutes on ice. Samples were stained with an anti-STAT1 pY701 Ab (BD Biosciences) for 20 at 4°C and analyzed with a FACSCalibur (BD Biosciences) and the CellQuest Pro Version 4.0.2 software (BD Biosciences).

MDA-MB-321 were stimulated with a serial dilution (100 ng/ml; 1 over 5) of mono- (PD-L1 VHH) and bi-specific (Clec9A and PD-L1 VHHs) chimeras for 15 minutes at 37°C. After fixation and permeabilization, cells were stained for phospho STAT1 and analyzed in FACS. Data illustrate that PD-L1 targeting (in mono- or bi-specific format) strongly increased STAT1 phosphorylation by chimeras having the IFN R149A mutation, but not the IFN R33A/E120R mutations. Untargeted (BcII10 VHH) chimera was unable to signal even at 100 ng/ml. *See* **FIG. 16**.

Example 9 Bi-specific Constructs with scFv Targeting Moieties

Expansion of the bi-specific concept beyond VHHs was undertaken. An anti-human CD20 scFv/anti-human CD3 scFv/IFN R149A of the following sequence was generated:

METDTLLLWVLLLWVPGSTG**QVQLQQSGAELARPGASVKMSCKASGYTF**
TRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSST
AYMQLSSLTSEDSAVYYCARYYDDHYSLDYWGQGTTLTVSSGSTGGGG
SGGGGSGGGGSDIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQ
KSGTSPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYC
QQWSSNPFTFGSGTKLEINRGGSGG**QIVLSQSPAILSASPGEKVTMTCRA**
SSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVRFSGSGSGTSYSLTIS

RVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRGSTGGGGSGGGGSGGG GSQVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYC ARSTYYGGDWYFNVWGAGTTVTVSSVDGGSGGSGGSGGSGGSGGSRS GGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSAAAMCDL PQTHSLGSRRTLMLLAQMRRISLFSCLKDRHDFGFPQEEFGNQFQKAETIP VLHEMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVT ETPLMKEDSILAVRKYFQRITLYLKEKKYSPCAWEVVRAEIMASFSLSTNLQ ESLRSKELEHHHHHH (SEQ ID NO: 367).

The signal peptide is underlined, scFvCD3 is bold, scFvCD20 is bold and underlined, hIFNa2(R149A)is shaded and linkers connect the targeting moieties and signaling agent.

The efficiency of targeting by the human scFV-based bi-specific chimera was examined by quantification of STAT1 phosphorylation in Daudi (CD20 positive), Jurkat (CD3) or Wish (negative control) cells. See **FIG. 17**.

Cells were stimulated with bi-specific chimeras as indicated for 15 minutes at 37°C in DMEM medium supplemented with 10% FBS. After stimulation, cells were fixed by adding 1 volume Fix Buffer I (BD Biosciences) for 10 minutes at 37°C, and permeabilised by resuspension in 2 volumes Perm III Buffer I (BD Biosciences) for 30 minutes on ice. Samples were stained with an anti-STAT1 pY701 Ab (BD Biosciences) for 20 minutes at 4°C and analyzed with a FACSCalibur (BD Biosciences) and the CellQuest Pro Version 4.0.2 software (BD Biosciences).

As shown in **FIG. 17**, Daudi, Jurkat and Wish cells were stimulated with a serial dilution (5000 ng/ml; 1 over 5) of scFv chimera for 15 minutes at 37°C. After fixation and permeabilization, cells were stained for phospho STAT1 and analyzed by FACS analysis. Data clearly illustrated that STAT1 phosphorylation was more efficient in Jurkat (CD3-expressing) and Daudi (CD20-expressing) cells compared to Wish cells due to CD3 and CD20 targeting, respectively.

Example 10. Modelled Human Anti-Tumor Effects

Selection of human cord blood

Stem cells were HLA-type matched with the expression of HLA-A2 by the tumor cells used during the experiments. To that end, only HLA-A2 positive cord bloods were selected to proceed with CD34 stem cell purification. Cells were stained with HLA-A2-FITC (BD Pharmingen) or HLA-ABC-PE (BD Pharmingen), the latter as a positive control. Samples were acquired on an Attune Nxt Acoustic Focusing Cytometer (Life Technologies).

Purification of $CD34^+$ stem cells from human cord blood

Viable mononuclear cells from human cord bloods were isolated using Fycoll (Lymphoprep, Stemcell technologies) gradient separation prior to $CD34^+$ MACS isolation using direct $CD34^+$ progenitor cell isolation kit (Miltenyi). Flow cytometric staining using human CD3-PE (BD Phamringen)/human-CD34-APC (BD Pharmingen)

was used to evaluate purity of the isolated stem cells. Samples were acquired on an Attune Nxt Acoustic Focusing Cytometer (Life Technologies). Purity of injected cells reached 95-98%.

Generation of humanized mice

Newborn NSG mice (1-2 days of age) were sublethally irradiated with 100 cGy prior to intrahepatic delivery of 10^5 $CD34^+$ human stem cells. At 6 weeks after CD34 transfer, peripheral blood is analyzed for the presence of both human and mouse CD45 (both BD) cells to analyze the effect of engraftment. Samples where acquired on a LSR flow cytometer (BD) and analyzed by FACS Diva software (BD).

The antitumor potential of chimeras was assessed in tumor-bearing humanized mice models. Newborn NSG mice (1-2 days of age) were sublethally irradiated with 100 cGy prior to intrahepatic delivery of 10^5 $CD34^+$ human stem cells (from HLA-A2 positive cord bloods). At week 13 after stem cell transfer mice were s.c. inoculated with 2.5 x 10^6 human RL follicular lymphoma cells. Mice were treated i.p. daily started at day 6 after tumor inoculation with 30 µl of Flt3L protein. Daily perilesional chimera delivery (30 µg) was started at day 10 after tumor inoculation, when a palpable tumor was reached. Treatment with the anti-human Clec9a VHH/anti-human PDL1 VHH/human IFN-R149A bi-specific chimera resulted in stabilization of the tumor growth, which was even more pronounced when combined with Flt3L injections in contrast to injection of PBS. The graph shows tumor growth as mean ± SEM.

Importantly, anti-human Clec9a VHH/anti-human PDL1 VHH/human IFN-R149A bi-specific chimera showed clear antitumor effects on a human tumor (RL) grown in humanized mice (mice with a reconstituted human immune system). See **FIG. 18**.

Example 11. Dendritic Cell Signaling Induced by Anti-human Bi-Specific VHH Chimeras

A dendritic cell pSTAT signaling assay was undertaken. Chimeras studied were anti-human Clec9A VHH/anti-human PD-L1 VHH/human IFN R149A and anti-human Clec9A VHH/ anti-human PD-L1 VHH/human IFN R33A/E120R. Two doses of the agents were studied: 100 ng/ml and 500 ng/ml.

Briefly, human PBMCs were isolated from blood obtained from healthy donors. Approximately 120 ml of blood was collected from each donor using heparin coated tubes (12 tubes). The blood was kept at room temperature and processed immediately Briefly, blood was diluted 1:1 with DPBS and 25 ml was gently layered onto 15 ml of Lympholyte H. After centrifugation, the mononuclear cell rings were collected and cells were washed three times with DPBS (PBS Dulbecco's Phosphate Buffered Saline, Wisent, catalog #311-425-LL) and counted. Dendritic cells were enriched from the PBMC population using "DC- enrichment kit" containing a combination of lineage specific monoclonal antibodies in PBS and a suspension of magnetic particles (STEMCELL Technologies Catalogue number 19251), according to manufacturer's instructions.

Dendritic cells (DC) were stimulated for 15 minutes in the presence or absence of test items and controls (PBS) and the level of phosphorylated-STAT1 (pSTAT1, specifically pY701-STAT1) was determined in isolated DC cell populations (Lin-(CD14/CD16/CD20/CD56/CD3)/HLA-DR+) by flow cytometry. Post stimulation, cells were fixed

(BD Cytofix fixation buffer, BD Bioscience, catalog #554655), then permeabilized with Perm buffer II (BD PhosFlow Perm Buffer, BD Bioscience, catalog #558052). Cells were then stained for phosphoSTAT1 and for DC surface markers (Lin-/HLA-DR+) (see table below). Both intra-cellular and surface staining were performed at the same time. Flow cytometry and data acquisition was performed after cell washing with DPBS.

Table showing list of antibodies for flow cytometry staining

Marker/Product Name	Fluorochrome	Clone	Purpose	Supplier-Catalog Number
pSTAT1	AlexaFluor647	4a	phospho-STAT1	BD-562070
Anti-human CD3	PE	UCHT1	T cells marker Lineage depletion	BD-561809
Anti-human CD14	PE	M5E2	Monocytes markers Lineage depletion	BD-555398
anti-human CD16	PE	B73.1	NK, Neutrophils, Monocytes marker Lineage depletion	BD-561313
anti-human CD19	PE	HIB19	B cells marker Lineage depletion	BD-555413
anti-human CD56	PE	B159	NK cells marker Lineage depletion	BD-555516
Anti-human HLA-DR	FITC	TU36	MHC II marker DC discrimination	BD-555560
Anti-human CD11c	BV421	B-Ly6	DC discrimination	BD-562561
LIVE/DEAD Fixable Aqua Dead Cell Stain	Aqua	N/Ap	Viability dye	ThermoFisher-L34957
Normal mouse IgG	N/Ap	N/Ap	Fc receptor blocker Blocking agent	ThermoFisher-10400C

FIG. 19 shows the data, expressed as a fold change of the percentage of $pSTAT^+$ dendritic cells.

This study clearly shows that a dual (bi-specific) human CLEC9A/PD-L1 antigen-targeting construct comprising an IFN signaling agent whose activity is recoverable upon cell targeting (IFN R149A) promotes IFN signaling in human dendritic cells (as determined by pSTAT1 induction). In contrast, no IFN signaling activation is observed with a bi-specific CLEC9A/PD-L1 antigen-targeting construct that incorporates an IFN signaling agent whose activity is not recoverable (IFN R33A/E120R). Thus, as observed for comparable IFN fusion constructs targeting mouse CLEC9A and PD-L1 antigens, targeting IFN to human dendritic cells using a targeting moiety directed at human CLEC9A antigen results in triggering of a pronounced IFN signal transduction.

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 invention 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 chimeric protein comprising:

(a) two or more targeting moieties, said targeting moieties comprising recognition domains which specifically bind to antigens or receptors of interest; and

(b) a modified signaling agent, said modified signaling agent having one or more mutations that confer improved safety as compared to a wild type signaling agent,

wherein the targeting moieties and modified signaling agent are optionally connected with one or more linkers.

2. The chimeric protein of claim 1, wherein one or more of the targeting moieties is directed against a tumor cell.

3. The chimeric protein of claim 1, wherein one or more of the targeting moieties is directed against an immune cell.

4. The chimeric protein of claim 3, wherein the immune cell is selected from a T cell, a B cell, a dendritic cell, a macrophage, a neutrophil, and a NK cell.

5. The chimeric protein of any of the above claims, wherein (i) one or more of the targeting moieties is directed against an immune cell selected from a T cell, a B cell, a dendritic cell, a neutrophil, a macrophage, and a NK cell and (ii) one or more of the targeting moieties is directed against a tumor cell.

6. The chimeric protein of any of the above claims, wherein the recognition domain is a full-length antibody, 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 (*e.g.* cysteine knot protein, knottin), a darpin, an anticalin, an adnectin, an aptamer, a Fv, a Fab, a Fab', a $F(ab')_2$, a peptide mimetic molecule, a natural ligand for a receptor, or a synthetic molecule.

7. The chimeric protein of any of the above claims, wherein the recognition domain is a single-domain antibody (VHH) or an scFv.

8. The chimeric protein of any of the above claims, wherein the recognition domain is a V_{HH}, humanized V_{HH}, or camelized V_{HH}.

9. The chimeric protein of any of the above claims, wherein the recognition domain functionally modulates the antigen or receptor of interest.

10. The chimeric protein of any of the above claims, wherein the recognition domain binds but does not functionally modulate the antigen or receptor of interest.

11. The chimeric protein of any of the above claims, wherein the antigen of interest is selected from one or more of CD8, CD3, CD33, SLAMF7 (CS1), Sirp1α, DNAM, and CLEC9A.

12. The chimeric protein of any of the above claims, wherein the antigen of interest is an immune checkpoint, optionally selected from PD-1, PD-L1, and PD-L2.

13. The chimeric protein of claim 1, wherein the modified signaling agent comprises one or more mutations conferring reduced affinity or activity for a receptor relative to a wild type signaling agent.

14. The chimeric protein of claim 1, wherein the modified signaling agent comprises one or more mutations conferring substantially reduced or ablated affinity or activity for a receptor relative to a wild type signaling agent.

15. The chimeric protein of claim 1, wherein the modified signaling agent comprises both (a) one or more mutations conferring substantially reduced or ablated affinity for a receptor relative to a wild type signaling agent and (b) one or more mutations conferring reduced affinity or activity for a receptor relative to a wild type signaling agent; and wherein the receptors are different.

16. The chimeric protein of claim 13, wherein the one or more mutations allow for attenuation of activity.

17. The chimeric protein of claim 16, wherein agonistic or antagonistic activity is attenuated.

18. The chimeric protein of claim 16 or 17, wherein the signaling agent comprises one or more mutations which convert its activity from agonistic to antagonistic.

19. The chimeric protein of claim 13, wherein the mutation confers reduced affinity or activity that is restorable by attachment to one or more targeting moiety.

20. The chimeric protein of claim 14, wherein the mutation confers substantially reduced or ablated affinity or activity that is not substantially restorable by attachment to one or more targeting moiety.

21. The chimeric protein of any of the above claims, wherein the chimeric protein has a molecular mass of about 50 KDa or higher.

22. The chimeric protein of any of the above claims, wherein the chimeric protein substantially evades filtration by the kidney.

23. The chimeric protein of any of the above claims, wherein the modified signaling agent is IFNα2a, optionally having one or more mutations at positions L153, R149, and M148.

24. The chimeric protein of any of the above claims, wherein the modified signaling agent is IL-1β, optionally having one or more mutations at positions R120 and H146.

25. The chimeric protein of any of the above claims, wherein the modified signaling agent is TNF, optionally having one or more mutations at positions Y87 and Y115.

26. The chimeric protein of any one of the above claims, wherein the chimeric protein is suitable for use in a patient having one or more of: cancer, infections, immune disorders, autoimmune diseases, cardiovascular diseases, wound, ischemia-related diseases, neurodegenerative diseases, and/or metabolic diseases.

27. A recombinant nucleic acid composition encoding one or chimeric proteins of any one of the above claims.

28. A host cell comprising a nucleic acid of claim 27.

29. A method for treating cancer, comprising administering an effective amount of the chimeric protein of any of the above claims to a patient in need thereof.

30. The method of claim 29, wherein the cancer is selected form one or more of 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.

31. A method for treating an autoimmune disease or disorder, comprising administering an effective amount of the chimeric protein of any of the above claims to a patient in need thereof.

32. The method of claim 31, wherein the autoimmune disease or disorder, is selected from one or more of Crohn's Disease, systemic lupus erythematosis, rheumatoid arthritis or juvenile rheumatoid arthritis, ulcerative colitis immune disorders such as eosinophilic fasciitis, hypoimmunoglobulinemia, or thymoma/thymic carcinoma, graft versus host disease, preleukemia, Nonhematologic syndrome (*e.g.* Down's, Dubowwitz, Seckel), Felty syndrome, hemolytic uremic syndrome, myelodysplasic syndrome, nocturnal paroxysmal hemoglobinuria, osteomyelofibrosis, pancytopenia, pure red-cell aplasia, Schoenlein-Henoch purpura, malaria, protein starvation, menorrhagia, systemic sclerosis, liver cirrhosis, hypometabolic states, and congestive heart failure.

33. A chimeric protein of any of the above claims for use in the treatment of one or more of: cancer, infections, immune disorders, autoimmune diseases, cardiovascular diseases, wound, ischemia-related diseases, neurodegenerative diseases, and/or metabolic diseases, as described herein.

34. Use of a chimeric protein of any of the above claims for the manufacture of a medicament for treating one or more of: cancer, infections, immune disorders, autoimmune diseases, cardiovascular diseases, wound, ischemia-related diseases, neurodegenerative diseases, and/or metabolic diseases, as described herein.

FIG. 1

A.

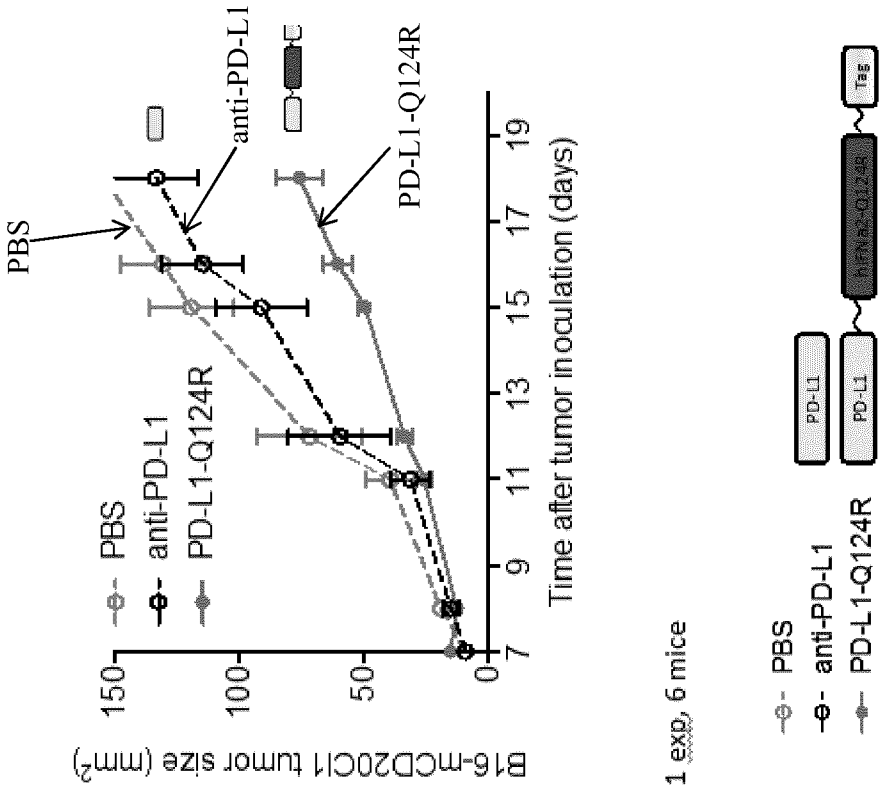
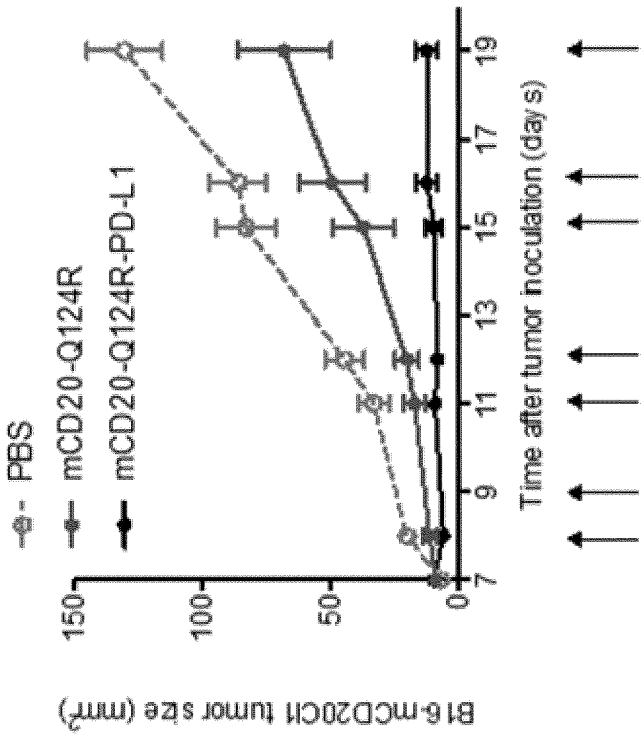

PBS
anti-PD-L1
PD-L1-Q124R
B16-mCD20C1 tumor size (mm²)
150
100
50
0
7
9
11
13
15
17
19
Time after tumor inoculation (days)
1 exp, 6 mice
PBS
anti-PD-L1
PD-L1-Q124R
PD-L1
PD-L1
Tag

FIG. 1 (CONT.)

