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(71) Applicant: **DANA-FARBER CANCER INSTITUTE, INC.** [US/US]; 450 Brookline Avenue, Boston, MA 02215-5450 (US).

(72) Inventors: **FREEMAN, Gordon, J.**; 305 Walnut Street, Brookline MA 02445 (GB). **CHAUDHRI, Apoorvi**; Mailbox 316, Vanderbilt Hall, 107 Avenue Louis Pasteur, Boston, MA 02115 (US).

(74) Agent: **SMITH, DeAnn, F.** et al.; Foley Hoag LLP, 155 Seaport Boulevard, Boston, MA 02210-2600 (US).

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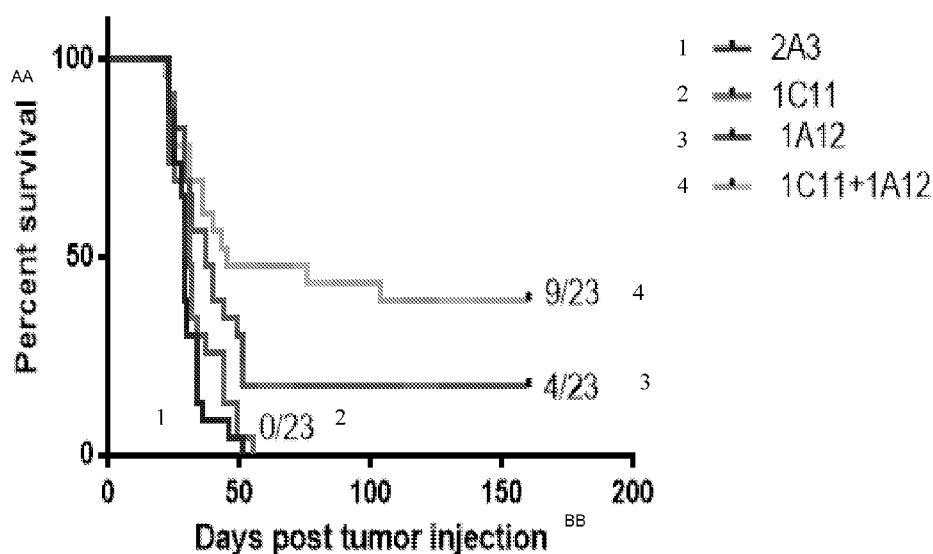
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(54) Title: METHODS FOR TREATING CANCER USING COMBINATIONS OF ANTI-CX3CR1 AND IMMUNE CHECKPOINT BLOCKADE AGENTS

FIG. 6



(57) Abstract: The present invention relates, in part, to methods of treating cancers using combinations of anti-CX3CR1 and anti-immune checkpoint therapies.

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METHODS FOR TREATING CANCER USING COMBINATIONS OF ANTI-CX3CR1 AND IMMUNE CHECKPOINT BLOCKADE AGENTS

Cross-Reference to Related Applications

5 This application claims the benefit of priority to U.S. Provisional Application Serial No. 62/840,678, filed on 30 April 2019; the entire contents of said application are incorporated herein in their entirety by this reference.

Statement of Rights

10 This invention was made with government support under Grant P01 AI112521 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

Background of the Invention

15 Immune checkpoints, such as CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, GITR, 4-1BB, OX-40, BTLA, SIRPalpha (CD47), CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, and A2aR and many more, negatively regulate immune response progression based on complex and
20 combinatorial interactions between numerous inputs. Great progress has been made in recent decades to treat cancers with immune checkpoint inhibitors, such as PD-1 inhibitors. PD-1 blockade therapy has shown response in a wide range of tumors. It is FDA-approved for advanced melanoma, non small lung carcinoma, kidney cancer, *etc.* It has also shown efficacy in multiple other tumor types including Hodgkin lymphoma, triple negative breast
25 cancer, and ovarian cancer (Brahmer *et al.* (2012) *N. Engl. J. Med.* 366:2455-2465, Berger *et al.* (2008) *Clin. Cancer Res.* 14:3044-3051). However, it is known that only about 30% of cancer patients respond to, for example, anti-PD-1 therapy. Therapeutically intervening in cancers has been a particular challenge for oncologists since cancers are generally resistant to many chemotherapy agents and surgical resection options. Many combination
30 therapies are being tested for better efficacy. Effective regimens include combining PD-1 therapy with CTLA-4 therapy, chemotherapy (Dolan *et al.* (2014) *Cancer control* 21:231-237) or radiation therapy with PD-1 (Deng *et al.* (2014) *J. Clin. Invest.* 124:687-695), PD-1 with immunostimulants, PD-1 with CAR-T therapy (John *et al.* (2013) *Oncoimmunology* 2:e26286) and cancer vaccines. Strategies such as targeting other inhibitory receptors, such

as LAG-3, TIM3, CD160, and TIGIT, are in progress (Freeman and Sharpe. (2012) *Nat. Immunol.* 13:113-115). Some of these combinations are under investigation in the clinic for prospective efficacy and safety. Accordingly, a great need exists in the art to identify therapeutic interventions to treat cancers.

5 Chemokines are chemotactic cytokines that activate seven-transmembrane G protein-coupled receptors (GPCR). They are divided into C, CC, CXC, and CX3C classes. CX3CL1 (also known as fractalkine; neurotactin; Chemokine C-X3-C Motif Ligand 1) is a chemokine that functions by binding to its receptor CX3CR1 (Chemokine C-X3-C Motif Receptor 1). CX3CL1 consists of an extracellular N-terminal domain, mucin like stalk,
10 transmembrane α helix, and a cytoplasmic tail (Jones *et al.* (2010) *Mol. Interv.* 10(5): 263–270). CX3CL1 exists as either a membrane-anchored pro-adhesive protein or a clipped soluble form (Ferretti *et al.* (2014) *Mediators Inflamm.* 480941). The chemokine CX3CL1 is secreted via cleavage by metalloproteinase (Bazan *et al.* (1997) *Nature* 385:640-644). CX3CL1 is expressed by many different cell types such as endothelial cells, epithelial cells,
15 neurons, smooth muscle cells, lymphocytes, microglial osteoblasts (Corcione *et al.* (2012) *J. Leukoc. Biol.* 92:51-58). The membrane bound form plays a role in leukocyte adhesion while the soluble form functions as a chemoattractant. CX3CR1 contains seven α helices forming loops across the transmembrane domain, CX3CL1 binds at the extracellular loops of the α helices (Wojdasiewicz *et al.* (2014) 62:395-403). CX3CR1 is expressed by
20 immune cells, natural killer cells, T cells, monocytes (Imai *et al.* (1997) *Cell* 91:521-530), macrophages, dendritic cells (Jung *et al.* (2000) *Mol. Cell Biol.* 20:4106-4114). Expression of CX3CR1 identifies distinct populations of monocytes, macrophages and dendritic cells (Hart *et al.* (2014) *Immunol. Cell Biol.* 92:499-508). On binding with its ligand CX3CR1 promotes cell adhesion and migration by activation of G protein-dependent signaling and
25 β integrins (Marchesi *et al.* (2008) *Cancer Res.* 68:9060-9069).

The CX3CR1-CX3CL1 axis has been highlighted in various inflammatory diseases (such as asthma, arthritis, atherosclerosis, parkinson's) and in cancer (Ferretti *et al.* (2014) *Mediators Inflamm.* 480941). CX3CL1 expression on endothelial cells is increased by inflammatory cytokines TNF- α and IL-1 β (Garcia *et al.* (2000) *J. Leukoc Biol.* 67:577-584).
30 For instance, CX3CL1 expressed on fibroblast and endothelial cells results in attraction and adhesion of immune cells to the particular site that releases inflammatory cytokines, leading to disease pathogenesis (Ferretti *et al.* (2014) *Mediators Inflamm.* 480941). High CX3CR1 expression in pancreatic tumors has been associated with tumor recurrence and peripheral

invasion. The CX3CR1/CX3CL1 axis has been indicated to be a target to prevent tumor dissemination (Marchesi *et al.* (2008) *Cancer Res.* 68:9060-9069). Breast cancer metastasis to the skeleton has been associated with CX3CR1 expression on breast cancer cells that interact with CX3CL1 on stromal cells. In a liver metastatic mouse model of colon cancer, mice lacking CX3CR1 developed fewer metastatic tumors and less macrophage infiltration (Zheng *et al.* (2013) *Mol. Cancer* 12:141). Since CX3CL1 is widely expressed in various tissues (*e.g.*, lung, bone marrow, and brain), these tissues may become metastatic sites for tumors with high CX3CR1 expression such as pancreatic (Marchesi *et al.* (2008) *Cancer Res.* 68:9060-9069), breast, or renal cell carcinoma (Yao *et al.* (2014) *Urol. Oncol.* 32:162-170). CX3CL1 also results in survival of CX3CR1 expressing microglia cells by activation of the PI3K pathway (Davis *et al.* (2006) *J. Pharmacol. Exp. Ther.* 316:356-363). Thus, through mechanisms of cell survival and metastasis, it may be an effective strategy to target the CX3CR1/CX3CL1 axis in the treatment of cancer.

Summary of the Invention

The present invention is based, at least in part, on the discovery that inhibiting or blocking both chemokine (C-X3-C motif) receptor 1 (CX3CR1) and an immune checkpoint (*e.g.*, PD-1) results in a synergistic therapeutic benefit for treating cancers that is unexpected given the lack of such benefit observed for inhibiting or blocking the signaling through the interaction of chemokine (C-X3-C motif) ligand 1 and CX3CR1 or inhibiting or blocking PD-1 signaling pathway alone. In particular, new CX3CR1 antibodies were generated and showed binding to an accessible extracellular motif of CX3CR1. It is described herein that the CX3CR1 antibody blockade increased the effectiveness of tumor immunotherapy in combination with anti-PD1 mAb. The tumor immune infiltrates were analyzed for the expression of CX3CR1 to understand the underlying mechanism of the CX3CR1 antibody blockade approach.

In one aspect, a method of treating a subject afflicted with a cancer comprising administering to the subject a therapeutically effective amount of at least one agent that inhibits or blocks both chemokine (C-X3-C motif) receptor 1 (CX3CR1) and an immune checkpoint (*e.g.*, PD-1), is provided.

Numerous embodiments are further provided that can be applied to any aspect of the present invention and/or combined with any other embodiment described herein. For example, in one embodiment, the at least one agent is a single agent that inhibits or blocks

both CX3CR1 and the immune checkpoint. In another embodiment, the at least one agent comprises, optionally in a synergistic combination, a first agent that selectively inhibits or blocks CX3CR1, chemokine (C-X3-C motif) ligand 1 (CX3CL1), and/or the interaction between CX3CR1 and CX3CL1, and a second agent that selectively inhibits or blocks the immune checkpoint described herein. In another embodiment, the at least one agent is a small molecule inhibitor that inhibits or blocks CX3CR1, CX3CL1, the interaction between CX3CR1 and CX3CL1, and/or the immune checkpoint. In still another embodiment, the at least one agent comprises an RNA interfering agent which inhibits expression of CX3CR1, CX3CL1, and/or the immune checkpoint (*e.g.*, wherein the RNA interfering agent is a small interfering RNA (siRNA), small hairpin RNA (shRNA), or a microRNA (miRNA)). In yet another embodiment, the at least one agent comprises an antisense oligonucleotide complementary to CX3CR1, CX3CL1, and/or the immune checkpoint. In another embodiment, the at least one agent comprises a peptide or peptidomimetic that inhibits or blocks CX3CR1, CX3CL1, the interaction between CX3CR1 and CX3CL1, and/or the immune checkpoint. In still another embodiment, the at least one agent comprises an aptamer that inhibits or blocks CX3CR1, CX3CL1, the interaction between CX3CR1 and CX3CL1, and/or the immune checkpoint. In yet another embodiment, the at least one agent is an antibody and/or intrabody, or an antigen binding fragment thereof, which specifically binds to CX3CR1, CX3CL1, and/or the immune checkpoint. In yet another embodiment, the at least one agent is an antibody, or antigen binding fragment thereof, selectively or specifically binds the extracellular domain of CX3CR1 and/or the immune checkpoint. The antibody and/or intrabody, or antigen binding fragment thereof, described herein may have any IgG isoform known in the art, including, *e.g.*, IgG2c, IgG1, IgG2, or IgG4 antibodies and/or intrabodies, or antigen binding fragment thereof. The antibody and/or intrabody, or antigen binding fragment thereof, described herein may be generated in any host cell or animal, such as a CX3CR1 knockout, CX3CL1 knockout, and/or immune checkpoint knockout host cell or animal.

In another embodiment, the antibody and/or intrabody, or antigen binding fragment thereof, is murine, chimeric, humanized, composite, or human. In still another embodiment, the antibody and/or intrabody, or antigen binding fragment thereof, is detectably labeled, comprises an effector domain, comprises an Fc domain, and/or is selected from the group consisting of Fv, Fav, F(ab')₂, Fab', dsFv, scFv, sc(Fv)₂, and diabodies fragments. In yet another embodiment, the antibody and/or intrabody, or antigen

binding fragment thereof, is conjugated to a cytotoxic agent. In another embodiment, the cytotoxic agent is selected from the group consisting of a chemotherapeutic agent, a biologic agent, a toxin, and a radioactive isotope.

In some embodiments, the at least one agent increases the expression and/or the function of the extracellular domain of CX3CR1 *in vitro*, *ex vivo*, and/or *in vivo*. In one embodiment, the at least one agent is a polypeptide comprising a soluble extracellular domain of CX3CR1. The function of such extracellular domain of CX3CR1 may include competing with endogenous CX3CR1 for binding to CX3CL1 and, thus, inhibit the CX3CL1-CX3CR1 signaling pathway. In another embodiment, the at least one agent is a polynucleotide encoding such polypeptide. In other embodiments, the at least one agent is a polypeptide comprising a mutated CX3CL1 comprising at least one amino acid substitution and/or modification. In one embodiment, such substitution and/or modification decreases or inhibits the function of the mutated CX3CL1 of binding and/or activating CX3CR1. In another embodiment, the at least one agent is a polynucleotide encoding such polypeptide.