B.


PBS
mCD20-Q124R
mCD20-Q124R-PD-L1
B16-mCD20Cl1 tumor size (mm²)
150
100
50
0
7
9
11
13
15
17
19
Time after tumor inoculation (days)

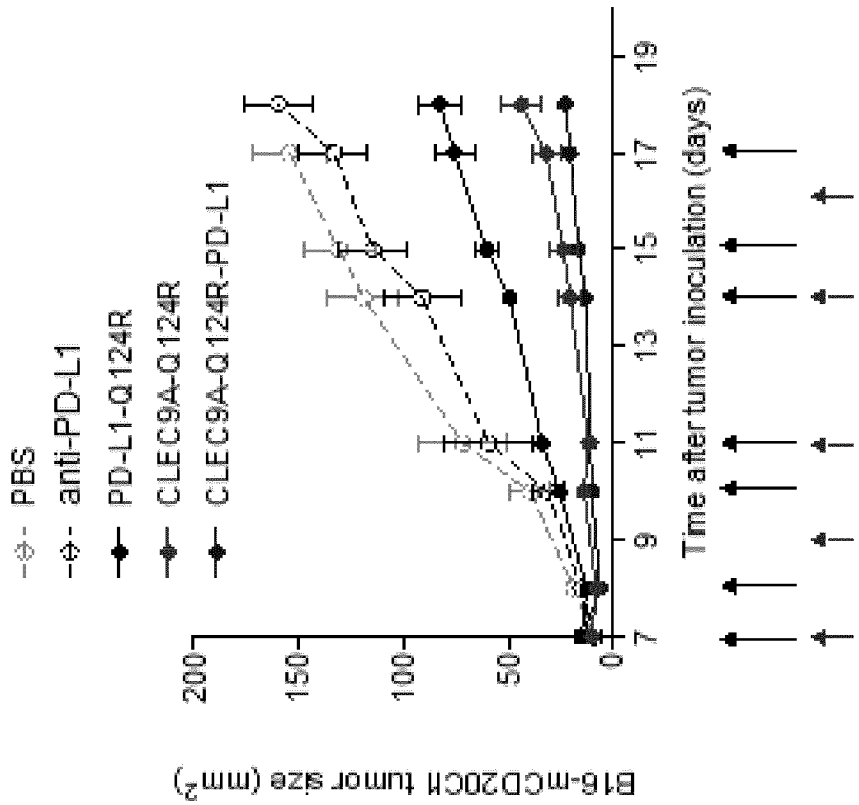
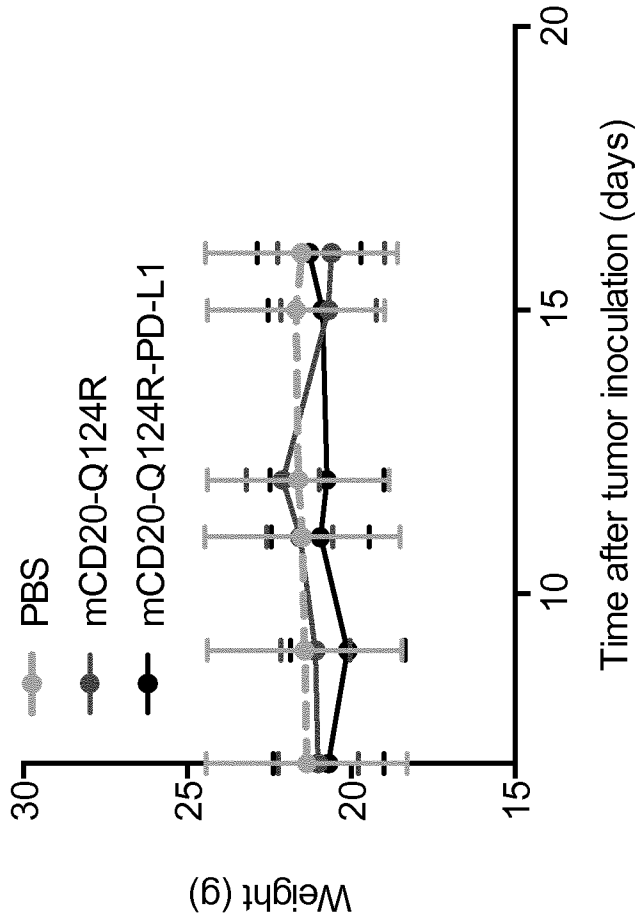

PBS
anti-PD-L1
PD-L1-Q124R
CLEC9A-Q124R
CLEC9A-Q124R-PD-L1
B16-mCD20CH tumor size (mm²)
200
150
100
50
0
7
9
11
13
15
17
19
Time after tumor inoculation (days)

FIG. 2

FIG. 3

A


PBS
mCD20-Q124R
mCD20-Q124R-PD-L1
Weight (g)
30
25
20
15
10
15
20
Time after tumor inoculation (days)

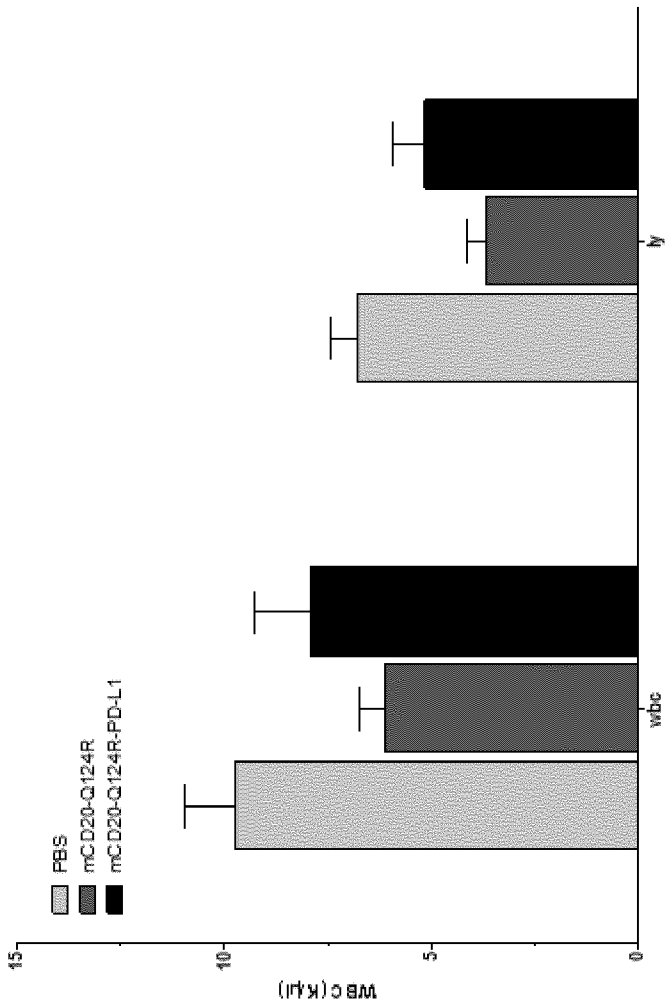


FIG. 3 (CONT.)
B.

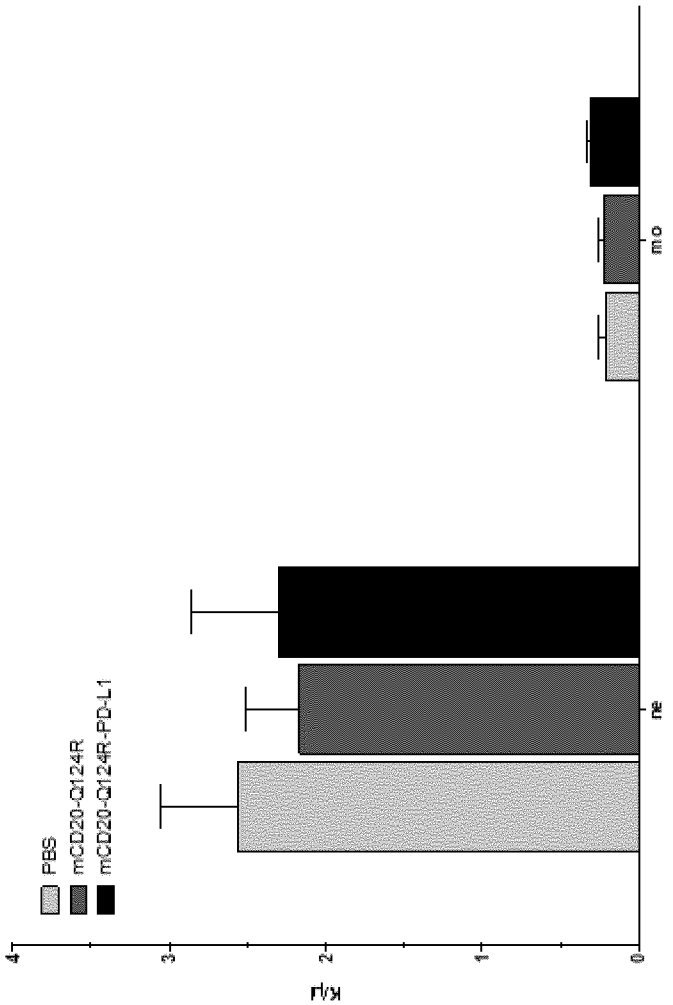


FIG. 3 (CONT.)
C.

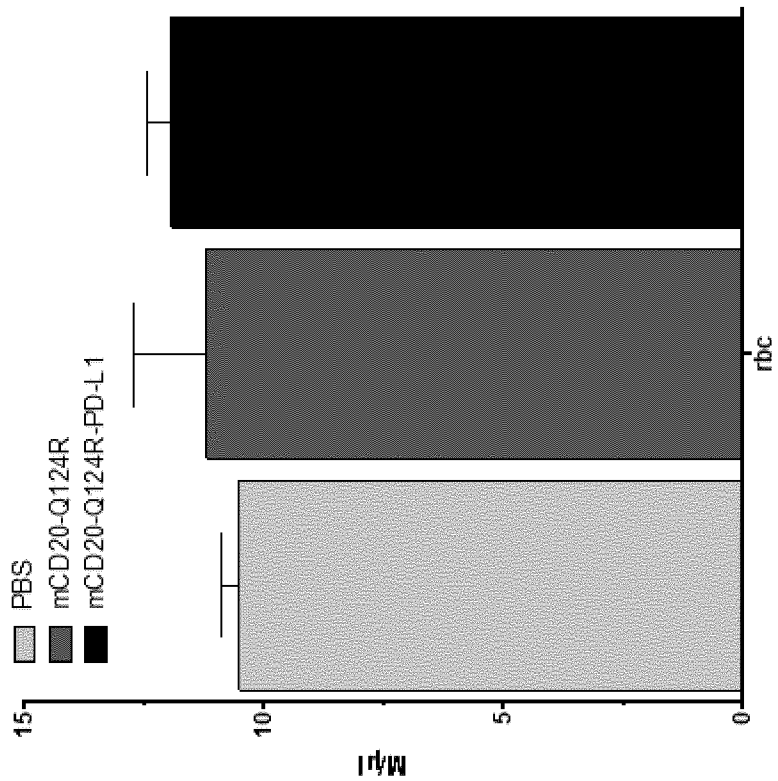


FIG. 3 (CONT.)
D.

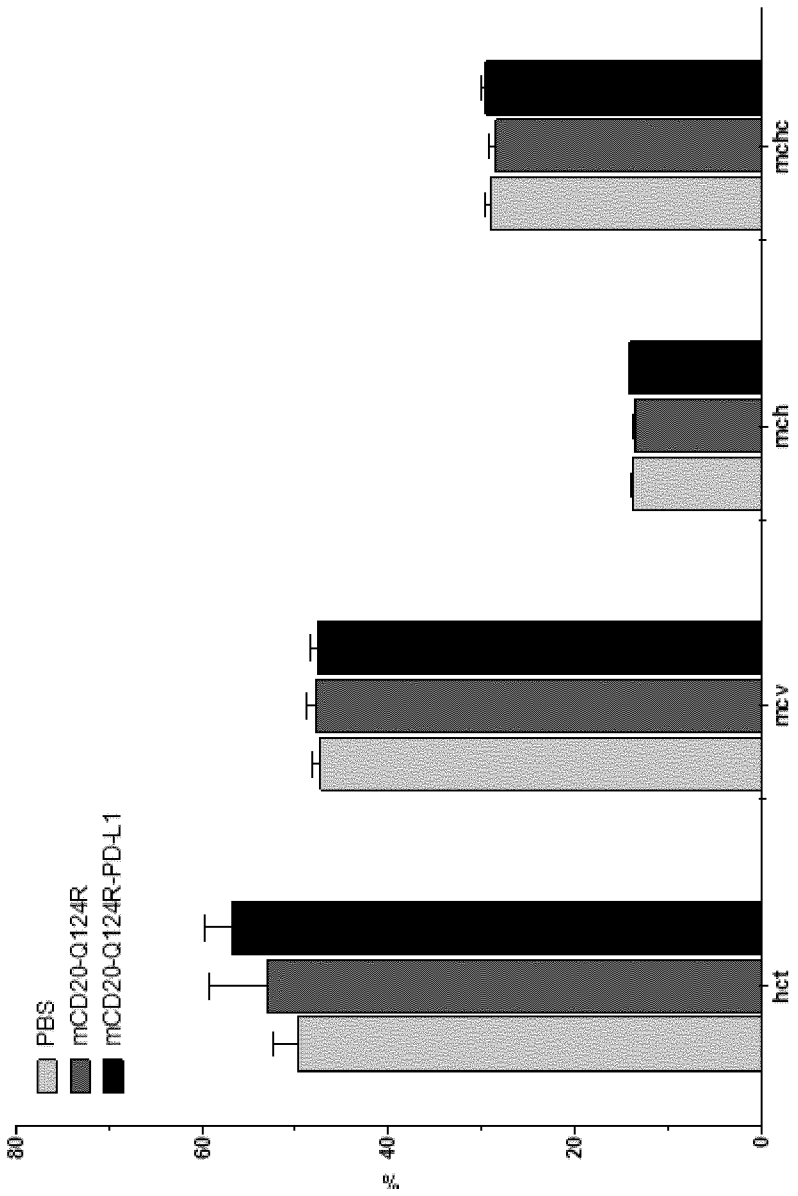


FIG. 3 (CONT.)
E.

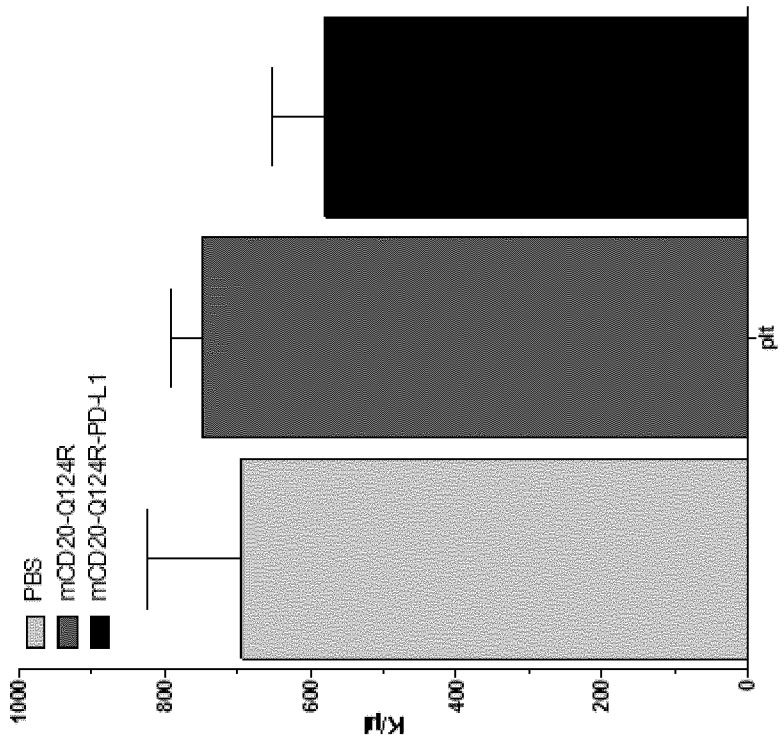

PBS
mCD20-Q124R
mCD20-Q124R-PD-L1
K/µl
1000
800
600
400
200
0
plt

FIG. 3 (CONT.)
F.

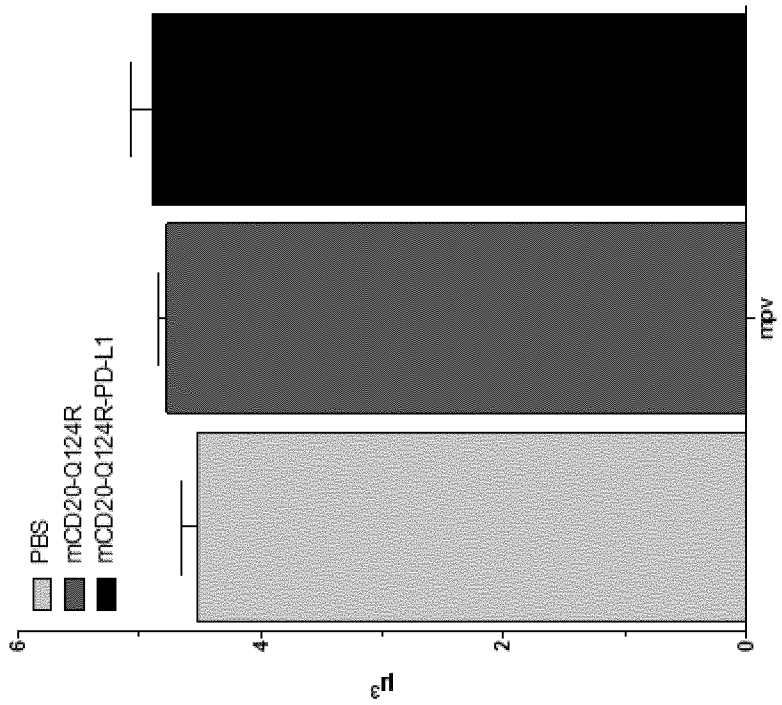


FIG. 3 (CONT.)
G.

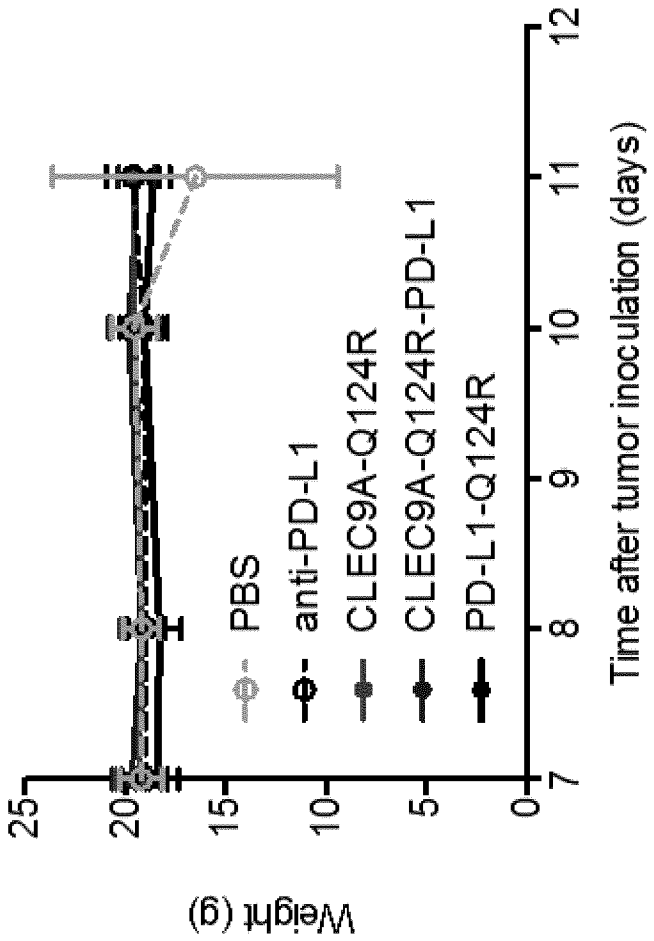

Weight (g)
25
20
15
10
5
0
7
8
9
10
11
12
Time after tumor inoculation (days)
PBS
anti-PD-L1
CLEC9A-Q124R
CLEC9A-Q124R-PD-L1
PD-L1-Q124R

FIG. 4
A.