In some embodiments, the immune checkpoint described herein is selected from the group consisting of CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, GITR, 4-1BB, OX-40, BTLA, SIRPalpha (CD47), CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, and A2aR. In one embodiment, the immune checkpoint is selected from the group consisting of CTLA-4, PD-1, PD-L1, PD-L2, TIM-3, and LAG-3. In another embodiment, the immune checkpoint is PD-1.

In some embodiments, the at least one agent described herein reduces the number of proliferating cells in the cancer and/or reduces the volume or size of a tumor of the cancer.

In some embodiments, the at least one agent is administered in a pharmaceutically acceptable formulation. In other embodiments, the method further comprises administering to the subject a therapeutic agent or regimen for treating the cancer.

The cancer described herein include any cancer cells in the subject expressing CX3CR1 and/or the immune checkpoint (*e.g.*, PD-1, PD-L1, etc.). In some embodiments, tumor immune infiltrating cells (*e.g.*, CD45⁺ cells) in the subject express CX3CR1, either alone or in combination with an immune checkpoint like PD-1. In certain embodiments, CD45⁺ cells are selected from the group consisting of B cells, NK cells, CD4 T cells, CD8 T cells, regulatory T cells (Tregs), M1 macrophages (M1), M2 macrophages (M2), and

dendritic cells (DC). In one embodiment, the cancer is selected from the group consisting of colorectal carcinoma, gliomas, neuroblastoma, prostate cancer, breast cancer, pancreatic ductal carcinoma, ovarian cancer like epithelial ovarian cancer, B-CLL, leukemia, B cell lymphoma, renal cell carcinoma, lung cancer, osteosarcoma, hepatoblastoma,

5 hepatocellular carcinoma, head and neck cancer, adenocarcinoma, gastric carcinoma, myeloma, squamous cell carcinoma, melanoma, and testicular germ cell tumor. In one embodiment, the cancer is colorectal cancer.

In some embodiments, the subject is an animal model of the cancer, such as an orthotopic xenograft animal model of a human-derived cancer and/or a mouse model. In
10 one embodiment, the subject is a mammal, such as a mouse or a human. In still another embodiment, the mammal is a human. In yet another embodiment, the subject described herein is a mouse model.

In some embodiments, the at least one agent is an antibody and/or an intrabody, or an antigen binding fragment thereof, which specifically binds to CX3CR1.

15 In one aspect, a monoclonal antibody, or antigen-binding fragment thereof, is provided, wherein the monoclonal antibody comprises: a) a heavy chain sequence with at least about 95% identity to a heavy chain sequence selected from the group consisting of the sequences listed in Table 2 or b) a light chain sequence with at least about 95% identity to a light chain sequence selected from the group consisting of the sequences listed in Table
20 2. In one embodiment, the monoclonal antibody, or antigen-binding fragment thereof, comprises: a) a heavy chain CDR sequence with at least about 95% identity to a heavy chain CDR sequence selected from the group consisting of the sequences listed in Table 2 or b) a light chain CDR sequence with at least about 95% identity to a light chain CDR sequence selected from the group consisting of the sequences listed in Table 2. In another
25 embodiment, the monoclonal antibody, or antigen-binding fragment thereof, comprises: a) a heavy chain sequence selected from the group consisting of the sequences listed in Table 2; or b) a light chain sequence selected from the group consisting of the sequences listed in Table 2. In still another embodiment, the monoclonal antibody, or antigen-binding fragment thereof, comprises: a) a heavy chain CDR sequence selected from the group
30 consisting of the sequences listed in Table 2; or b) a light chain CDR sequence selected from the group consisting the sequences listed in Table 2. In yet another embodiment, the monoclonal antibody, or antigen-binding fragment thereof, is chimeric, humanized, composite, murine, or human. In another embodiment, the monoclonal antibody, or

antigen-binding fragment thereof, is detectably labeled, comprises an effector domain, comprises an Fc domain, and/or is selected from the group consisting of Fv, Fav, F(ab')₂, Fab', dsFv, scFv, sc(Fv)₂, and diabodies fragments. In still another embodiment, the monoclonal antibody, or antigen-binding fragment thereof, inhibits the binding of
5 commercial antibody to CX3CR1. In yet another embodiment, the monoclonal antibody, or antigen-binding fragment thereof, is obtainable from hybridoma _____ deposited under deposit accession number _____.

In another aspect, an immunoglobulin heavy and/or light chain of any monoclonal antibody, or antigen-binding fragment thereof, described herein, is provided.

10 In still another aspect, an isolated nucleic acid molecule that hybridizes, under stringent conditions, with the complement of a nucleic acid encoding a polypeptide selected from the group consisting of the sequences listed in Table 2, or a sequence with at least about 95% homology to a nucleic acid encoding a polypeptide selected from the group consisting of the sequences listed in Table 2, is provided.

15 Any monoclonal antibody, or antigen-binding fragment or immunoglobulin heavy and/or light chain thereof, described herein can be used according to the methods of the present invention.

In yet another aspect, a vector comprising such an isolated nucleic acid is provided.

20 In another aspect, a host cell which comprises an isolated nucleic acid, a vector, expresses a monoclonal antibody, or antigen-binding fragment thereof or is accessible under deposit access number _____, described herein, is provided.

In still another aspect, a device or kit comprising at least one monoclonal antibody or antigen-binding fragment thereof, described herein is provided, wherein said device or kit optionally comprising a label to detect the at least one monoclonal antibody or antigen-
25 binding fragment thereof, or a complex comprising the monoclonal antibody or antigen-binding fragment thereof.

In yet another aspect, a method of producing an antibody, or antigen-binding fragment thereof, described herein, which method comprises the steps of: (i) culturing a transformed host cell which has been transformed by a nucleic acid comprising a sequence
30 encoding a monoclonal antibody, described herein, under conditions suitable to allow expression of said antibody, or antigen-binding fragment thereof; and (ii) recovering the expressed antibody, or antigen-binding fragment thereof.

In another aspect, a method of detecting the presence or level of a CX3CR1 polypeptide is provided, wherein said method comprises obtaining a sample and detecting said polypeptide in a sample by use of at least one monoclonal antibody, or antigen-binding fragment thereof, described herein. In one embodiment, the at least one monoclonal
5 antibody, or antigen-binding fragment thereof, forms a complex with a CX3CR1 polypeptide and the complex is detected in the form of an enzyme linked immunosorbent assay (ELISA), radioimmune assay (RIA), immunochemically, or using an intracellular flow assay.

In still another aspect, a method for monitoring the progression of a disorder
10 associated with aberrant CX3CR1 expression in a subject is provided, wherein the method comprises: a) detecting in a subject sample at a first point in time the level of expression of CX3CR1 using at least one monoclonal antibody, or antigen-binding fragment thereof, described herein; b) repeating step a) at a subsequent point in time; and c) comparing the level of expression of said CX3CR1 detected in steps a) and b) to monitor the progression
15 of the disorder in the subject. In one method, the subject has undergone treatment to ameliorate the disorder between the first point in time and the subsequent point in time.

In yet another aspect, a method for predicting the clinical outcome of a subject afflicted with a disorder associated with aberrant CX3CR1 is provided, wherein the method comprises: a) determining the level of expression of CX3CR1 in a patient sample using at
20 least one monoclonal antibody, or antigen-binding fragment thereof, described herein; b) determining the level of expression of CX3CR1 in a sample from a control subject having a good clinical outcome using at least one monoclonal antibody, or antigen-binding fragment thereof, described herein; and c) comparing the level of expression of CX3CR1 in the patient sample and in the sample from the control subject; wherein a significantly higher
25 level of expression in the patient sample as compared to the expression level in the sample from the control subject is an indication that the patient has a poor clinical outcome.

In another aspect, a method of assessing the efficacy of a therapy for a disorder associated with aberrant CX3CR1 in a subject is provided, wherein the method comprises comparing: a) the level of expression of CX3CR1 using at least one monoclonal antibody,
30 or antigen-binding fragment thereof, described herein, in a first sample obtained from the subject prior to providing at least a portion of the therapy to the subject, and b) the level of expression of CX3CR1 in a second sample obtained from the subject following provision of the portion of the therapy, wherein a significantly lower level of expression of CX3CR1 in

the second sample, relative to the first sample, is an indication that the therapy is efficacious for inhibiting the disorder in the subject.

In still another aspect, a method of assessing the efficacy of a test compound for inhibiting a disorder associated with aberrant CX3CR1 in a subject is provided, wherein the method comprises comparing: a) the level of expression of CX3CR1 using at least one monoclonal antibody, or antigen-binding fragment thereof, described herein, in a first sample obtained from the subject and exposed to the test compound; and b) the level of expression of CX3CR1 in a second sample obtained from the subject, wherein the second sample is not exposed to the test compound, and a significantly lower level of expression of CX3CR1, relative to the second sample, is an indication that the test compound is efficacious for inhibiting the disorder in the subject. In one embodiment, the first and second samples are portions of a single sample obtained from the subject or portions of pooled samples obtained from the subject.

For any aspect of the present invention described herein, certain specific embodiments are contemplated. For example, in one embodiment, the disorder is a cancer, such as colorectal cancer, gliomas, neuroblastoma, prostate cancer, breast cancer, pancreatic ductal carcinoma, ovarian cancer like an epithelial ovarian cancer, B-CLL, leukemia, B cell lymphoma, renal cell carcinoma, lung cancer, osteosarcoma, hepatoblastoma, hepatocellular carcinoma, head and neck cancer, adenocarcinoma, gastric carcinoma, myeloma, squamous cell carcinoma, melanoma, and testicular germ cell tumor. In another embodiment, the sample comprises cells, serum, peritumoral tissue, and/or intratumoral tissue obtained from the subject. In still another embodiment, a significant increase comprises an at least twenty percent increase between the level of expression of CX3CR1 in the subject sample relative to the normal level of expression of CX3CR1 in the sample from the control subject. In yet another embodiment, the subject is a human.

Brief Description of the Drawings

FIG. 1 shows that anti-mouse CX3CR1 (anti-mCX3CR1) clone 1C11 binds to cell surface expressed mCX3CR1. Jurkat cells transfected with mCX3CR1 were treated with the anti-mCX3CR1 monoclonal antibodies, 1C11 and 2E8 beginning at 10 µg/ml and two-fold dilutions. Isotype control mouse IgG1 (mIgG1) was used at the same concentrations. The secondary antibody was 10 µg/ml goat anti-mIgG-PE.

FIG. 2 shows that anti-mCX3CR1 antibodies block the interaction of CX3CL1 with CX3CR1. Jurkat cells transfected with mCX3CR1 were incubated with hybridoma supernatant of anti-mCX3CR1 clone 1C11 and 2E8 in a two-fold dilution series and CX3CL1-human Fc fusion protein was added at 2 µg/ml. The secondary goat anti-hIgG PE antibody was used at 10 µg/ml.

FIG. 3A - FIG. 3B show cell surface expression of CX3CR1 and PD-L1 on CT26 colon carcinoma tumor cell line by flow cytometry. The CT26 tumor cell line was incubated with anti-mCX3CR1 antibody clone 1C11 and isotype control mouse IgG2a (mIgG2a) (FIGS. 3A) or anti-mouse PD-L1 (anti-mPD-L1) antibody clone 8E2 (FIGS. 3B) and isotype control mIgG1, at 10 µg/ml. The secondary antibody was 10 µg/ml goat anti-mIgG PE.

FIG. 4A - FIG. 4B show *ex vivo* expression of CX3CR1 on the surface of mouse tumor cells (FIG. 4B), as well as on the surface of infiltrating immune cells in subcutaneous CT26 tumor (1 cm in size) (FIG. 4A). Infiltrating immune cells were gated on live CD45-positive cells and tumor cells were gated on live CD45-negative cells. Representative histograms show CX3CR1 expression. The light gray curve represents isotype control and the dark gray curve represents CX3CR1 expression.

FIG. 5A - FIG. 5B show *ex vivo* expression of CX3CR1 on the cell surface of mouse infiltrating immune cells in subcutaneous tumor (1 cm in size). FIG. 5A shows the CX3CR1 positive cell population as a percentage of the indicated cell population. FIG. 5B shows the CX3CR1 positive cell population as a percentage of total CD45+ immune cells. N = 5 mice.

FIG. 6 show that anti-CX3CR1 mAb in combination with anti-PD1 mAb improved animal survival. CT26 tumor cells were injected at 0.2-0.25 million cells per mouse to mice and antibody treatment (200 µg per dose) began at day 7 for a total of 5 treatments at 3 day intervals (FIG. 6). Treatment groups included anti-mCX3CR1 monoclonal antibody clone 1C11, anti-mouse PD-1 (anti-mPD-1) monoclonal antibody clone 1A12, a rat IgG2a isotype control monoclonal antibody clone 2A3, and a combination of the 1C11 and 1A12 monoclonal antibodies. N = 23 mice per group.

FIG. 7 shows Western blot results of CX3CR1 expression on CT26 tumor cell line cells. A representative Western blot for CX3CR1 expression by CT26 cells is shown. Primary antibody, anti-mCX3CR1 455.1C11, was used at 1 µg/ml. Secondary antibody, goat anti-mouse IgG HRP, was used at 1:5000. Size in kDa is indicated.

FIG. 8 shows cell surface expression of CX3CR1 on 4T1 and GL261 tumor cell lines by flow cytometry. Tumor cell lines 4T1 and GL261 were incubated with anti-mCX3CR1, 1C11 or isotype control mIgG2a at 10 µg/ml. The secondary antibody was goat anti-mIgG PE.

FIG. 9 shows Western blot results of CX3CR1 expression on 4T1, ID8, and GL261 tumor cell line cells. A representative Western blot for CX3CR1 expression by 4T1, ID8, and GL261 cells is shown. Primary antibody, anti-mCX3CR1 455.1C11, was used at 1 µg/ml. Secondary antibody, goat anti-mouse IgG HRP, was used at 1:5000. Size in kDa is indicated.