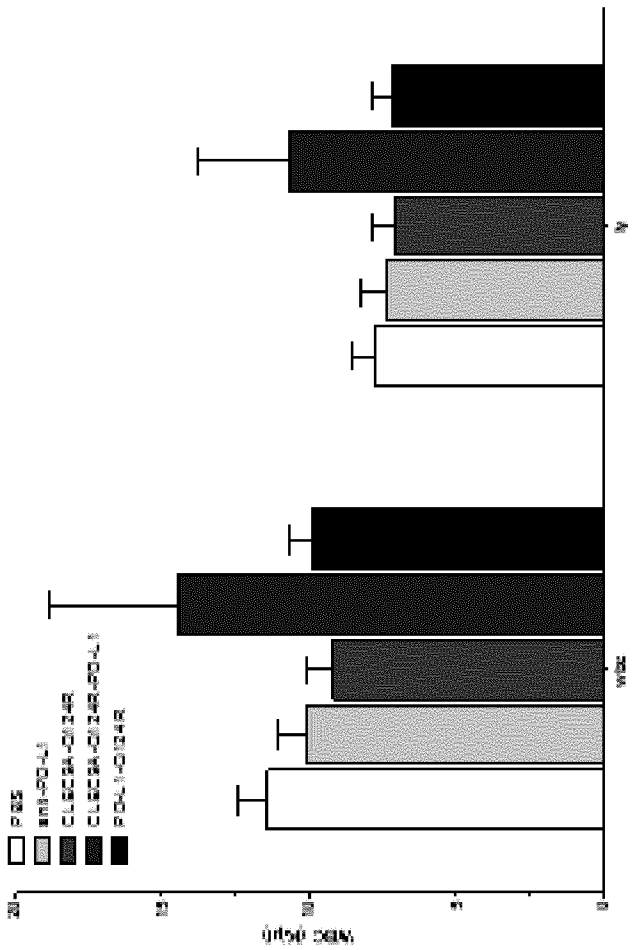


FIG. 4 (CONT.)
B.

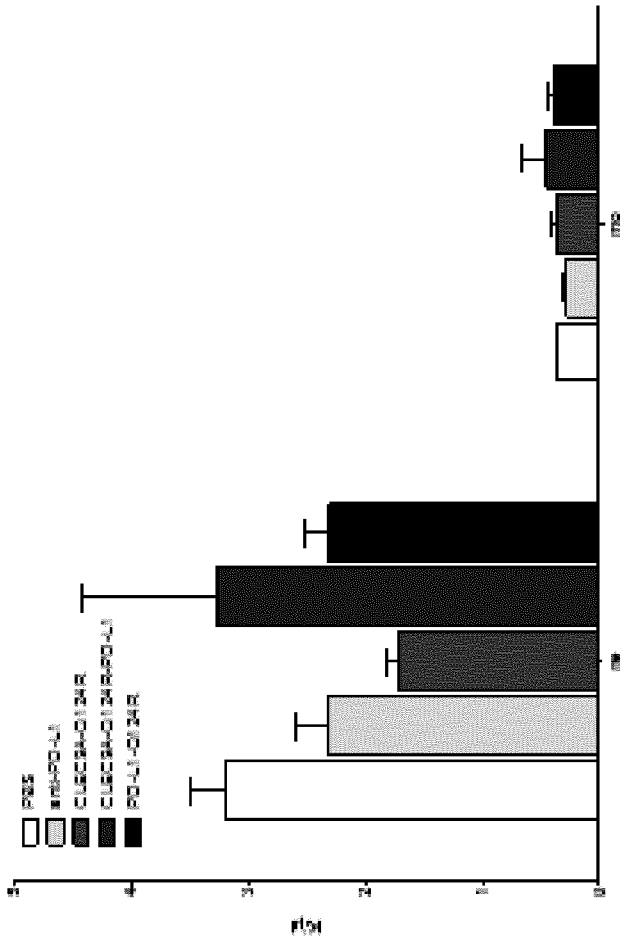


FIG. 4 (CONT.)
C.

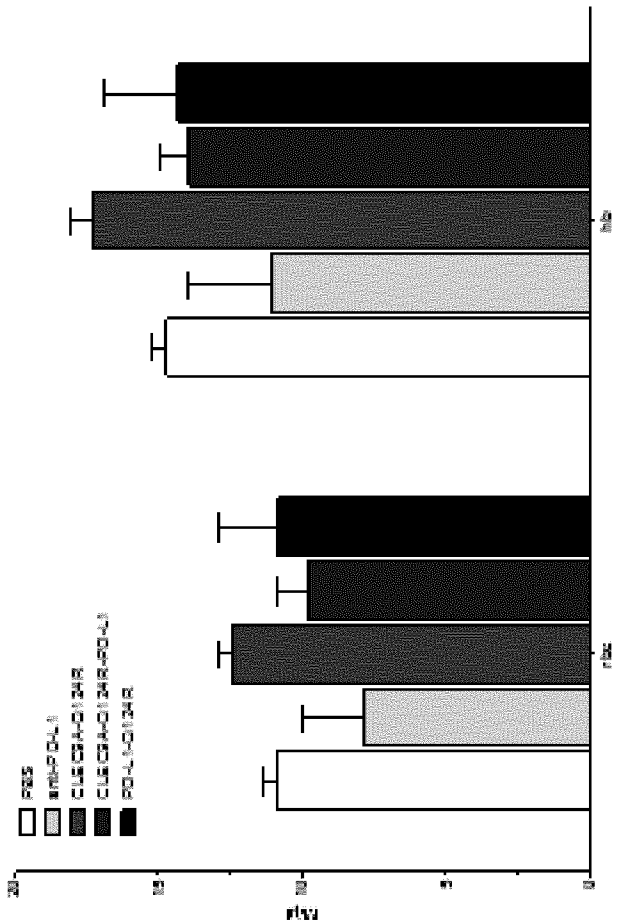


FIG. 4 (CONT.)
D.

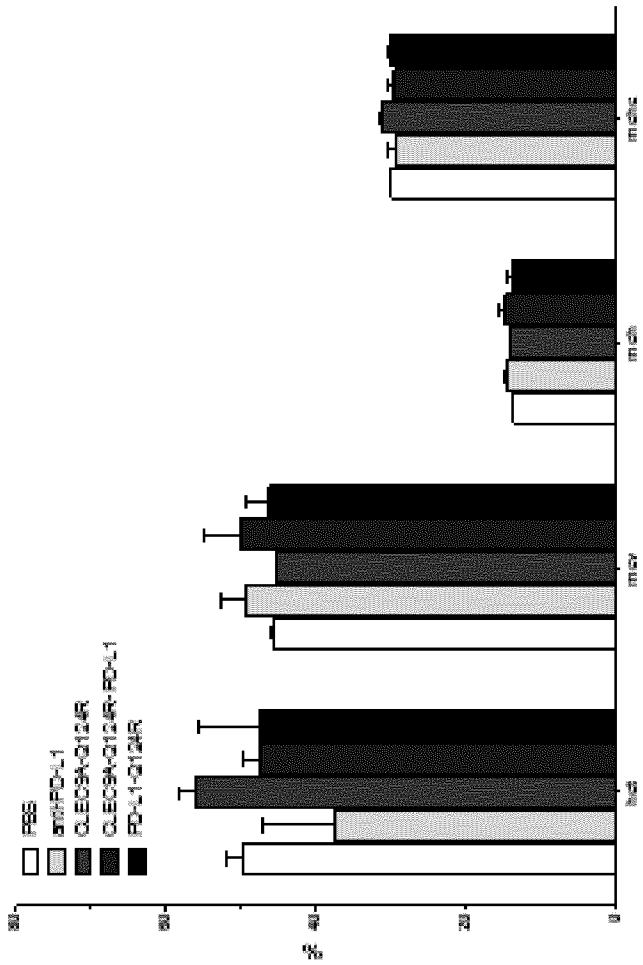

PBS
anti-PD-L1
Q-ECSA-Q12-R
Q-ECSA-Q12-R-PD-L1
PD-L1-Q12-R
med
mcv
mch
mchc

FIG. 4 (CONT.)
E.

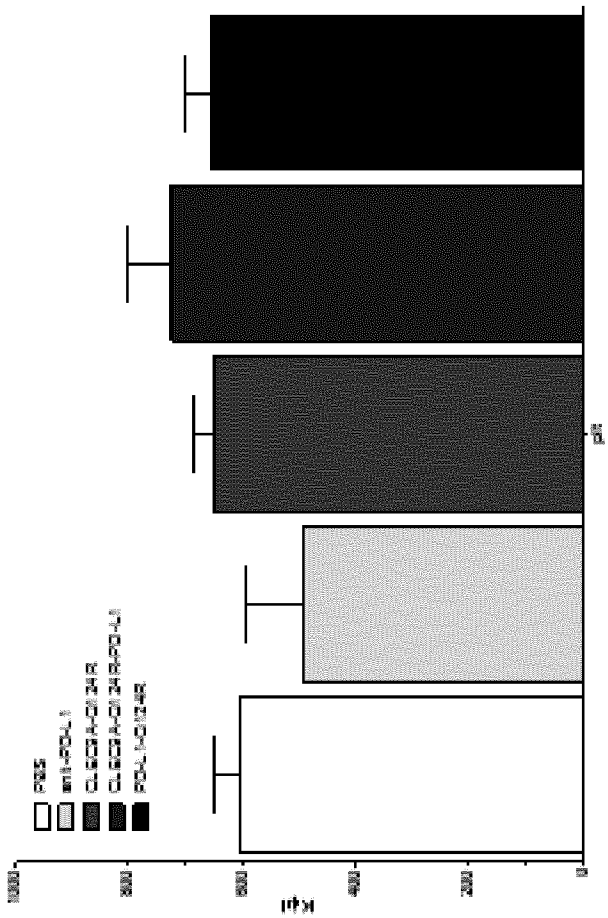


FIG. 4 (CONT.)
F.

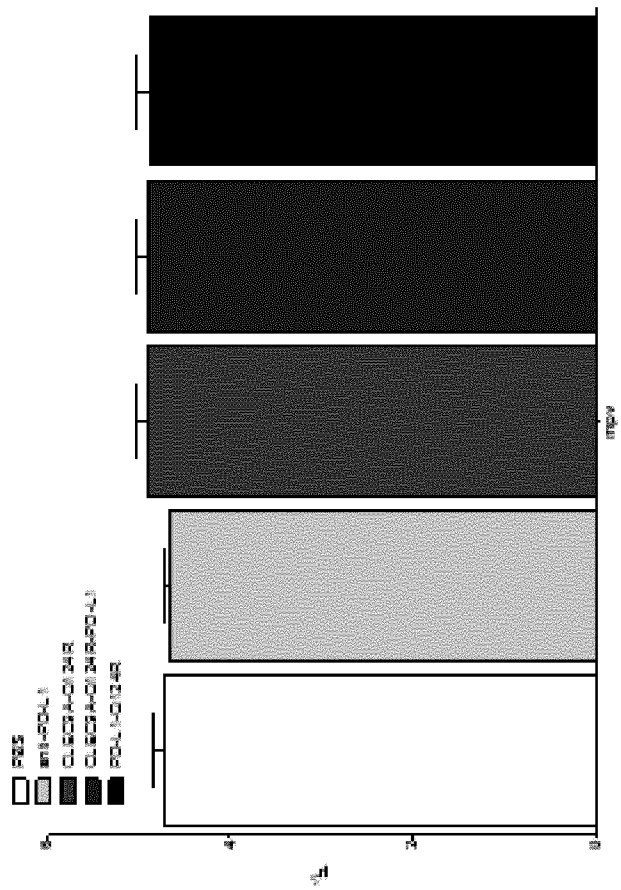
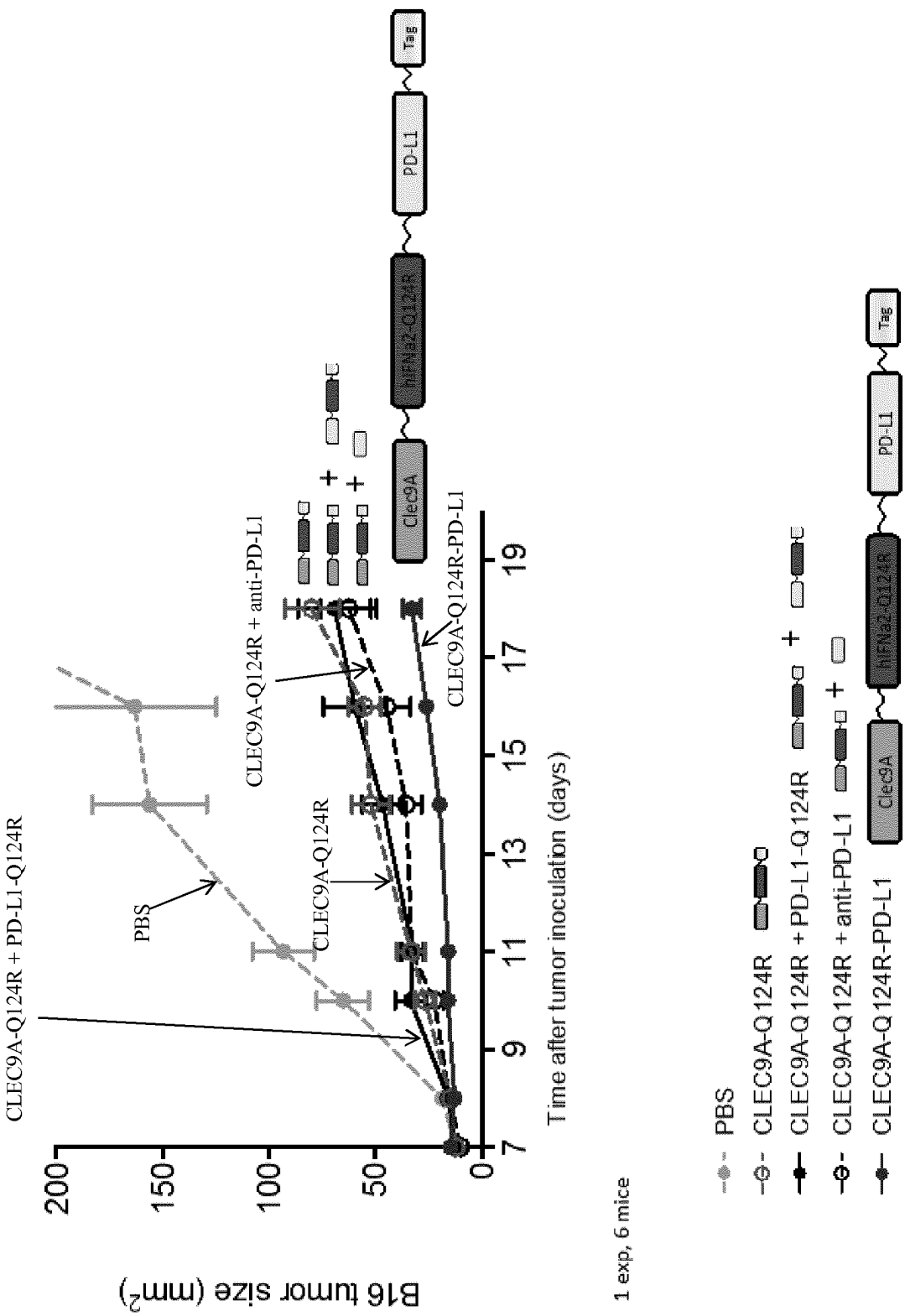


FIG. 4 (CONT.)
G.

FIG. 5


B16 tumor size (mm²)
200
150
100
50
0
7
9
11
13
15
17
19
Time after tumor inoculation (days)
1 exp, 6 mice
PBS
CLEC9A-Q124R + PD-L1-Q124R
CLEC9A-Q124R
CLEC9A-Q124R + anti-PD-L1
CLEC9A-Q124R-PD-L1
PBS
CLEC9A-Q124R
CLEC9A-Q124R + PD-L1-Q124R
CLEC9A-Q124R + anti-PD-L1
CLEC9A-Q124R-PD-L1
Clec9A
hIFNa2-Q124R
PD-L1
Tag

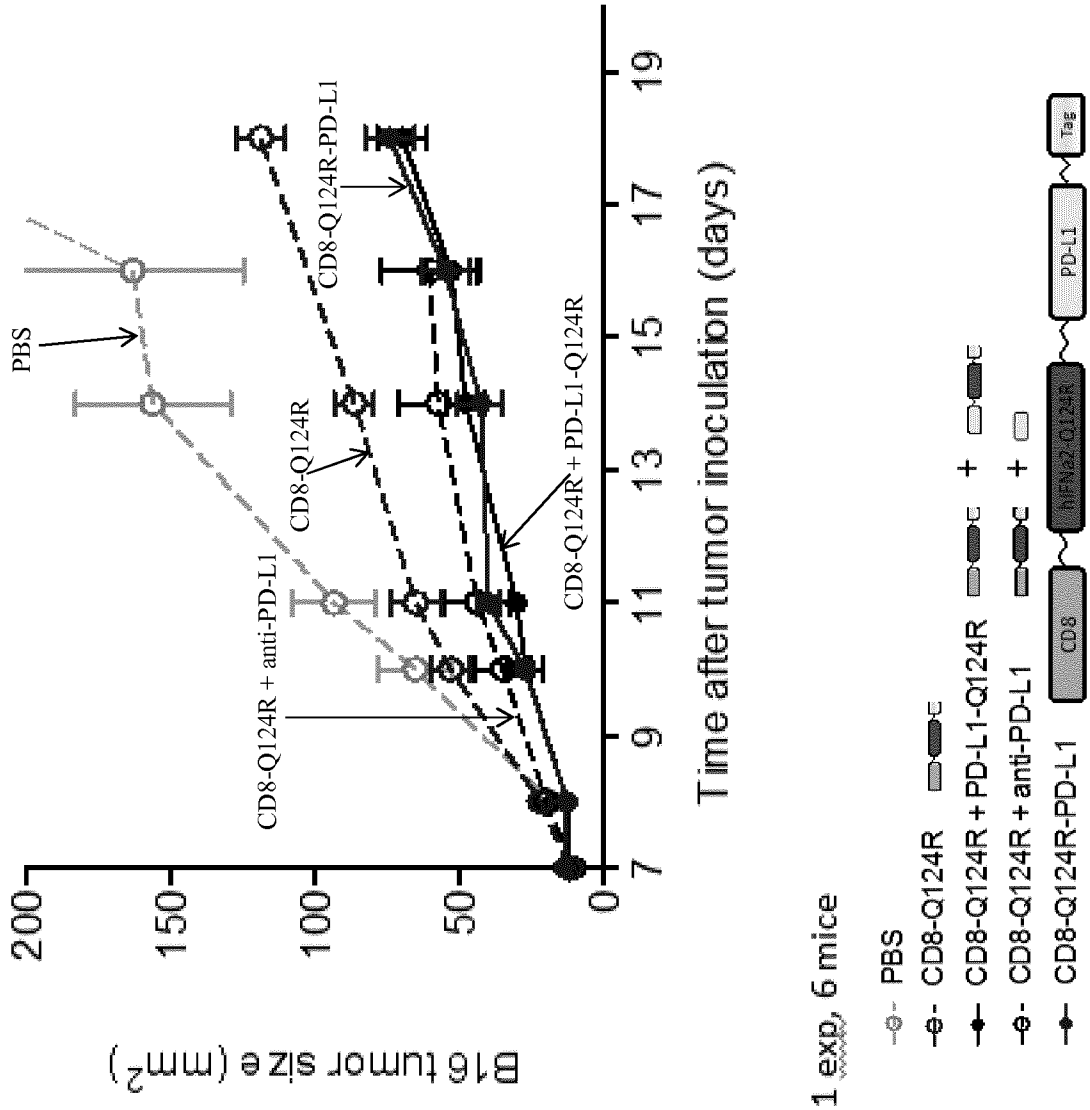

B16 tumor size (mm²)
200
150
100
50
0
7
9
11
13
15
17
19
Time after tumor inoculation (days)
PBS
CD8-Q124R + anti-PD-L1
CD8-Q124R
CD8-Q124R-PD-L1
CD8-Q124R + PD-L1-Q124R
1 exp, 6 mice
PBS
CD8-Q124R
CD8-Q124R + PD-L1-Q124R
CD8-Q124R + anti-PD-L1
CD8-Q124R-PD-L1
CD8
IFNα2-Q124R
PD-L1
Tag