For any figure showing a bar histogram, curve, or other data associated with a legend, the bars, curve, or other data presented from left to right for each indication correspond directly and in order to the boxes from top to bottom, or from left to right, of the legend.

Detailed Description of the Invention

It has been determined herein that cancers, such as colon carcinoma, can overcome a lack of response to an immune checkpoint inhibitor, such as PD-1, through treatment with a combination of the immune checkpoint inhibitor (*e.g.*, anti-PD-1) and an inhibitor of CX3CR1 (*i.e.*, anti-CX3CR1/immune checkpoint therapy). CX3CR1 is a seven-transmembrane G protein coupled receptor (GPCR) whose ligand is CX3CL1, also known as fractalkine. CX3CL1 is an adhesion molecule, but also functions as a chemokine to modulate the migration of immune cells and possibly other cells, such as tumor cells and tumor infiltrating cells. Chemokines, like CX3CL1, draw cells into a tumor microenvironment and is synthesized in both a transmembrane protein form and a soluble protein form through proteolytic cleavage. Thus, the combination of an immune checkpoint inhibitor and a CX3CR1 inhibitor to treat cancer is surprising because blocking CX3CR1 would be expected to inhibit immune cells from infiltrating a tumor microenvironment and thereby inhibit an anti-cancer immune response by preventing access of the immune cells to target and destroy the cancer cells. In another aspect, the present invention provides anti-CX3CR1 monoclonal antibody compositions, which may also be used in the combination therapy and other methods described herein. Also, as described herein, CX3CR1 and/or an immune checkpoint can be inhibited in a number of contemplated ways, such as by direct inhibition of CX3CR1 and/or an immune checkpoint and/or indirect inhibition of these

targets, such as by inhibiting a CX3CR1 ligand like CX3CL1 and/or an immune checkpoint ligand like PD-L1 or PD-L2 for the PD-1 immune checkpoint receptor.

I. Definitions

5 The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

 The term “altered amount” or “altered level” refers to increased or decreased copy number (*e.g.*, germline and/or somatic) of a biomarker nucleic acid, *e.g.*, increased or
10 decreased expression level in a cancer sample, as compared to the expression level or copy number of the biomarker nucleic acid in a control sample. The term “altered amount” of a biomarker also includes an increased or decreased protein level of a biomarker protein in a sample, *e.g.*, a cancer sample, as compared to the corresponding protein level in a normal, control sample. Furthermore, an altered amount of a biomarker protein may be determined
15 by detecting posttranslational modification such as methylation status of the marker, which may affect the expression or activity of the biomarker protein.

 The amount of a biomarker in a subject is “significantly” higher or lower than the normal amount of the biomarker, if the amount of the biomarker is greater or less, respectively, than the normal level by an amount greater than the standard error of the assay
20 employed to assess amount, and preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 300%, 350%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or than that amount. Alternately, the amount of the biomarker in the subject can be considered “significantly” higher or lower than the normal amount if the amount is at least about two, and preferably at least about three, four, or five times, higher or lower,
25 respectively, than the normal amount of the biomarker. Such “significance” can also be applied to any other measured parameter described herein, such as for expression, inhibition, cytotoxicity, cell growth, and the like.

 The term “altered level of expression” of a biomarker refers to an expression level or copy number of the biomarker in a test sample, *e.g.*, a sample derived from a patient
30 suffering from cancer, that is greater or less than the standard error of the assay employed to assess expression or copy number, and is preferably at least twice, and more preferably three, four, five or ten or more times the expression level or copy number of the biomarker in a control sample (*e.g.*, sample from a healthy subjects not having the associated disease)

and preferably, the average expression level or copy number of the biomarker in several control samples. The altered level of expression is greater or less than the standard error of the assay employed to assess expression or copy number, and is preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 300%, 350%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more times the expression level or copy number of the biomarker in a control sample (*e.g.*, sample from a healthy subjects not having the associated disease) and preferably, the average expression level or copy number of the biomarker in several control samples. In some embodiments, the level of the biomarker refers to the level of the biomarker itself, the level of a modified biomarker (*e.g.*, phosphorylated biomarker), or to the level of a biomarker relative to another measured variable, such as a control (*e.g.*, phosphorylated biomarker relative to an unphosphorylated biomarker).

The term “altered activity” of a biomarker refers to an activity of the biomarker which is increased or decreased in a disease state, *e.g.*, in a cancer sample, as compared to the activity of the biomarker in a normal, control sample. Altered activity of the biomarker may be the result of, for example, altered expression of the biomarker, altered protein level of the biomarker, altered structure of the biomarker, or, *e.g.*, an altered interaction with other proteins involved in the same or different pathway as the biomarker or altered interaction with transcriptional activators or inhibitors.

The term “altered structure” of a biomarker refers to the presence of mutations or allelic variants within a biomarker nucleic acid or protein, *e.g.*, mutations which affect expression or activity of the biomarker nucleic acid or protein, as compared to the normal or wild-type gene or protein. For example, mutations include, but are not limited to substitutions, deletions, or addition mutations. Mutations may be present in the coding or non-coding region of the biomarker nucleic acid.

Unless otherwise specified here within, the terms “antibody” and “antibodies” broadly encompass naturally-occurring forms of antibodies (*e.g.* IgG, IgA, IgM, IgE) and recombinant antibodies such as single-chain antibodies, chimeric and humanized antibodies and multi-specific antibodies, as well as fragments and derivatives of all of the foregoing, which fragments and derivatives have at least an antigenic binding site. Antibody derivatives may comprise a protein or chemical moiety conjugated to an antibody.

The term “antibody” as used herein also includes an “antigen-binding portion” of an antibody (or simply “antibody portion”). The term “antigen-binding portion”, as used

herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, a biomarker polypeptide or fragment thereof). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term “antigen-binding portion” of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent polypeptides (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Osbourn *et al.* 1998, *Nature Biotechnology* 16: 778). Such single chain antibodies are also intended to be encompassed within the term “antigen-binding portion” of an antibody. Any VH and VL sequences of specific scFv can be linked to human immunoglobulin constant region cDNA or genomic sequences, in order to generate expression vectors encoding complete IgG polypeptides or other isotypes. VH and VL can also be used in the generation of Fab, Fv or other fragments of immunoglobulins using either protein chemistry or recombinant DNA technology. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R. J., *et al.* (1994) *Structure* 2:1121-1123).

Still further, an antibody or antigen-binding portion thereof may be part of larger immunoadhesion polypeptides, formed by covalent or noncovalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion polypeptides include use of the streptavidin core region to make a

tetrameric scFv polypeptide (Kipriyanov, S.M., *et al.* (1995) *Human Antibodies and Hybridomas* 6:93-101) and use of a cysteine residue, biomarker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv polypeptides (Kipriyanov, S.M., *et al.* (1994) *Mol. Immunol.* 31:1047-1058). Antibody portions, such as Fab and F(ab')₂ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion polypeptides can be obtained using standard recombinant DNA techniques, as described herein.

By contrast, antigen-binding portions can be adapted to be expressed within cells as “intracellular antibodies.” (Chen *et al.* (1994) *Human Gene Ther.* 5:595-601). Methods are well-known in the art for adapting antibodies to target (*e.g.*, inhibit) intracellular moieties, such as the use of single-chain antibodies (scFvs), modification of immunoglobulin VL domains for hyperstability, modification of antibodies to resist the reducing intracellular environment, generating fusion proteins that increase intracellular stability and/or modulate intracellular localization, and the like. Intracellular antibodies can also be introduced and expressed in one or more cells, tissues or organs of a multicellular organism, for example for prophylactic and/or therapeutic purposes (*e.g.*, as a gene therapy) (see, at least PCT Publs. WO 08/020079, WO 94/02610, WO 95/22618, and WO 03/014960; U.S. Pat. No. 7,004,940; Cattaneo and Biocca (1997) *Intracellular Antibodies: Development and Applications* (Landes and Springer-Verlag publs.); Kontermann (2004) *Methods* 34:163-170; Cohen *et al.* (1998) *Oncogene* 17:2445-2456; Auf der Maur *et al.* (2001) *FEBS Lett.* 508:407-412; Shaki-Loewenstein *et al.* (2005) *J. Immunol. Meth.* 303:19-39).

Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or modified forms thereof (*e.g.* humanized, chimeric, etc.). Antibodies may also be fully human. Preferably, antibodies of the present invention bind specifically or substantially specifically to a biomarker polypeptide or fragment thereof. The terms “monoclonal antibodies” and “monoclonal antibody composition”, as used herein, refer to a population of antibody polypeptides that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term “polyclonal antibodies” and “polyclonal antibody composition” refer to a population of antibody polypeptides that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody composition typically displays a single binding affinity for a particular antigen with which it immunoreacts.

Antibodies may also be “humanized”, which is intended to include antibodies made by a non-human cell having variable and constant regions which have been altered to more closely resemble antibodies that would be made by a human cell. For example, by altering the non-human antibody amino acid sequence to incorporate amino acids found in human germline immunoglobulin sequences. The humanized antibodies of the present invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs. The term “humanized antibody”, as used herein, also includes antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term “assigned score” refers to the numerical value designated for each of the biomarkers after being measured in a patient sample. The assigned score correlates to the absence, presence or inferred amount of the biomarker in the sample. The assigned score can be generated manually (*e.g.*, by visual inspection) or with the aid of instrumentation for image acquisition and analysis. In certain embodiments, the assigned score is determined by a qualitative assessment, for example, detection of a fluorescent readout on a graded scale, or quantitative assessment. In one embodiment, an “aggregate score,” which refers to the combination of assigned scores from a plurality of measured biomarkers, is determined. In one embodiment the aggregate score is a summation of assigned scores. In another embodiment, combination of assigned scores involves performing mathematical operations on the assigned scores before combining them into an aggregate score. In certain, embodiments, the aggregate score is also referred to herein as the “predictive score.”

The term “biomarker” refers to a measurable entity of the present invention that has been determined to be predictive of CX3CR1 and immune checkpoint (*e.g.*, PD-1) combinatorial inhibitor therapy effects on a cancer. Biomarkers can include, without limitation, nucleic acids and proteins, including those shown in the Tables, the Examples, the Figures, and otherwise described herein. As described herein, any relevant characteristic of a biomarker can be used, such as the copy number, amount, activity, location, modification (*e.g.*, phosphorylation), and the like.

A “blocking” antibody or an antibody “antagonist” is one which inhibits or reduces at least one biological activity of the antigen(s) it binds. In certain embodiments, the

blocking antibodies or antagonist antibodies or fragments thereof described herein substantially or completely inhibit a given biological activity of the antigen(s).

The term “body fluid” refers to fluids that are excreted or secreted from the body as well as fluids that are normally not (*e.g.* amniotic fluid, aqueous humor, bile, blood and blood plasma, cerebrospinal fluid, cerumen and earwax, cowper’s fluid or pre-ejaculatory fluid, chyle, chyme, stool, female ejaculate, interstitial fluid, intracellular fluid, lymph, menses, breast milk, mucus, pleural fluid, pus, saliva, sebum, semen, serum, sweat, synovial fluid, tears, urine, vaginal lubrication, vitreous humor, vomit).

The terms “cancer” or “tumor” or “hyperproliferative” refer to the presence of cells possessing characteristics typical of cancer-causing cells, such as uncontrolled proliferation, immortality, metastatic potential, rapid growth and proliferation rate, and certain characteristic morphological features. Unless otherwise stated, the terms include metaplasias. In some embodiments, such cells exhibit such characteristics in part or in full due to the expression and activity of the CX3CL1-CX3CR1 signaling pathway. Cancer cells are often in the form of a tumor, but such cells may exist alone within an animal, or may be a non-tumorigenic cancer cell, such as a leukemia cell. As used herein, the term “cancer” includes premalignant as well as malignant cancers. Cancers include, but are not limited to, B cell cancer, *e.g.*, multiple myeloma, Waldenström's macroglobulinemia, the heavy chain diseases, such as, for example, alpha chain disease, gamma chain disease, and mu chain disease, benign monoclonal gammopathy, and immunocytic amyloidosis, melanomas, breast cancer, lung cancer, bronchus cancer, colorectal cancer, prostate cancer, pancreatic cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cervical cancer, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, testicular cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid gland cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, cancer of hematologic tissues, and the like. Other non-limiting examples of types of cancers applicable to the methods encompassed by the present invention include human sarcomas and carcinomas, *e.g.*, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, colorectal cancer, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma,

basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, liver cancer, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, bone cancer, brain tumor, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, *e.g.*, acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease. In some embodiments, cancers are epithelial in nature and include but are not limited to, bladder cancer, breast cancer, cervical cancer, colon cancer, gynecologic cancers, renal cancer, laryngeal cancer, lung cancer, oral cancer, head and neck cancer, ovarian cancer, pancreatic cancer, prostate cancer, or skin cancer. In other embodiments, the cancer is breast cancer, prostate cancer, lung cancer, or colon cancer. In still other embodiments, the epithelial cancer is non-small-cell lung cancer, nonpapillary renal cell carcinoma, cervical carcinoma, ovarian carcinoma (*e.g.*, serous ovarian carcinoma), or breast carcinoma. The epithelial cancers may be characterized in various other ways including, but not limited to, serous, endometrioid, mucinous, clear cell, Brenner, or undifferentiated.

In certain embodiments, the cancer encompasses colorectal cancer (*e.g.*, colorectal carcinoma), gliomas, neuroblastoma, prostate cancer, breast cancer, pancreatic ductal carcinoma, ovarian cancer like epithelial ovarian cancer, B-CLL, leukemia, B cell lymphoma, renal cell carcinoma, lung cancer, osteosarcoma, hepatoblastoma, hepatocellular carcinoma, head and neck cancer, adenocarcinoma, gastric carcinoma, myeloma, squamous cell carcinoma, melanoma, and testicular germ cell tumor.

The term “colorectal cancer” as used herein, is meant to include cancer of cells of the intestinal tract below the small intestine (*e.g.*, the large intestine (colon), including the cecum, ascending colon, transverse colon, descending colon, and sigmoid colon, and rectum). Additionally, as used herein, the term “colorectal cancer” is meant to further include cancer of cells of the duodenum and small intestine (jejunum and ileum).