FIG. 6

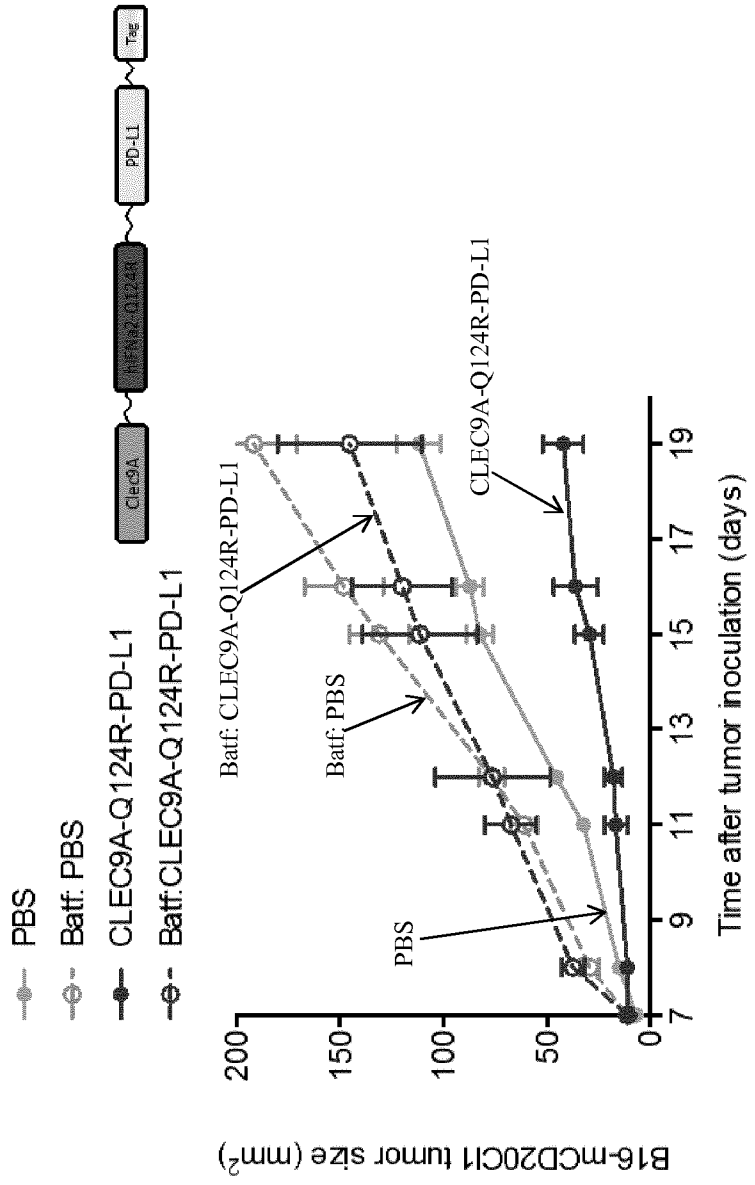

PBS
Batf: PBS
CLEC9A-Q124R-PD-L1
Batf:CLEC9A-Q124R-PD-L1
CLEC9A
PD-L1
Tag
Batf: CLEC9A-Q124R-PD-L1
Batf: PBS
PBS
CLEC9A-Q124R-PD-L1
B16-mCD20Cl1 tumor size (mm²)
200
150
100
50
0
7
9
11
13
15
17
19
Time after tumor inoculation (days)

FIG. 7

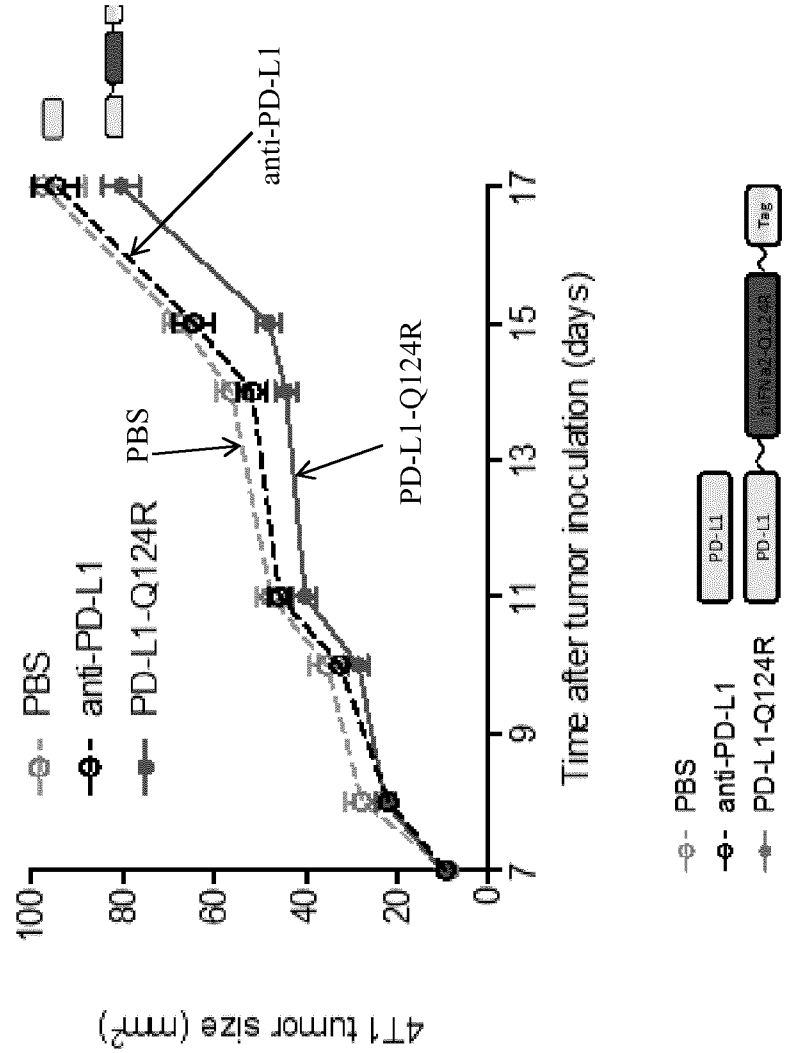

PBS
anti-PD-L1
PD-L1-Q124R
4T1 tumor size (mm²)
100
80
60
40
20
0
7
9
11
13
15
17
Time after tumor inoculation (days)
PBS
anti-PD-L1
PD-L1-Q124R
PBS
anti-PD-L1
PD-L1-Q124R
PD-L1
PD-L1
Tag

FIG. 8
A.

FIG. 8 (CONT.)
B.

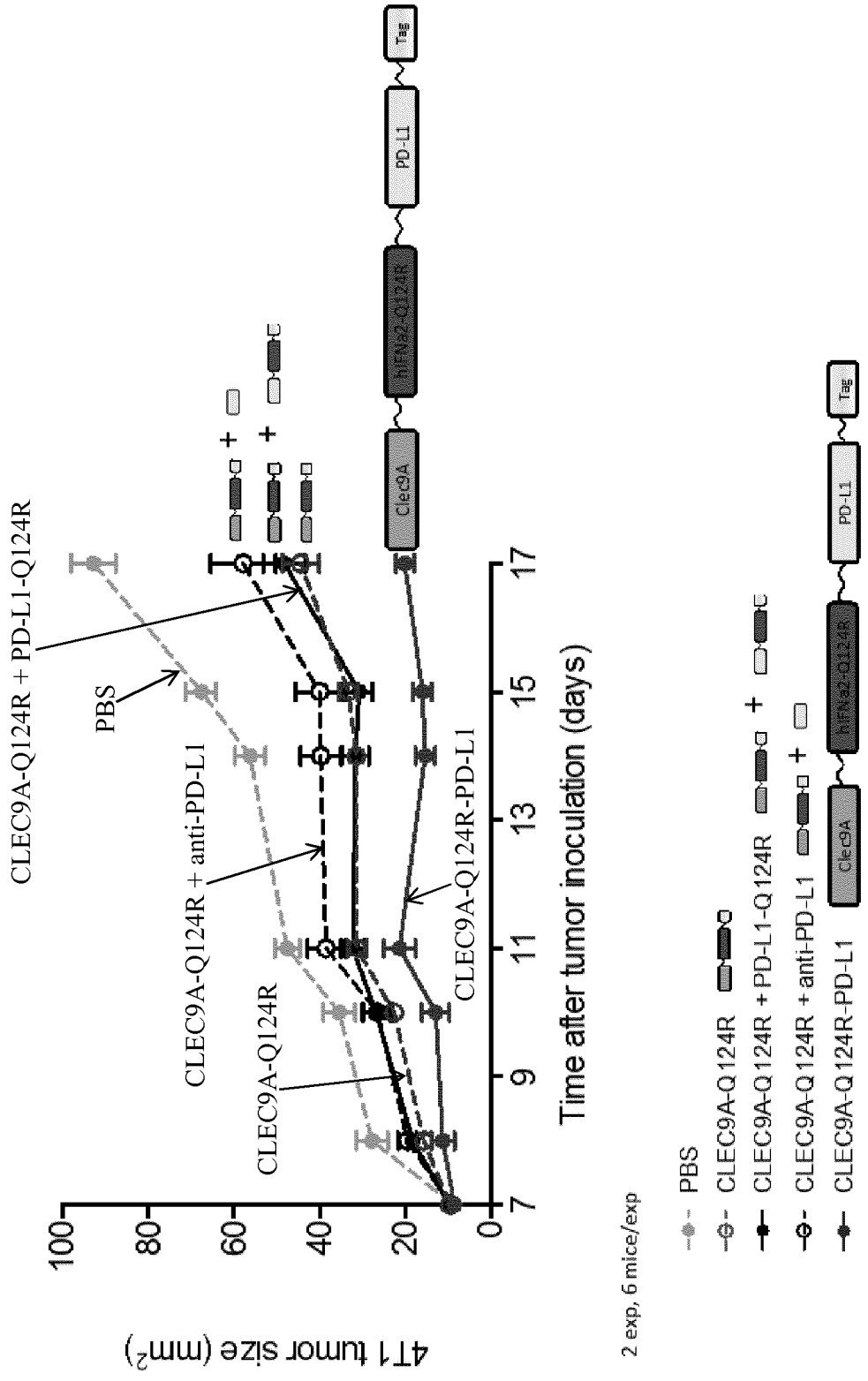
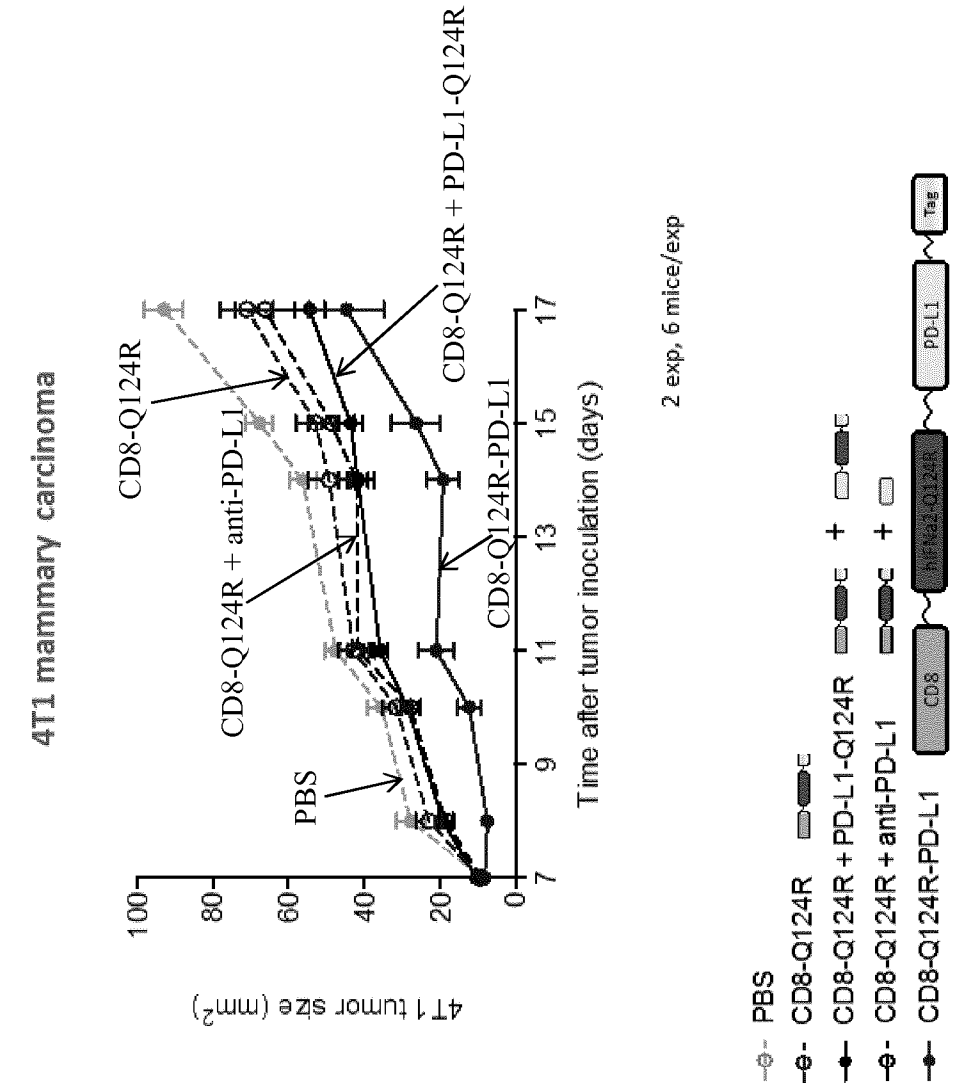

4T1 tumor size (mm²)
Time after tumor inoculation (days)
CLEC9A-Q124R + PD-L1-Q124R
PBS
CLEC9A-Q124R + anti-PD-L1
CLEC9A-Q124R
CLEC9A-Q124R-PD-L1
2 exp, 6 mice/exp
PBS
CLEC9A-Q124R
CLEC9A-Q124R + PD-L1-Q124R
CLEC9A-Q124R + anti-PD-L1
CLEC9A-Q124R-PD-L1
Clec9A
hFN-β2-Q124R
PD-L1
Tag

FIG. 8 (CONT.)
C.


4T1 mammary carcinoma
CD8-Q124R
CD8-Q124R + anti-PD-L1
PBS
CD8-Q124R + PD-L1-Q124R
CD8-Q124R-PD-L1
4T1 tumor size (mm2)
Time after tumor inoculation (days)
2 exp, 6 mice/exp
PBS
CD8-Q124R
CD8-Q124R + PD-L1-Q124R
CD8-Q124R + anti-PD-L1
CD8-Q124R-PD-L1
CD8
PD-L1
Tag

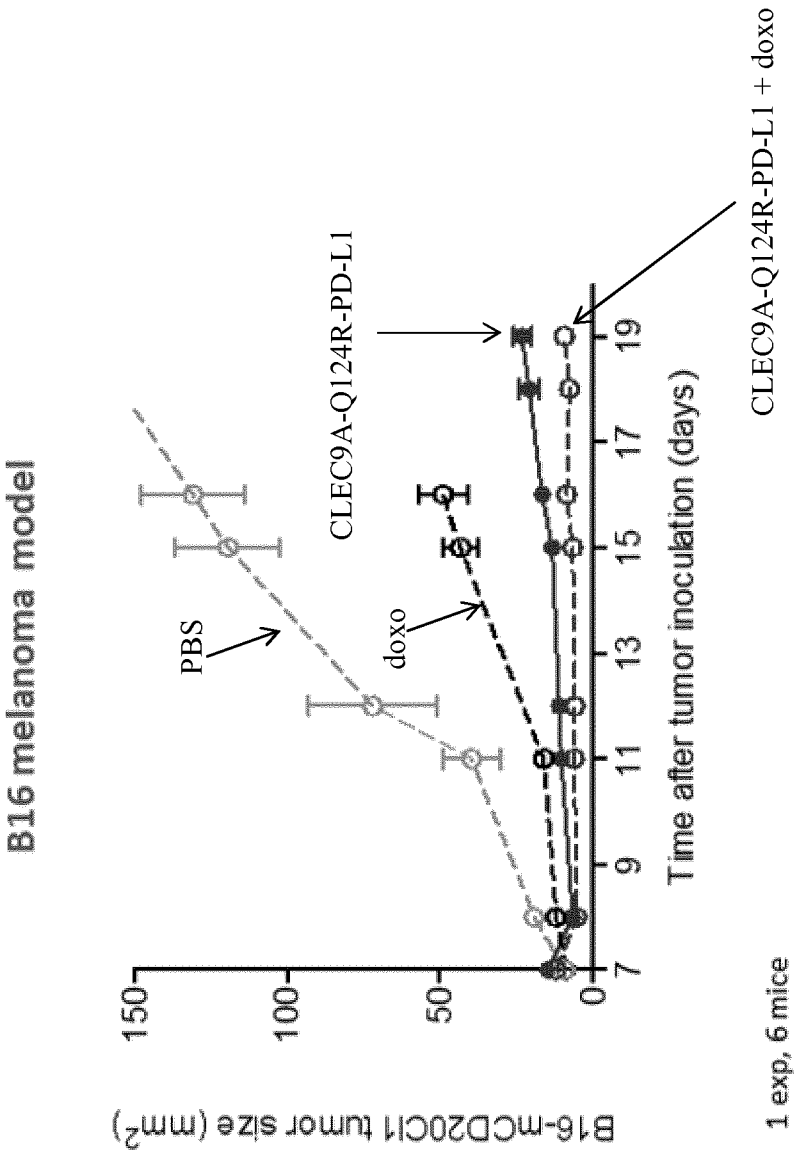

B16 melanoma model
B16-mCD20Cl1 tumor size (mm²)
150
100
50
0
7
9
11
13
15
17
19
Time after tumor inoculation (days)
PBS
doxo
CLEC9A-Q124R-PD-L1
CLEC9A-Q124R-PD-L1 + doxo
1 exp, 6 mice

FIG. 9
A.

FIG. 9 (CONT.)

B.

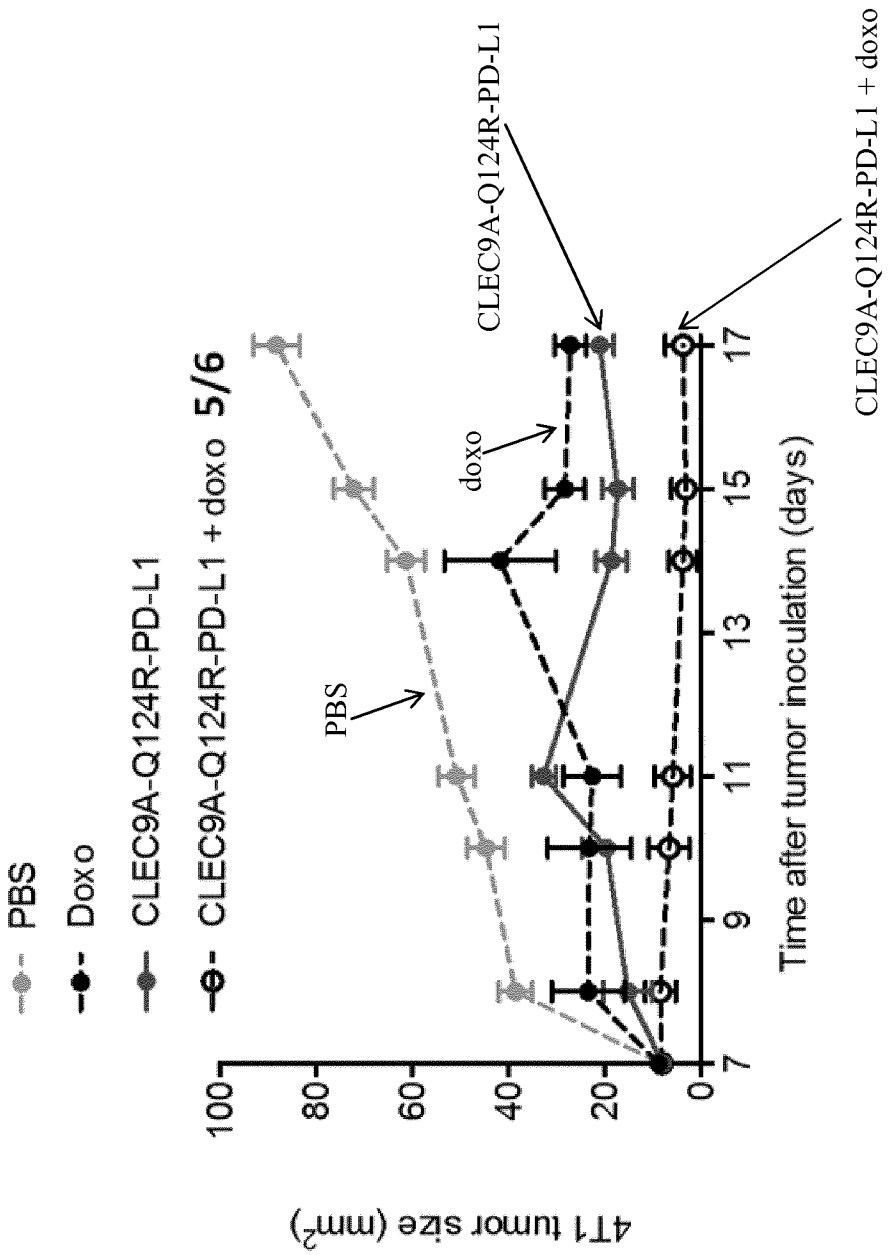
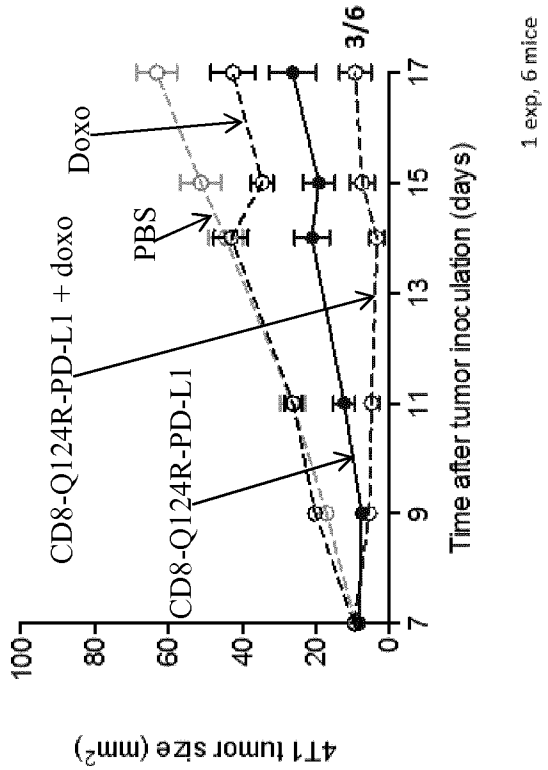

PBS
Doxo
CLEC9A-Q124R-PD-L1
CLEC9A-Q124R-PD-L1 + doxo 5/6
4T1 tumor size (mm²)
Time after tumor inoculation (days)
PBS
doxo
CLEC9A-Q124R-PD-L1
CLEC9A-Q124R-PD-L1 + doxo

FIG. 9 (CONT.)

C.


4T1 mammary carcinoma
4T1 tumor size (mm²)
Time after tumor inoculation (days)
CD8-Q124R-PD-L1 + doxo
Doxo
PBS
CD8-Q124R-PD-L1
3/6
1 exp, 6 mice
PBS
Doxo
CD8-Q124R-PD-L1
CD8-Q124R-PD-L1 + doxo

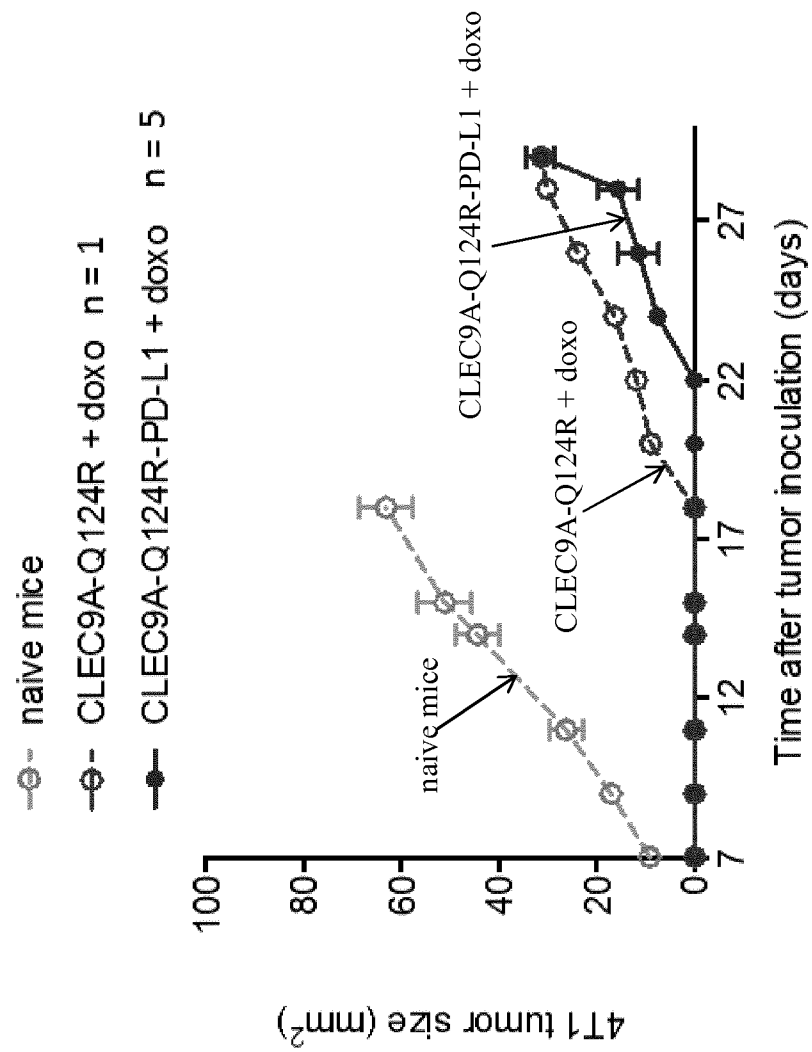

naive mice
CLEC9A-Q124R + doxo n = 1
CLEC9A-Q124R-PD-L1 + doxo n = 5
4T1 tumor size (mm²)
100
80
60
40
20
0
7
12
17
22
27
Time after tumor inoculation (days)
naive mice
CLEC9A-Q124R + doxo
CLEC9A-Q124R-PD-L1 + doxo

FIG. 10

FIG. 11

A.

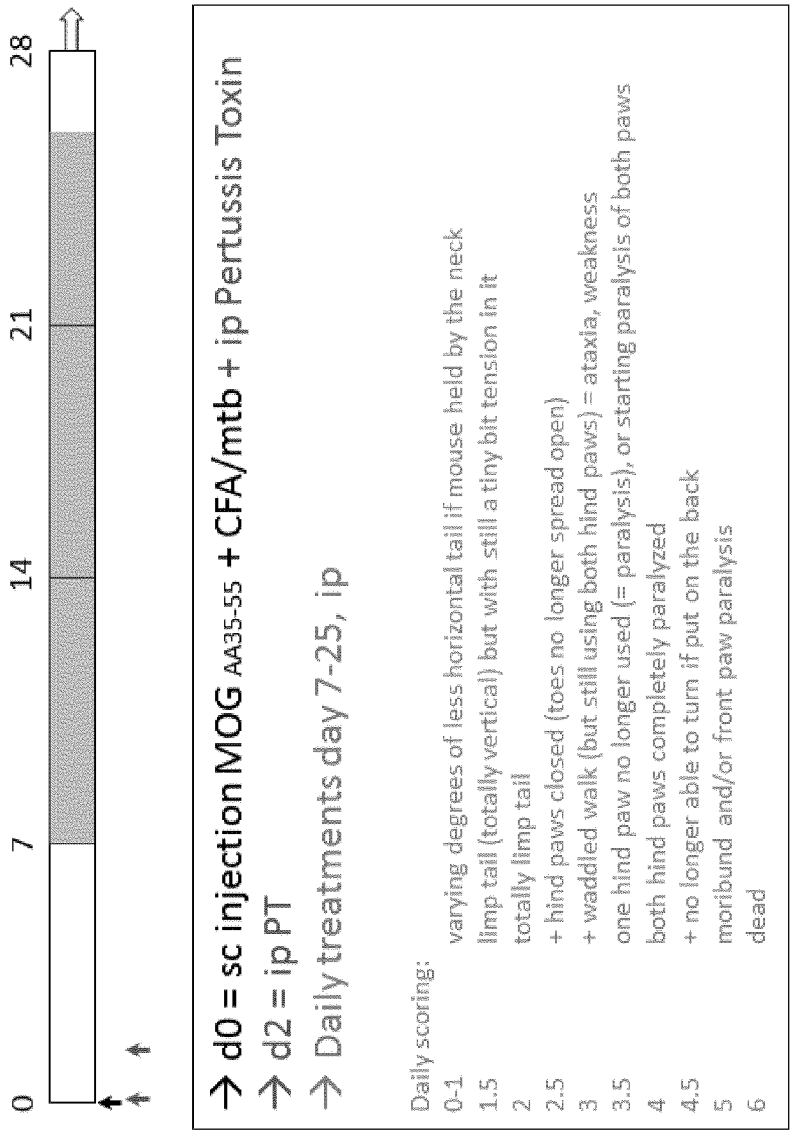

0
7
14
21
28
→ d0 = sc injection MOG AA35-55 + CFA/mtb + ip Pertussis Toxin
→ d2 = ip PT
→ Daily treatments day 7-25, ip
Daily scoring:
0-1 varying degrees of less horizontal tail if mouse held by the neck
1.5 limp tail (totally vertical) but with still a tiny bit tension in it
2 totally limp tail
2.5 + hind paws closed (toes no longer spread open)
3 + waddled walk (but still using both hind paws) = ataxia, weakness
3.5 one hind paw no longer used (= paralysis), or starting paralysis of both paws
4 both hind paws completely paralyzed
4.5 + no longer able to turn if put on the back
5 moribund and/or front paw paralysis
6 dead

FIG. 11 (CONT.)

B.

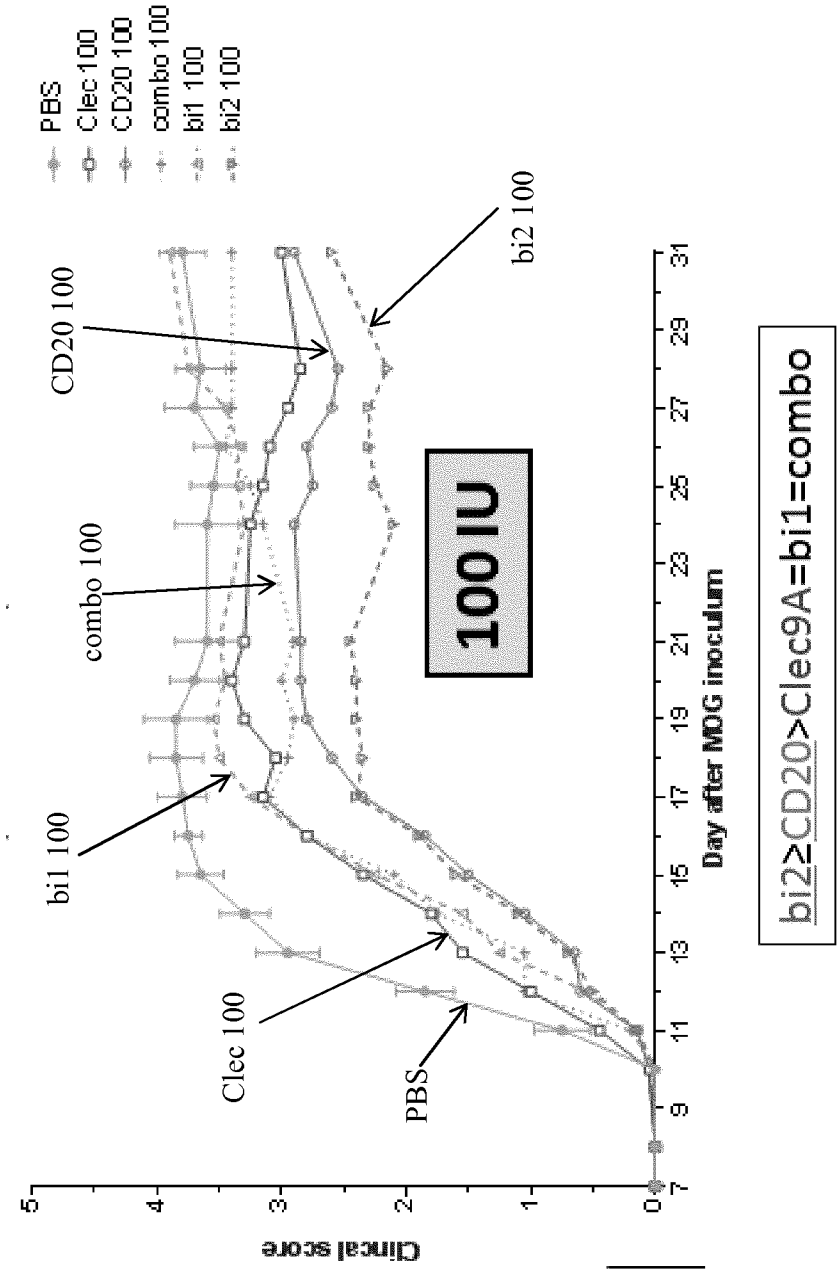
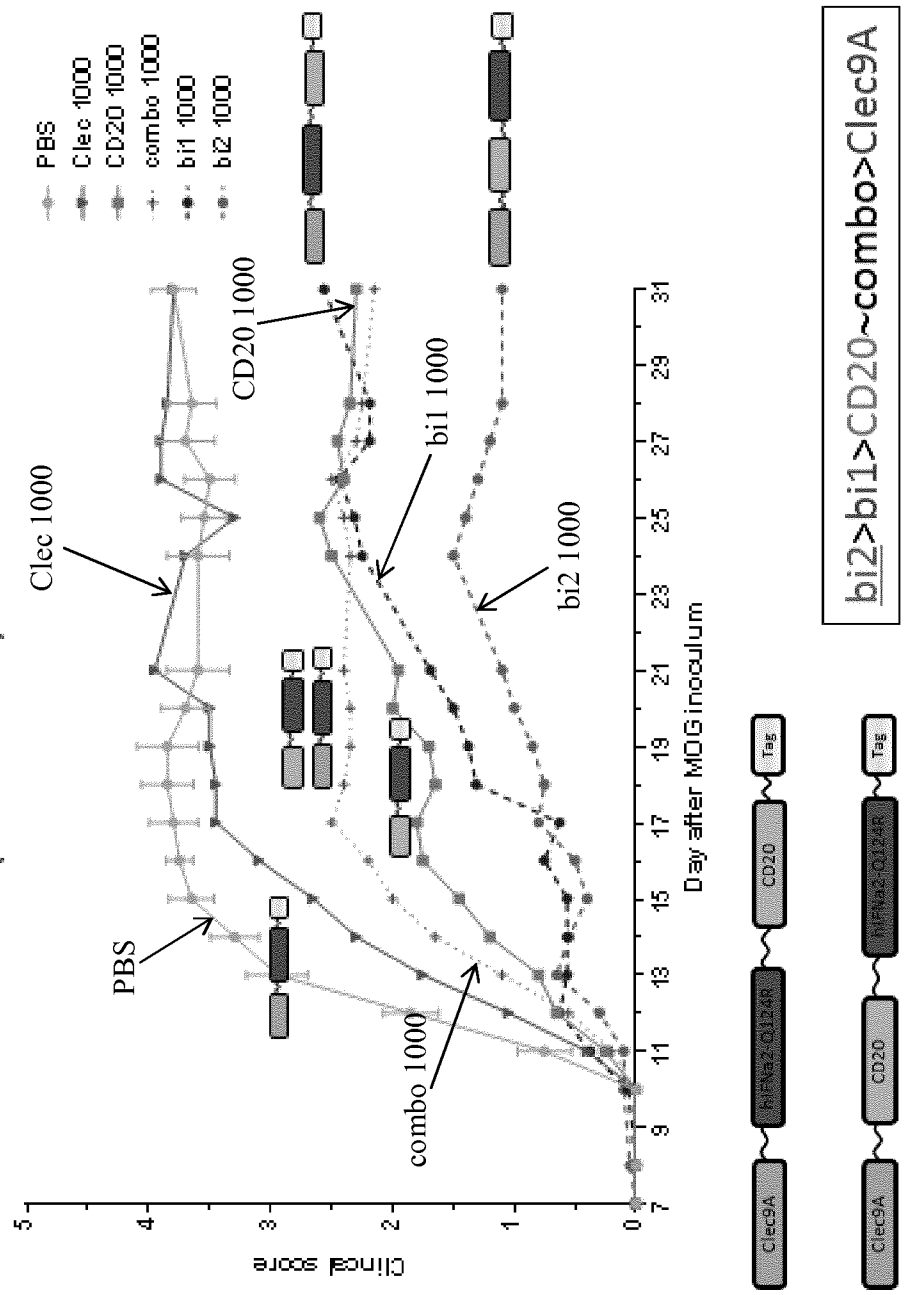

PBS
Clec100
CD20 100
combo100
bi1 100
bi2 100
CD20 100
combo 100
bi1 100
bi2 100
100 IU
Clec 100
PBS
Clinical score
Day after MOG inoculum
bi2≥CD20>Clec9A=bi1=combo

FIG. 11 (CONT.)