Colorectal cancer also includes neoplastic diseases involving proliferation of a single clone of cells of the colon and includes adenocarcinoma and carcinoma of the colon whether in a primary site or metastasized.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and ranks
5 second in cancer mortality. Extensive genetic and genomic analysis of human CRC has uncovered germline and somatic mutations relevant to CRC biology and malignant transformation (Fearon *et al.* (1990) *Cell* 61, 759-767). These mutations have been linked to well-defined disease stages from aberrant crypt proliferation or hyperplastic lesions to benign adenomas, to carcinoma *in situ*, and finally to invasive and metastatic disease,
10 thereby establishing a genetic paradigm for cancer initiation and progression. Genetic and genomic instability are catalysts for colon carcinogenesis (Lengauer *et al.* (1998) *Nature* 396:643-649). CRC can present with two distinct genomic profiles that have been termed (i) chromosomal instability neoplasia (CIN), characterized by rampant structural and numerical chromosomal aberrations driven in part by telomere dysfunction (Artandi *et al.* (2000) *Nature* 406:641-645; Fodde *et al.* (2001) *Nat. Rev. Cancer* 1:55-67; Maser and DePinho (2002) *Science* 297:565-569; Rudolph *et al.* (2001) *Nat. Genet.* 28:155-159) and mitotic aberrations (Lengauer *et al.* (1998) *Nature* 396:643-649) and (ii) microsatellite instability neoplasia (MIN), characterized by near diploid karyotypes with alterations at the nucleotide level due to mutations in mismatch repair (MMR) genes (Fishel *et al.* (1993)
20 *Cell* 75:1027-1038; Ilyas *et al.* (1999) *Eur. J. Cancer* 35:335-351; Modrich (1991) *Annu. Rev. Genet.* 25:229-253; Parsons *et al.* (1995) *Science* 268:738-740; Parsons *et al.* (1993) *Cell* 75:1227-1236). Germline MMR mutations are highly penetrant lesions which drive the MIN phenotype in hereditary nonpolyposis colorectal cancers, accounting for 1-5% of CRC cases (de la Chapelle (2004) *Nat. Rev. Cancer* 4:769-780; Lynch and de la Chapelle (1999) *J. Med. Genet.* 36:801-818; Umar *et al.* (2004) *Nat. Rev. Cancer* 4:153-158). While
25 CIN and MIN are mechanistically distinct, their genomic and genetic consequences emphasize the requirement of dominant mutator mechanisms to drive intestinal epithelial cells towards a threshold of oncogenic changes needed for malignant transformation.

A growing number of genetic mutations have been identified and functionally
30 validated in CRC pathogenesis. Activation of the WNT signaling pathway is an early requisite event for adenoma formation. Somatic alterations are present in *APC* in greater than 70% of nonfamilial sporadic cases and appear to contribute to genomic instability and induce the expression of *c-myc* and *Cyclin D1* (Fodde *et al.* (2001) *Nat. Rev. Cancer* 1:55-

67), while activating β -catenin mutations represent an alternative means of WNT pathway deregulation in CRC (Morin (1997) *Science* 275:1787-1790). *K-Ras* mutations occur early in neoplastic progression and are present in approximately 50% of large adenomas (Fearon and Gruber (2001) *Molecular abnormalities in colon and rectal cancer*, ed. J. Mendelsohn, P.H., M. Israel, and L. Liotta, W.B. Saunders, Philadelphia). The *BRAF* serine/threonine kinase and *PIK3CA* lipid kinase are mutated in 5-18% and 28% of sporadic CRCs, respectively (Samuels *et al.* (2004) *Science* 304:554; Davies *et al.* (2002) *Nature* 417:949-954; Rajagopalan *et al.* (2002) *Nature* 418:934; Yuen *et al.* (2002) *Cancer Res.* 62:6451-6455). *BRAF* and *K-ras* mutations are mutually exclusive in CRC, suggesting over-lapping oncogenic activities (Davies *et al.* (2002) *Nature* 417:949-954; Rajagopalan *et al.* (2002) *Nature* 418:934). Mutations associated with CRC progression, specifically the adenoma-to-carcinoma transition, target the *TP53* and the TGF- β pathways (Markowitz *et al.* (2002) *Cancer Cell* 1:233-236). Greater than 50% of CRCs harbor *TP53* inactivating mutations (Fearon and Gruber (2001) *Molecular abnormalities in colon and rectal cancer*, ed. J. Mendelsohn, P.H., M. Israel, and L. Liotta, W.B. Saunders, Philadelphia) and 30% of cases possess *TGF β -RII* mutations (Markowitz (2000) *Biochim. Biophys. Acta* 1470:M13-M20; Markowitz *et al.* (1995) *Science* 268:1336-1338). MIN cancers consistently inactivate *TGF β -RII* by frameshift mutations, whereas CIN cancers target the pathway via inactivating somatic mutations in the *TGF β -RII* kinase domain (15%) or in the downstream signaling components of the pathway, including *SMAD4* (15%) or *SMAD2* (5%) transcription factors (Markowitz (2000) *Biochim. Biophys. Acta* 1470:M13-M20). In some embodiments, the colorectal cancer is microsatellite instable (MSI) colorectal cancer (Llosa *et al.* (2014) *Cancer Disc.* CD-14-0863; published online Oct. 30, 2014). MSI represents about 15% of sporadic CRC and about 5-6% of stage IV CRCs. MSI is caused by epigenetic silencing or mutation of DNA mismatch repair genes and typically presents with lower stage disease than microsatellite stable subset (MSS) CRC. MSI highly express immune checkpoints, such as PD-1, PD-L1, CTLA-4, LAG-3, and IDO. In other embodiments, the colorectal cancer is MSS CRC.

The term “coding region” refers to regions of a nucleotide sequence comprising codons which are translated into amino acid residues, whereas the term “noncoding region” refers to regions of a nucleotide sequence that are not translated into amino acids (*e.g.*, 5' and 3' untranslated regions).

The term “complementary” refers to the broad concept of sequence complementarity between regions of two nucleic acid strands or between two regions of the same nucleic acid strand. It is known that an adenine residue of a first nucleic acid region is capable of forming specific hydrogen bonds (“base pairing”) with a residue of a second nucleic acid region which is antiparallel to the first region if the residue is thymine or uracil. Similarly, it is known that a cytosine residue of a first nucleic acid strand is capable of base pairing with a residue of a second nucleic acid strand which is antiparallel to the first strand if the residue is guanine. A first region of a nucleic acid is complementary to a second region of the same or a different nucleic acid if, when the two regions are arranged in an antiparallel fashion, at least one nucleotide residue of the first region is capable of base pairing with a residue of the second region. Preferably, the first region comprises a first portion and the second region comprises a second portion, whereby, when the first and second portions are arranged in an antiparallel fashion, at least about 50%, and preferably at least about 75%, at least about 90%, or at least about 95% of the nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion. More preferably, all nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion.