C.


clinical score
(treatment d7-28)
PBS
Clec 1000
CD20 1000
combo 1000
bi1 1000
bi2 1000
Clinical score
Day after MOG inoculum
bi2>bi1>CD20~combo>Clec9A
Clec9A
CD20
Tag

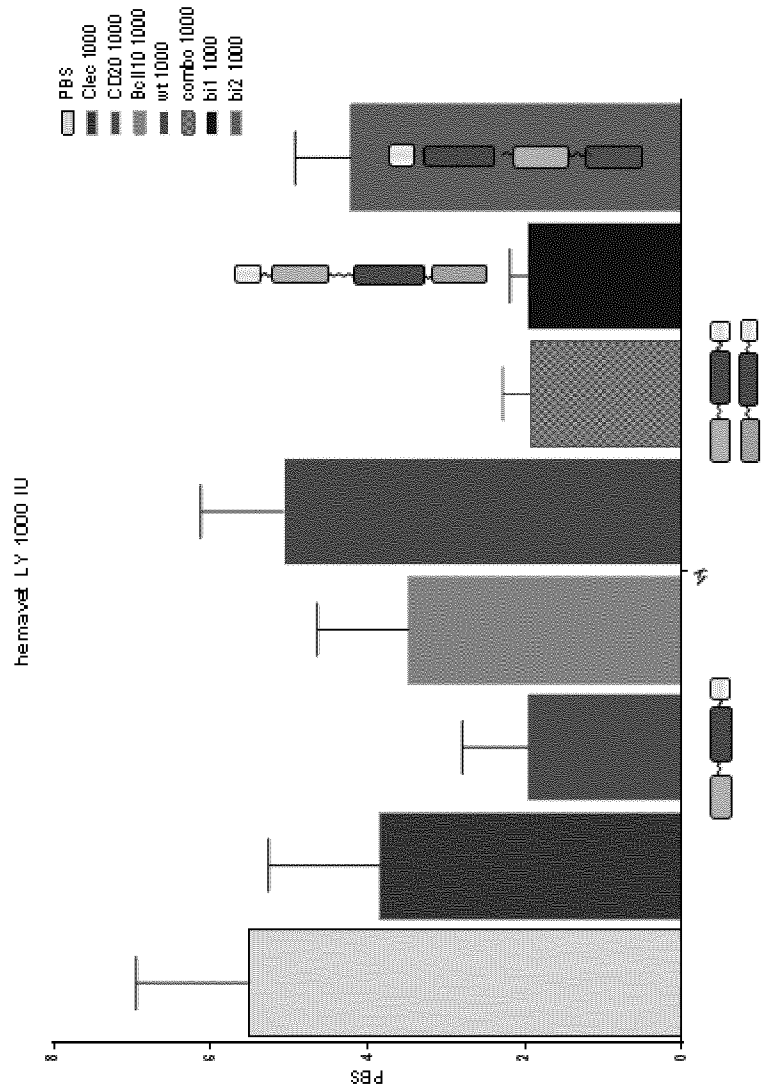

PBS
Clec 1000
CD20 1000
Bcl10 1000
wt 1000
combo 1000
bi1 1000
bi2 1000
hemavet LY 1000 IU
PBS
0
2
4
6
8

FIG. 11 (CONT.)

D.

FIG. 11 (CONT.)

E.

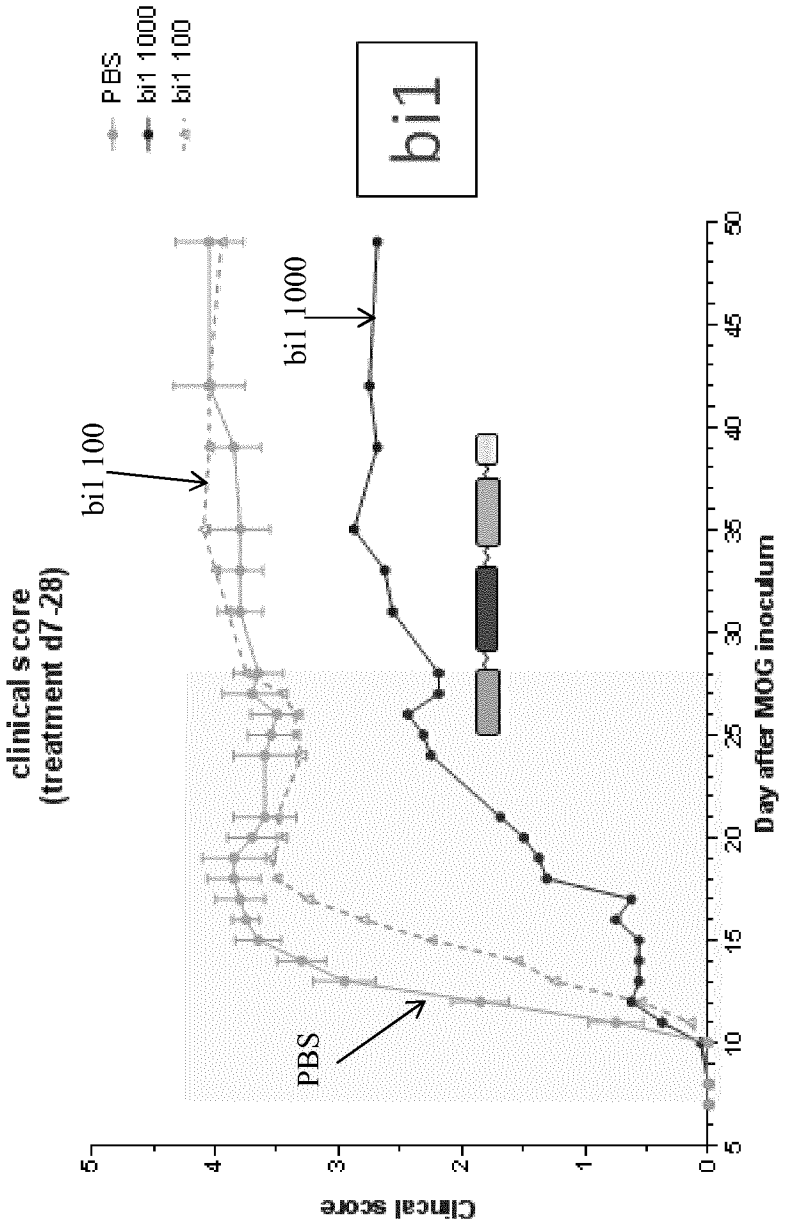

PBS
bi1 1000
bi1 100
bi1
clinical score
(treatment d7-28)
bi1_100
bi1_1000
PBS
Clinical score
Day after MOG inoculum
0
1
2
3
4
5
5
10
15
20
25
30
35
40
45
50

FIG. 11 (CONT.)

F.

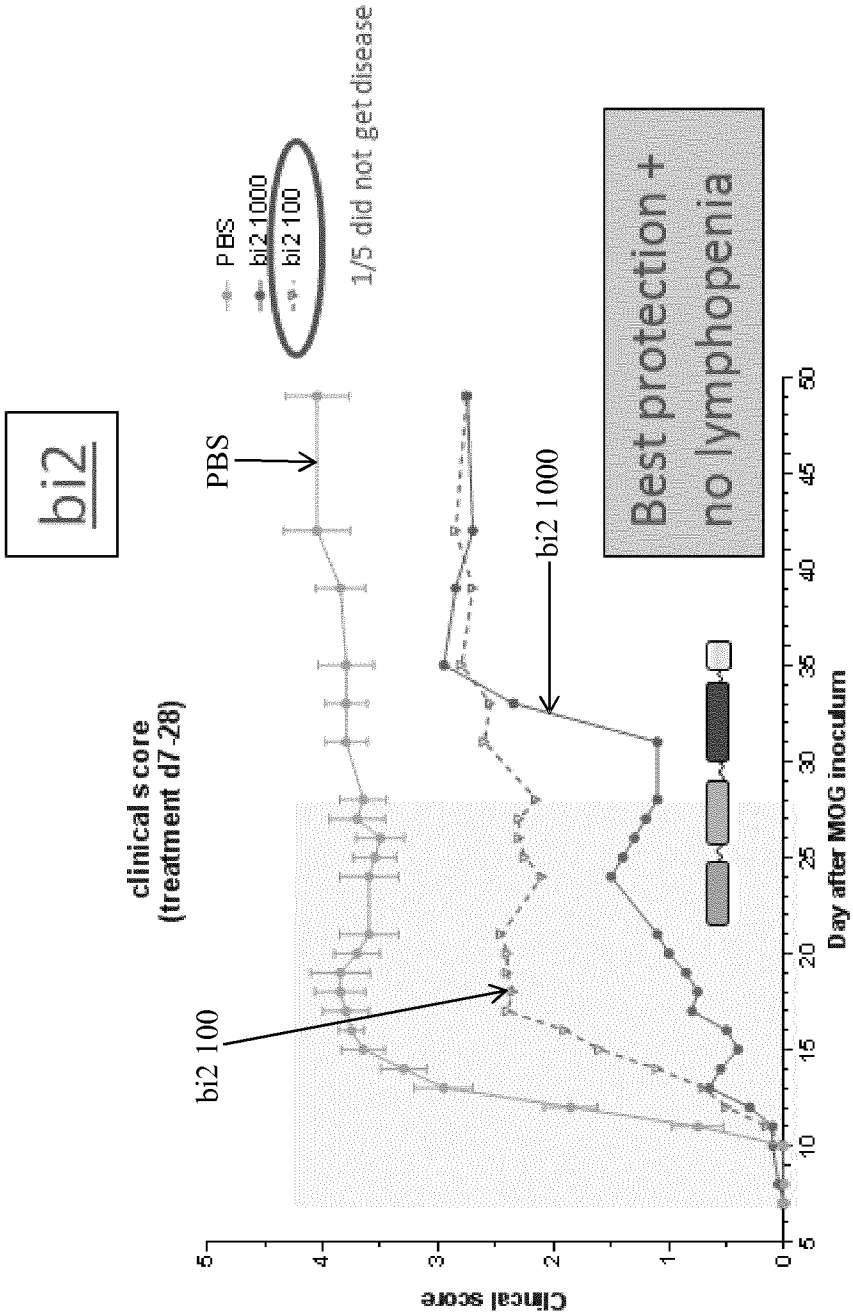

bi2
clinical score
(treatment d7-28)
PBS
bi2 1000
bi2 100
1/5 did not get disease
PBS
bi2 100
bi2 1000
Best protection +
no lymphopenia
Clinical score
Day after MOG inoculum
0
1
2
3
4
5
5
10
15
20
25
30
35
40
45
50

FIG. 12

A.

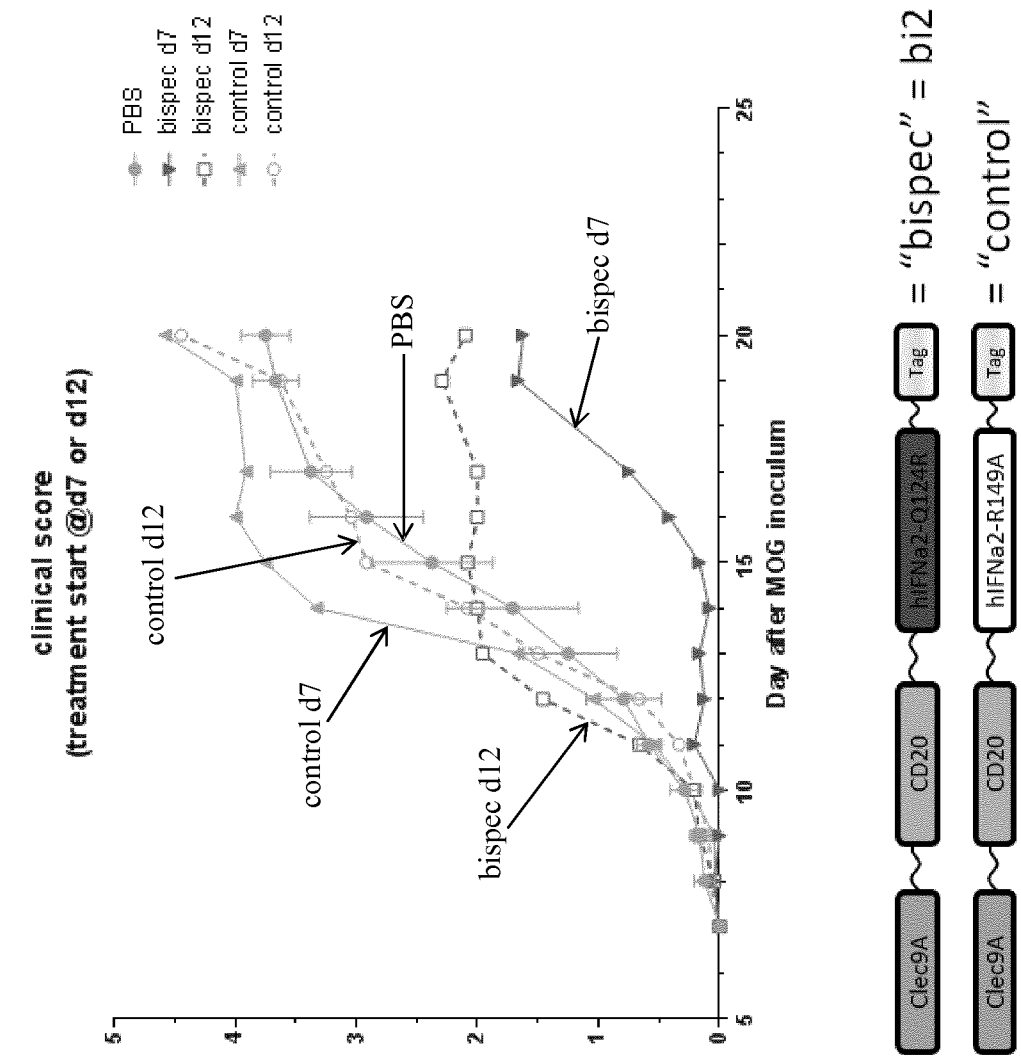

clinical score
(treatment start @d7 or d12)
PBS
bispec d7
bispec d12
control d7
control d12
Clinical score
Day after MOG inoculum
control d12
control d7
bispec d12
PBS
bispec d7
Clec9A
CD20
hIFNa2-Q124R
Tag
= "bispec" = bi2
Clec9A
CD20
hIFNa2-R149A
Tag
= "control"

FIG. 12

B.

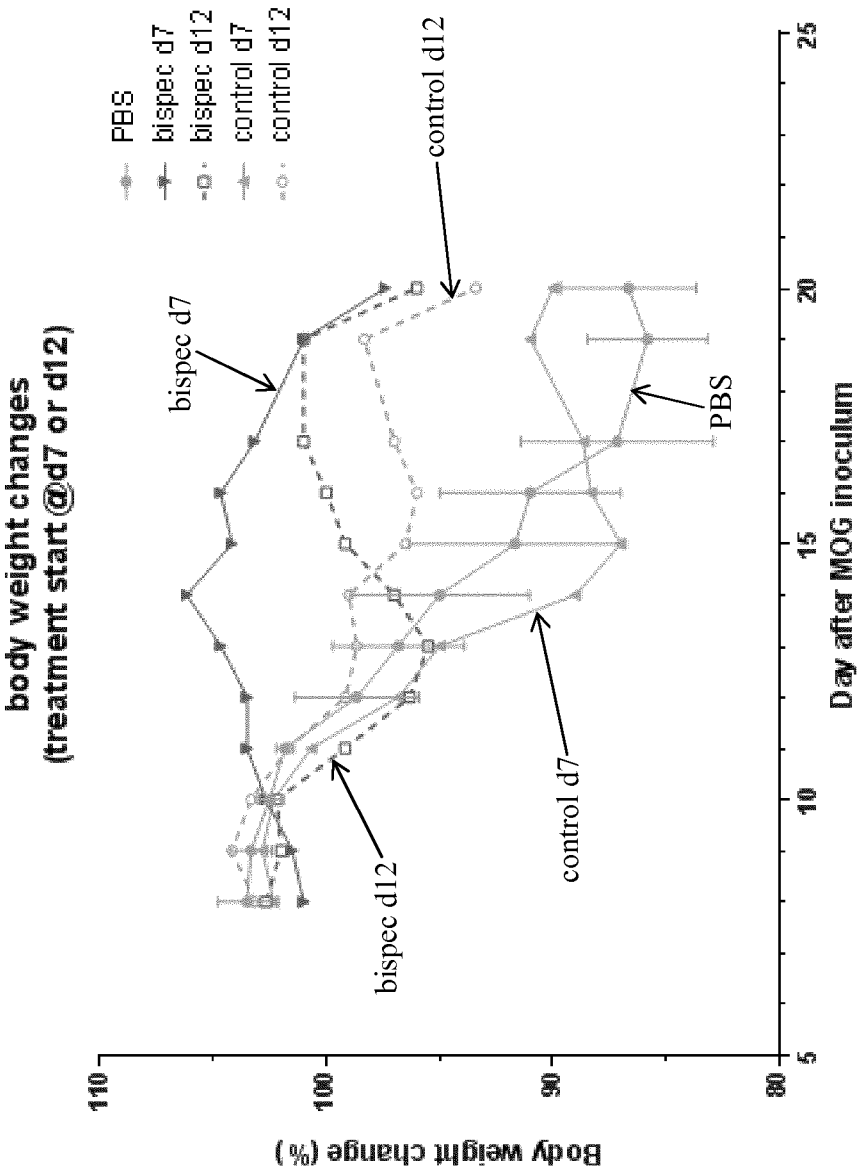
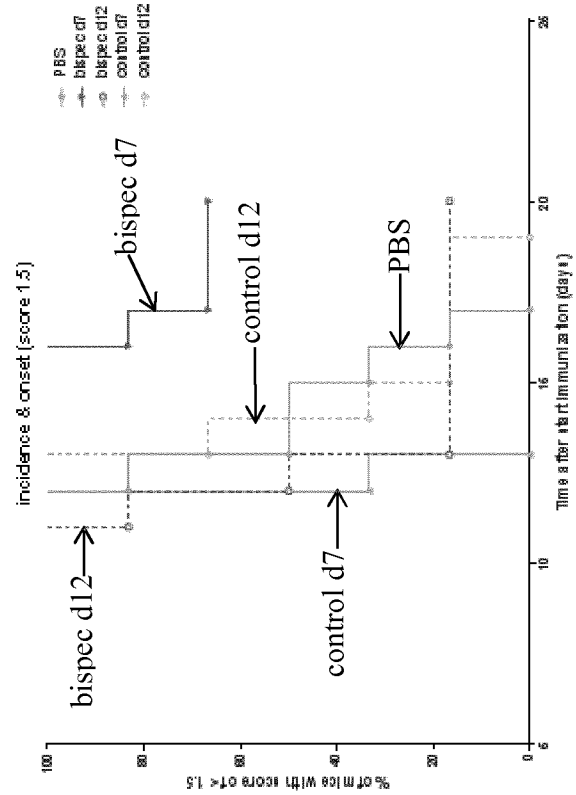

body weight changes
(treatment start @d7 or d12)
PBS
bispec d7
bispec d12
control d7
control d12
bispec d7
control d12
bispec d12
control d7
PBS
Body weight change (%)
110
100
90
80
5
10
15
20
25
Day after MOG inoculum

FIG. 12

C.


PBS
bispec d7
bispec d12
control d7
control d12
incidence & onset (score 1.5)
bispec d12
bispec d7
control d12
control d7
PBS
% of mice with score of< 1.5
Time after start immunization (days)

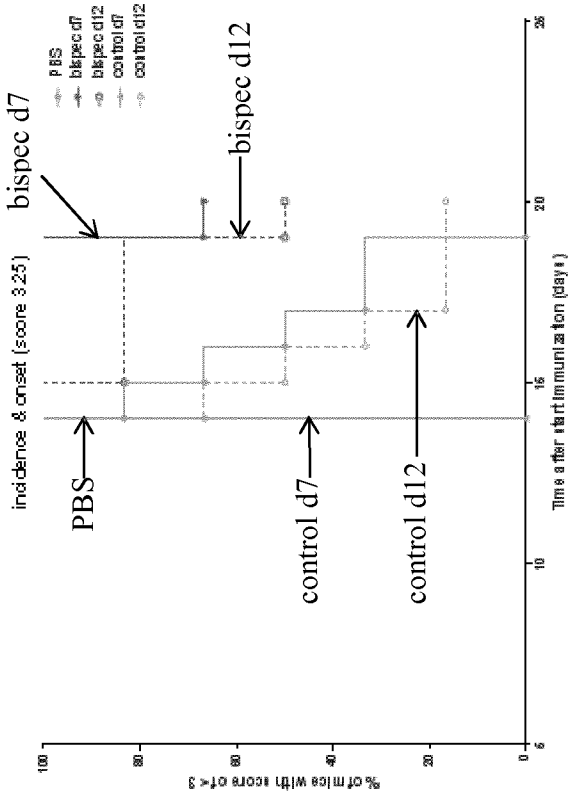

PBS
bispec d7
bispec d12
control d7
control d12
incidence & onset (score 3.25)
PBS
bispec d7
control d7
bispec d12
control d12
% of mice with score of< 3
Time after start immunization (days)

FIG. 13
A.

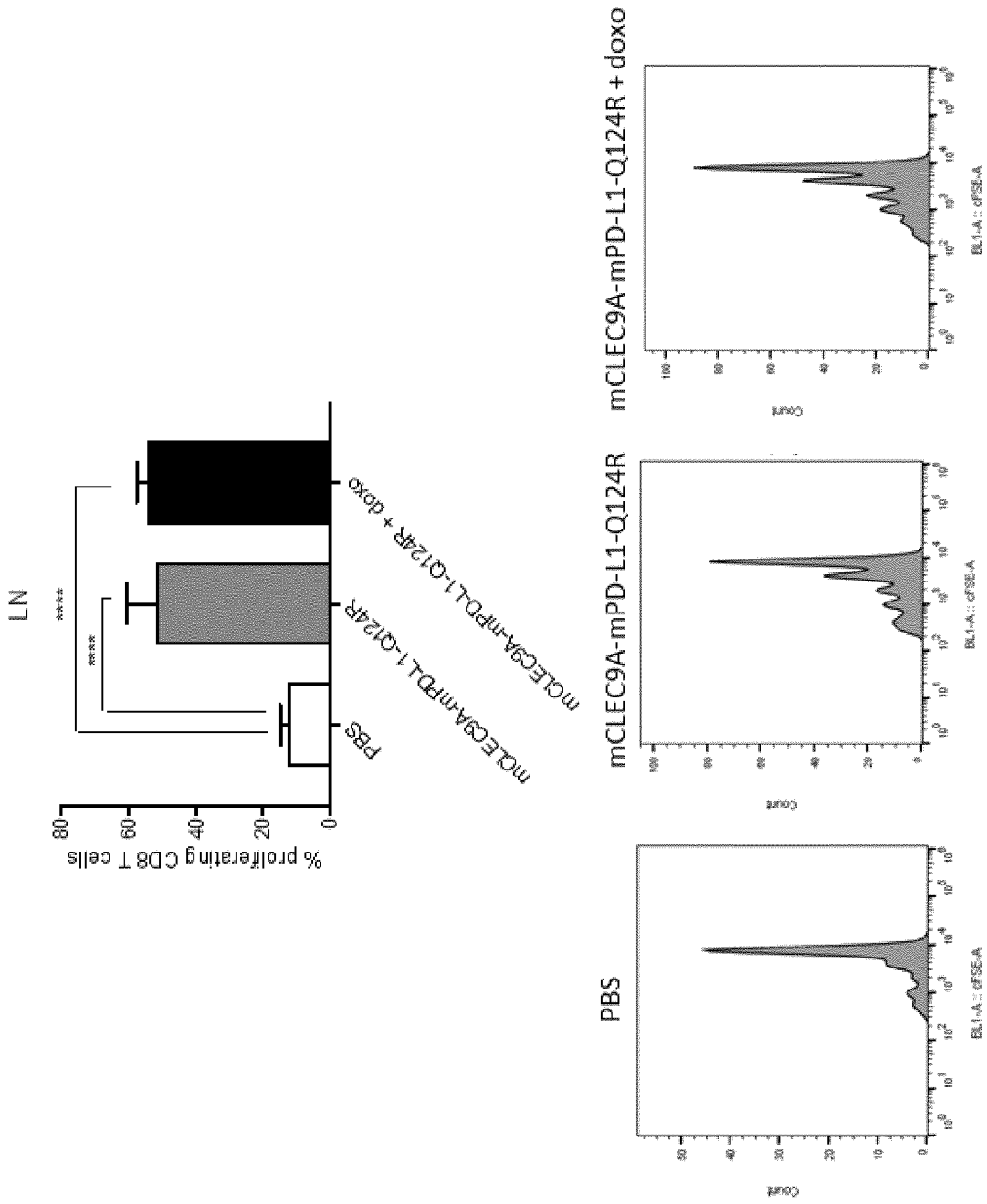
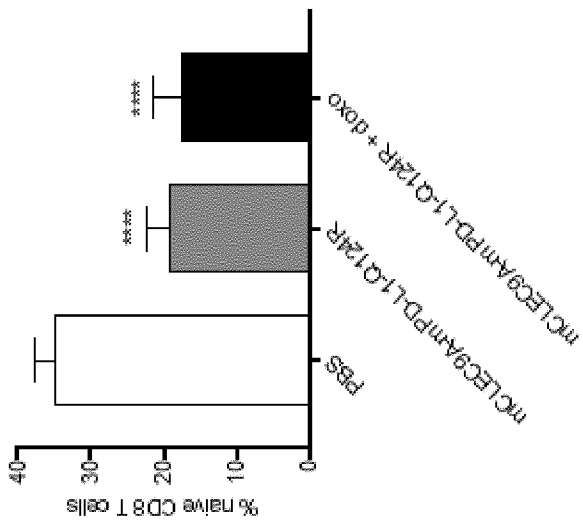
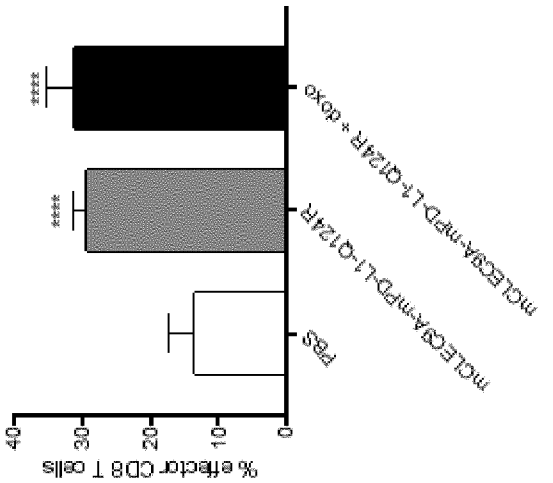

LN
% proliferating CD8 T cells
0
20
40
60
80
****
****
PBS
mCLEC9A-mPD-L1-Q124R
mCLEC9A-mPD-L1-Q124R + doxo
PBS
mCLEC9A-mPD-L1-Q124R
mCLEC9A-mPD-L1-Q124R + doxo
Count
BL1-A :: CFSE-A

FIG. 13 (CONT.)
B.


% naive CD8 T cells
0
10
20
30
40
PBS
mCLEC9A-mPDL1-Q12AR
mCLEC9A-mPDL1-Q12AR + doxo
****
****


% effector CD8 T cells
0
10
20
30
40
PBS
mCLEC9A-mPDL1-Q12AR
mCLEC9A-mPDL1-Q12AR + doxo
****
****

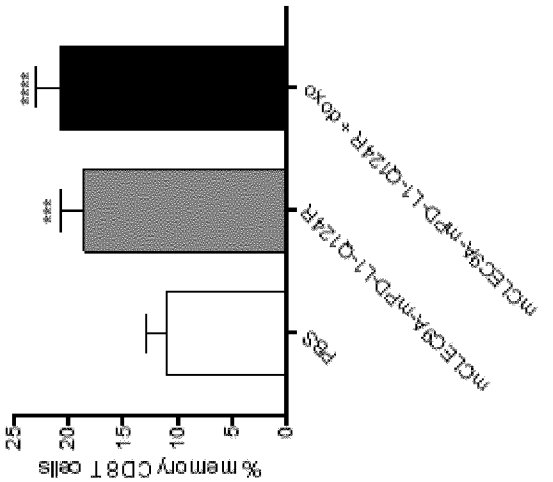

% memory CD8 T cells
0
5
10
15
20
25
PBS
mCLEC9A-mPDL1-Q12AR
mCLEC9A-mPDL1-Q12AR + doxo
***
****

FIG. 13 (CONT.)
C.

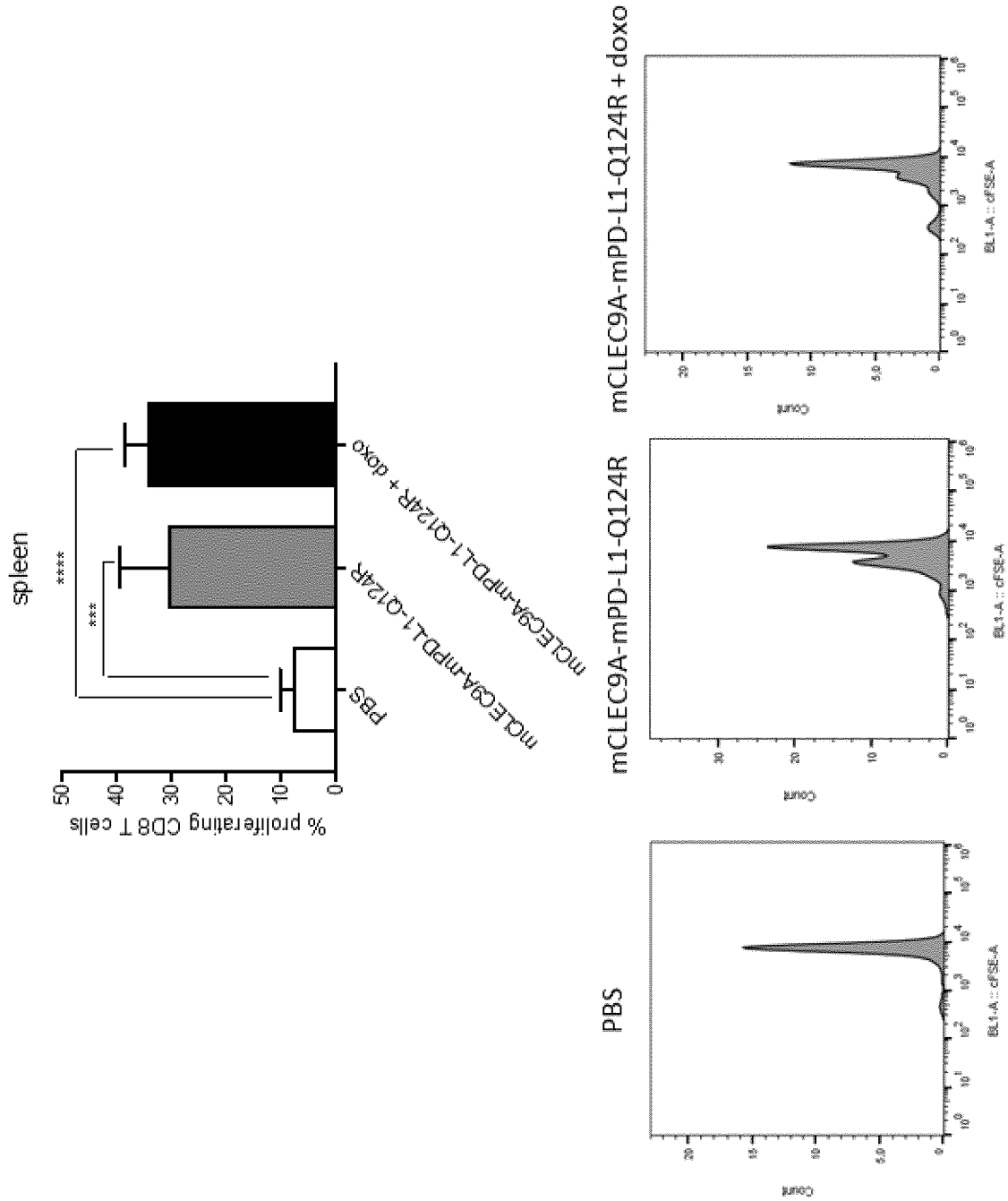
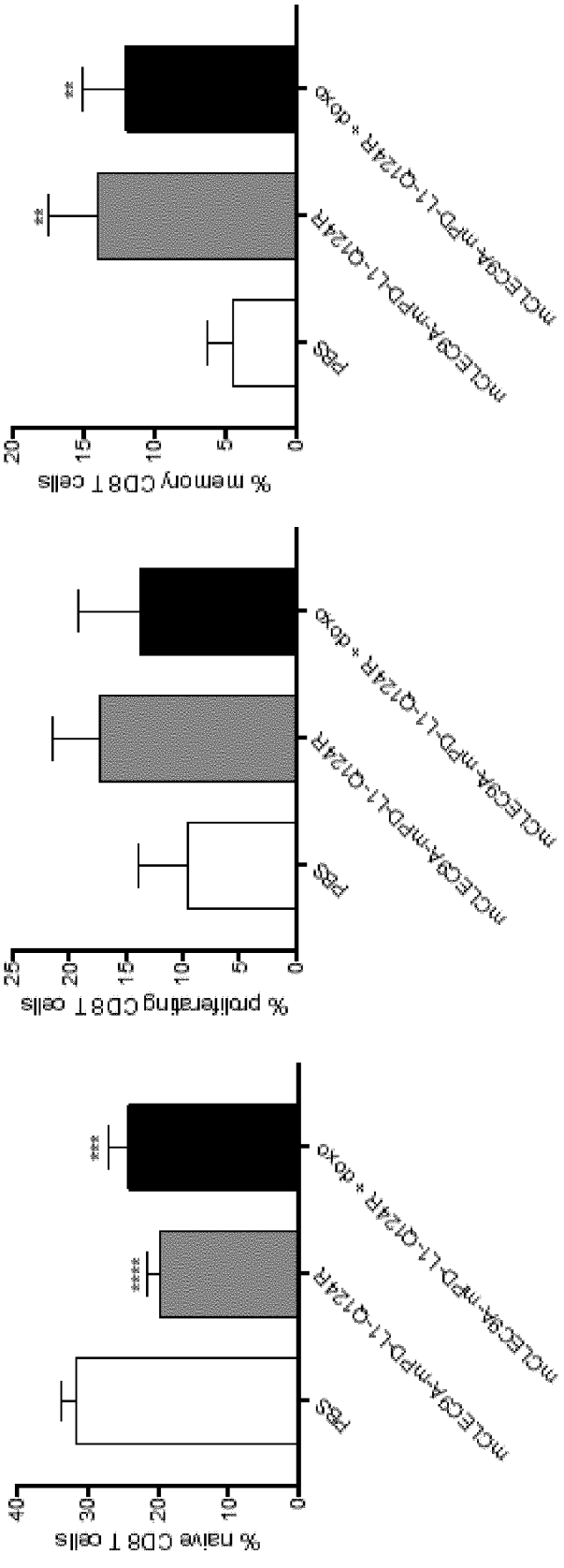

spleen
% proliferating CD8 T cells
0
10
20
30
40
50
****
***
PBS
mCLEC9A-mPD-L1-Q124R
mCLEC9A-mPD-L1-Q124R + doxo
PBS
mCLEC9A-mPD-L1-Q124R
mCLEC9A-mPD-L1-Q124R + doxo
Count
BL1-A :: cFSE-A

FIG. 13 (CONT.)
D.


% memory CD8 T cells
0
5
10
15
20
PBS
mCLEC9A-mPD-L1-Q124R
mCLEC9A-mPD-L1-Q124R + doxo
**
**
% proliferating CD8 T cells
0
5
10
15
20
25
PBS
mCLEC9A-mPD-L1-Q124R
mCLEC9A-mPD-L1-Q124R + doxo
% naive CD8 T cells
0
10
20
30
40
PBS
mCLEC9A-mPD-L1-Q124R
mCLEC9A-mPD-L1-Q124R + doxo
****
***

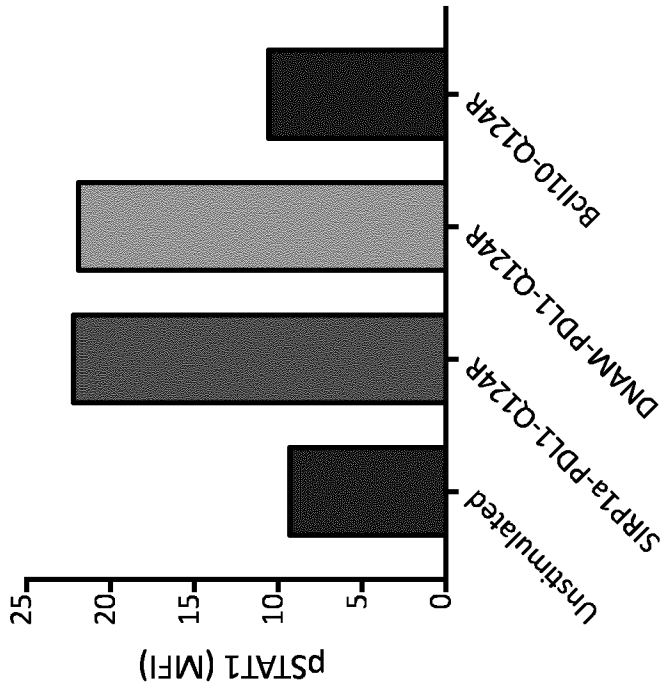

pSTAT1 (MFI)
0
5
10
15
20
25
Unstimulated
SIRP1a-PDL1-Q124R
DNAM-PDL1-Q124R
Bcl10-Q124R

FIG. 14

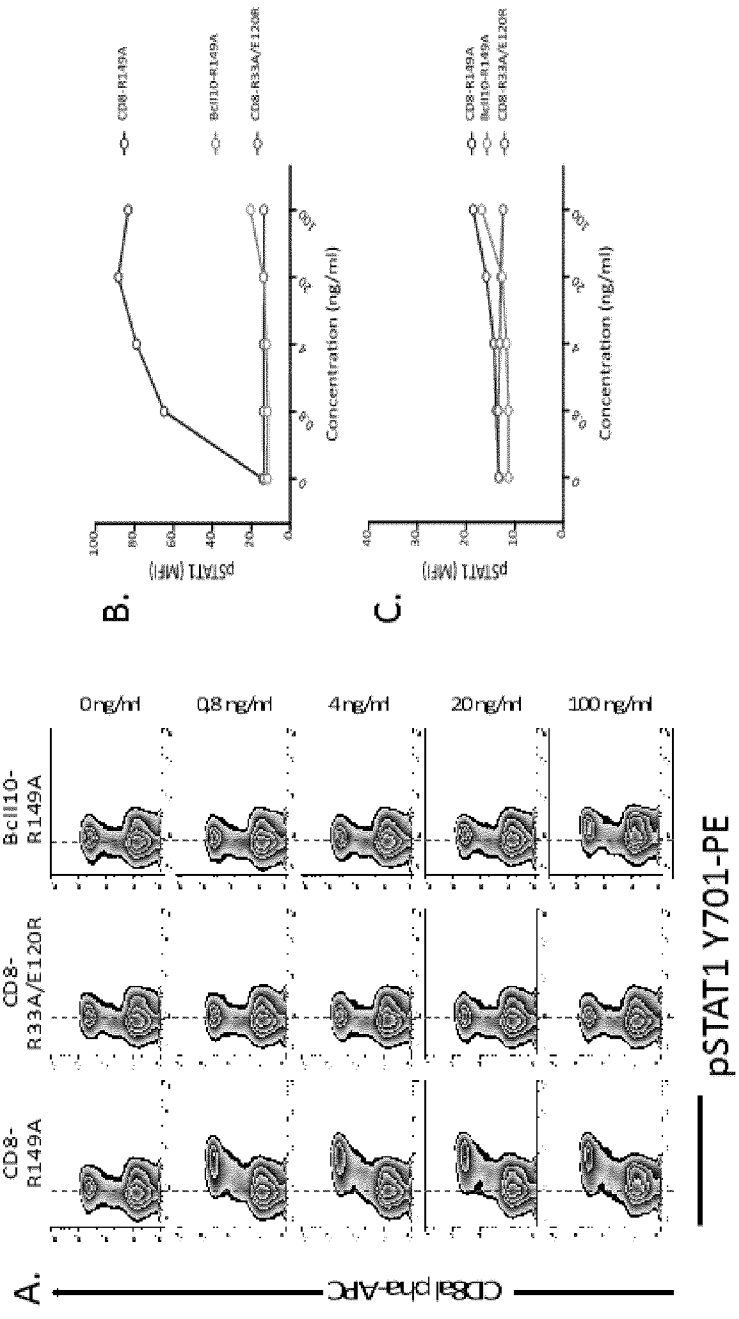

A.
CD8a! pha-APC
pSTAT1 Y701-PE
CD8-R149A
CD8-R33A/E120R
Bcll10-R149A
0ng/ml
0,8 ng/ml
4ng/ml
20ng/ml
100 ng/ml
B.
pSTAT1 (MFI)
Concentration (ng/ml)
CD8-R149A
Bcll10-R149A
CD8-R33A/E120R
C.
pSTAT1 (MFI)
Concentration (ng/ml)
CD8-R149A
Bcll10-R149A
CD8-R33A/E120R

FIG. 15

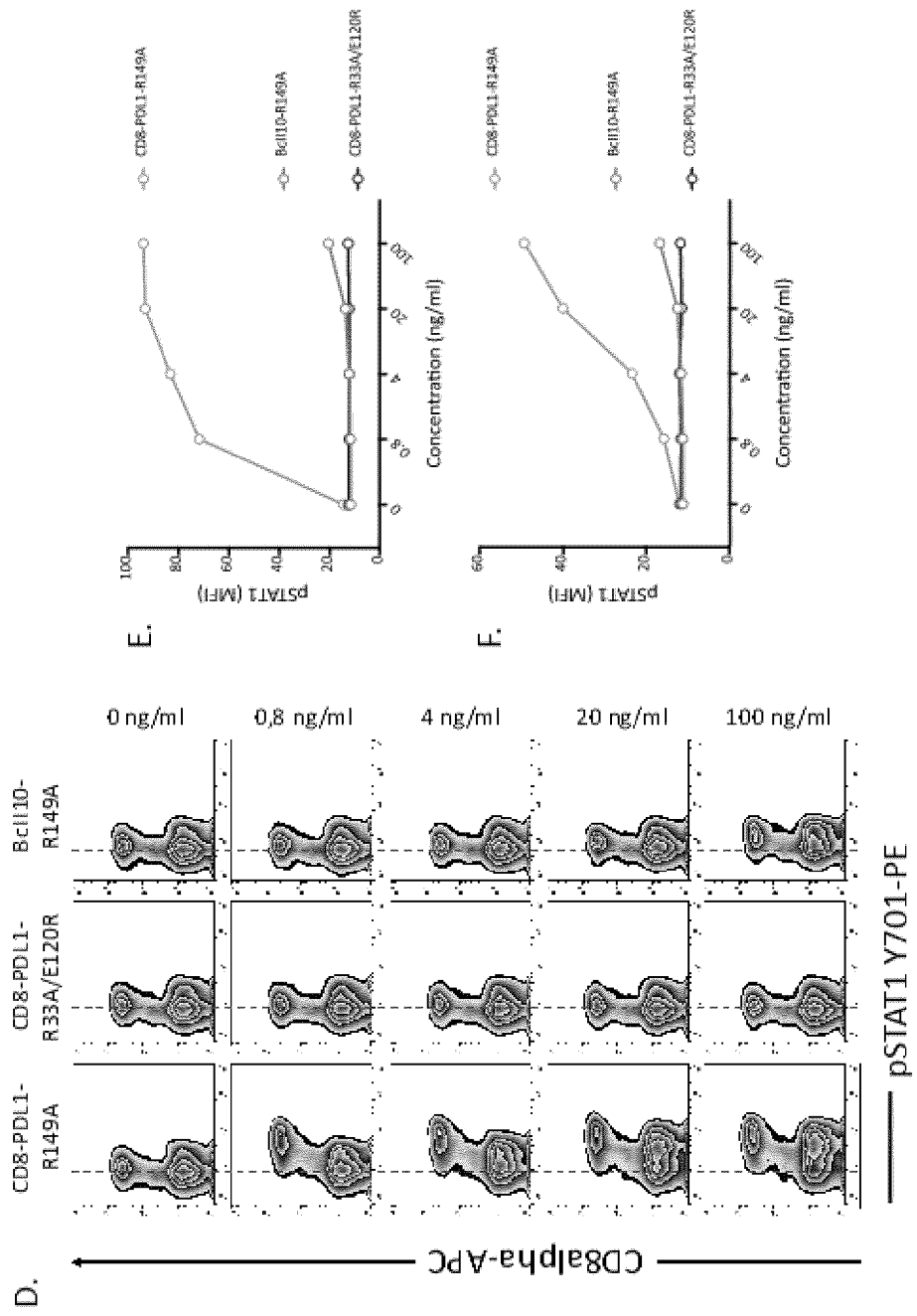

D.
CD8-PDL1-R149A
CD8-PDL1-R33A/E120R
BcII10-R149A
0 ng/ml
0,8 ng/ml
4 ng/ml
20 ng/ml
100 ng/ml
CD8alpha-APC
pSTAT1 Y701-PE
E.
pSTAT1 (MFI)
Concentration (ng/ml)
F.
pSTAT1 (MFI)
Concentration (ng/ml)

FIG. 15 (CONT.)

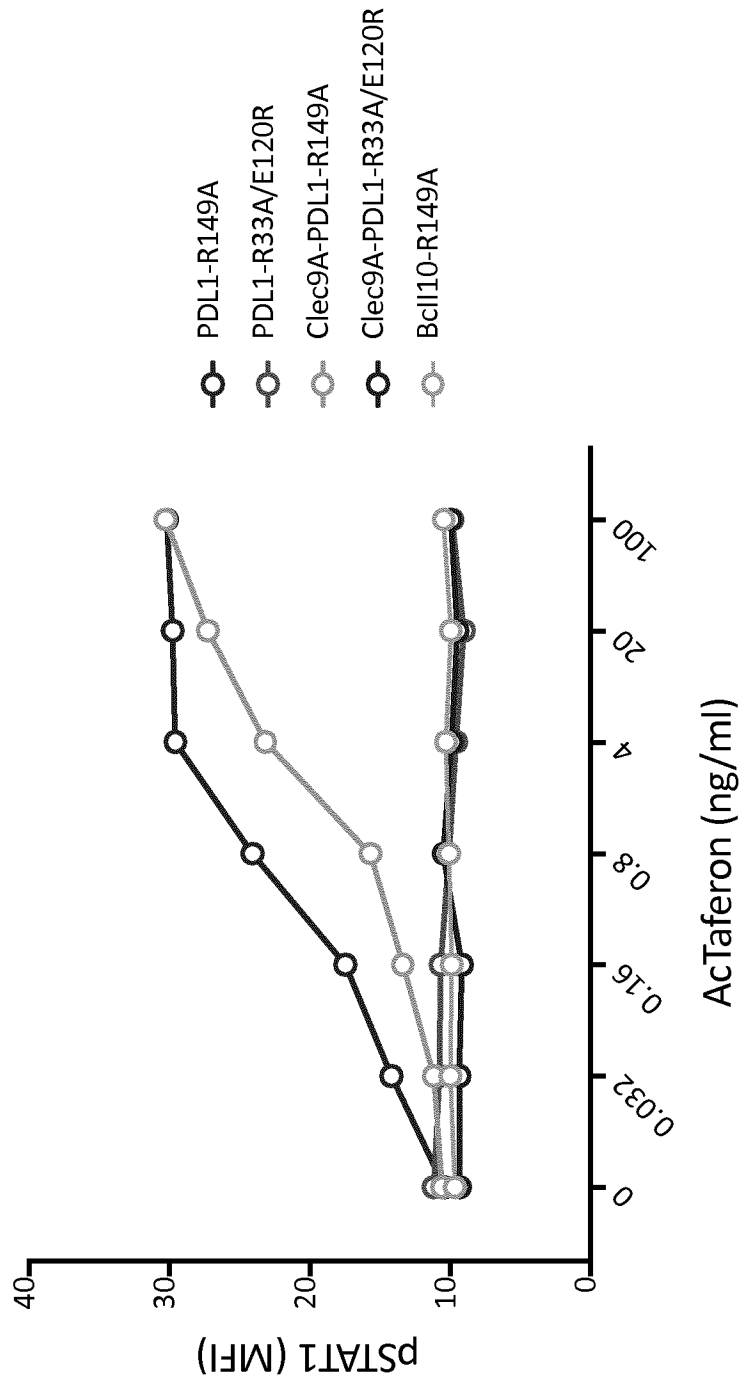

PDL1-R149A
PDL1-R33A/E120R
Clec9A-PDL1-R149A
Clec9A-PDL1-R33A/E120R
Bcll10-R149A
pSTAT1 (MFI)
0
10
20
30
40
0
0.032
0.16
0.8
4
20
100
AcTaferon (ng/ml)

FIG. 16

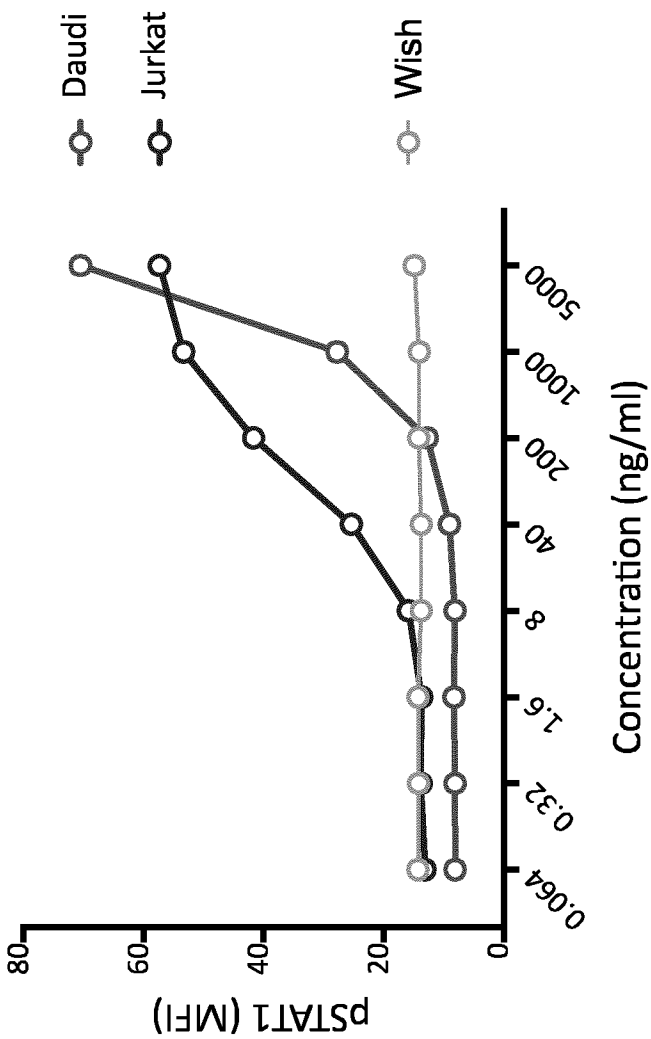

Daudi
Jurkat
Wish
pSTAT1 (MFI)
0
20
40
60
80
0.064
0.32
1.6
8
40
200
1000
5000
Concentration (ng/ml)

FIG. 17

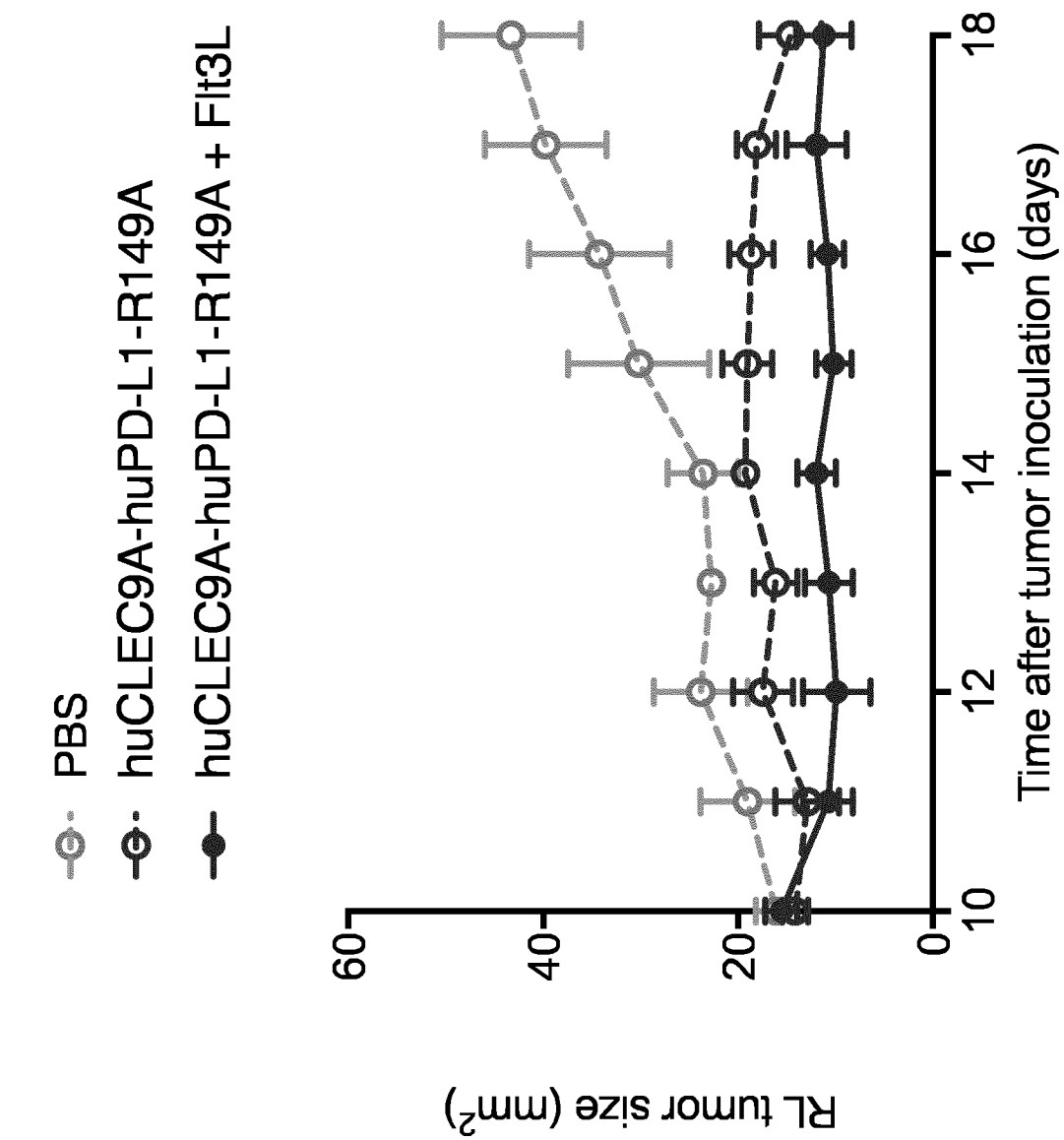

PBS
huCLEC9A-huPD-L1-R149A
huCLEC9A-huPD-L1-R149A + Flt3L
RL tumor size (mm²)
60
40
20
0
10
12
14
16
18
Time after tumor inoculation (days)

FIG. 18

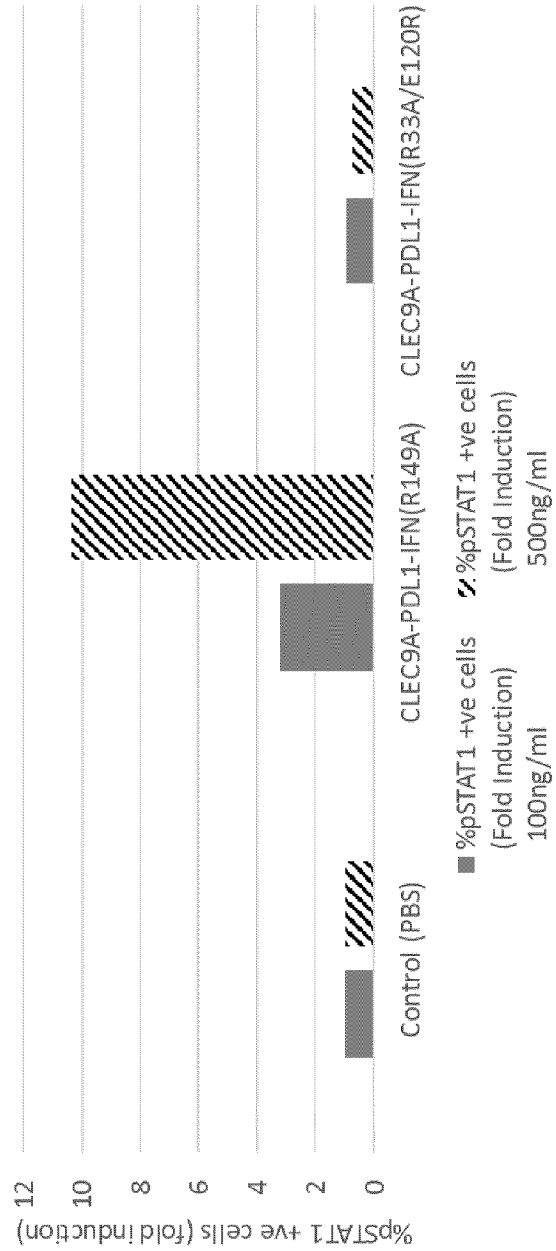

%pSTAT1 +ve cells (fold induction)
12
10
8
6
4
2
0
Control (PBS)
CLEC9A-PDL1-IFN(R149A)
CLEC9A-PDL1-IFN(R33A/E120R)
%pSTAT1 +ve cells (Fold Induction) 100ng/ml
%pSTAT1 +ve cells (Fold Induction) 500ng/ml

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/052550

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/020273 A1 (CHANG CHIEN-HSING [US] ET AL) 27 January 2011 (2011-01-27) paragraph [0019]; claim 5 -----	1-34
X	US 2013/230517 A1 (GREWAL IQBAL [US] ET AL) 5 September 2013 (2013-09-05) table 1 ----- -/--	1-23, 26-34

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 April 2017	15/05/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Scheffzyk, Irmgard

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/052550

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R Kircheis ET AL: "Biological activity of mutants of human tumour necrosis factor-alpha", Immunology, 1 July 1992 (1992-07-01), pages 433-438, XP55361626, ENGLAND Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1421674/pdf/immunology00106-0087.pdf abstract -----	25
A	JOHN PUSKAS ET AL: "Development of an attenuated interleukin-2 fusion protein that can be activated by tumour-expressed proteases", IMMUNOLOGY, vol. 133, no. 2, 23 June 2011 (2011-06-23), pages 206-220, XP055073392, ISSN: 0019-2805, DOI: 10.1111/j.1365-2567.2011.03428.x abstract -----	24

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/052550

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011020273 A1	27-01-2011	NONE	
US 2013230517 A1	05-09-2013	AU 2013230484 A1	12-02-2015
		CA 2866126 A1	12-09-2013
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		EP 2822575 A1	14-01-2015
		JP 2015515453 A	28-05-2015
		US 2013230517 A1	05-09-2013
		US 2015139951 A1	21-05-2015
		US 2017073388 A1	16-03-2017
		WO 2013134138 A1	12-09-2013