The term “control” refers to any reference standard suitable to provide a comparison to the expression products in the test sample. In one embodiment, the control comprises obtaining a “control sample” from which expression product levels are detected and compared to the expression product levels from the test sample. Such a control sample may comprise any suitable sample, including but not limited to a sample from a control cancer patient (can be stored sample or previous sample measurement) with a known outcome; normal tissue or cells isolated from a subject, such as a normal patient or the cancer patient, cultured primary cells/tissues isolated from a subject such as a normal subject or the cancer patient, adjacent normal cells/tissues obtained from the same organ or body location of the cancer patient, a tissue or cell sample isolated from a normal subject, or a primary cells/tissues obtained from a depository. In another preferred embodiment, the control may comprise a reference standard expression product level from any suitable source, including but not limited to housekeeping genes, an expression product level range from normal tissue (or other previously analyzed control sample), a previously determined expression product level range within a test sample from a group of patients, or a set of patients with a certain outcome (for example, survival for one, two, three, four years, etc.) or receiving a

certain treatment (for example, standard of care cancer therapy). It will be understood by those of skill in the art that such control samples and reference standard expression product levels can be used in combination as controls in the methods of the present invention. In one embodiment, the control may comprise normal or non-cancerous cell/tissue sample. In another preferred embodiment, the control may comprise an expression level for a set of patients, such as a set of cancer patients, or for a set of cancer patients receiving a certain treatment, or for a set of patients with one outcome versus another outcome. In the former case, the specific expression product level of each patient can be assigned to a percentile level of expression, or expressed as either higher or lower than the mean or average of the reference standard expression level. In another preferred embodiment, the control may comprise normal cells, cells from patients treated with combination chemotherapy, and cells from patients having benign cancer. In another embodiment, the control may also comprise a measured value for example, average level of expression of a particular gene in a population compared to the level of expression of a housekeeping gene in the same population. Such a population may comprise normal subjects, cancer patients who have not undergone any treatment (*i.e.*, treatment naive), cancer patients undergoing standard of care therapy, or patients having benign cancer. In another preferred embodiment, the control comprises a ratio transformation of expression product levels, including but not limited to determining a ratio of expression product levels of two genes in the test sample and comparing it to any suitable ratio of the same two genes in a reference standard; determining expression product levels of the two or more genes in the test sample and determining a difference in expression product levels in any suitable control; and determining expression product levels of the two or more genes in the test sample, normalizing their expression to expression of housekeeping genes in the test sample, and comparing to any suitable control. In particularly preferred embodiments, the control comprises a control sample which is of the same lineage and/or type as the test sample. In another embodiment, the control may comprise expression product levels grouped as percentiles within or based on a set of patient samples, such as all patients with cancer. In one embodiment a control expression product level is established wherein higher or lower levels of expression product relative to, for instance, a particular percentile, are used as the basis for predicting outcome. In another preferred embodiment, a control expression product level is established using expression product levels from cancer control patients with a known outcome, and the expression product levels from the test sample are

compared to the control expression product level as the basis for predicting outcome. As demonstrated by the data below, the methods of the present invention are not limited to use of a specific cut-point in comparing the level of expression product in the test sample to the control.

5 The “copy number” of a biomarker nucleic acid refers to the number of DNA sequences in a cell (*e.g.*, germline and/or somatic) encoding a particular gene product. Generally, for a given gene, a mammal has two copies of each gene. The copy number can be increased, however, by gene amplification or duplication, or reduced by deletion. For example, germline copy number changes include changes at one or more genomic loci,
10 wherein said one or more genomic loci are not accounted for by the number of copies in the normal complement of germline copies in a control (*e.g.*, the normal copy number in germline DNA for the same species as that from which the specific germline DNA and corresponding copy number were determined). Somatic copy number changes include changes at one or more genomic loci, wherein said one or more genomic loci are not
15 accounted for by the number of copies in germline DNA of a control (*e.g.*, copy number in germline DNA for the same subject as that from which the somatic DNA and corresponding copy number were determined).

 The “normal” copy number (*e.g.*, germline and/or somatic) of a biomarker nucleic acid or “normal” level of expression of a biomarker nucleic acid or protein is the
20 activity/level of expression or copy number in a biological sample, *e.g.*, a sample containing tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow, from a subject, *e.g.*, a human, not afflicted with cancer, or from a corresponding non-cancerous tissue in the same subject who has cancer.

 As used herein, the term “costimulate” with reference to activated immune cells
25 includes the ability of a costimulatory molecule to provide a second, non-activating receptor mediated signal (a “costimulatory signal”) that induces proliferation or effector function. For example, a costimulatory signal can result in cytokine secretion, *e.g.*, in a T cell that has received a T cell-receptor-mediated signal. Immune cells that have received a cell-receptor mediated signal, *e.g.*, via an activating receptor are referred to herein as
30 “activated immune cells.”

 The term “determining a suitable treatment regimen for the subject” is taken to mean the determination of a treatment regimen (*i.e.*, a single therapy or a combination of different therapies that are used for the prevention and/or treatment of the cancer in the

subject) for a subject that is started, modified and/or ended based or essentially based or at least partially based on the results of the analysis according to the present invention. One example is starting an adjuvant therapy after surgery whose purpose is to decrease the risk of recurrence, another would be to modify the dosage of a particular chemotherapy. The determination can, in addition to the results of the analysis according to the present invention, be based on personal characteristics of the subject to be treated. In most cases, the actual determination of the suitable treatment regimen for the subject will be performed by the attending physician or doctor.

The term “diagnosing cancer” includes the use of the methods, systems, and code of the present invention to determine the presence or absence of a cancer or subtype thereof in an individual. The term also includes methods, systems, and code for assessing the level of disease activity in an individual.

A molecule is “fixed” or “affixed” to a substrate if it is covalently or non-covalently associated with the substrate such that the substrate can be rinsed with a fluid (*e.g.* standard saline citrate, pH 7.4) without a substantial fraction of the molecule dissociating from the substrate.

The term “expression signature” or “signature” refers to a group of one or more coordinately expressed biomarkers related to a measured phenotype. For example, the genes, proteins, metabolites, and the like making up this signature may be expressed in a specific cell lineage, stage of differentiation, or during a particular biological response. The biomarkers can reflect biological aspects of the tumors in which they are expressed, such as the cell of origin of the cancer, the nature of the non-malignant cells in the biopsy, and the oncogenic mechanisms responsible for the cancer. Expression data and gene expression levels can be stored on computer readable media, *e.g.*, the computer readable medium used in conjunction with a microarray or chip reading device. Such expression data can be manipulated to generate expression signatures.

“Homologous” as used herein, refers to nucleotide sequence similarity between two regions of the same nucleic acid strand or between regions of two different nucleic acid strands. When a nucleotide residue position in both regions is occupied by the same nucleotide residue, then the regions are homologous at that position. A first region is homologous to a second region if at least one nucleotide residue position of each region is occupied by the same residue. Homology between two regions is expressed in terms of the proportion of nucleotide residue positions of the two regions that are occupied by the same

nucleotide residue. By way of example, a region having the nucleotide sequence 5'-ATTGCC-3' and a region having the nucleotide sequence 5'-TATGGC-3' share 50% homology. Preferably, the first region comprises a first portion and the second region comprises a second portion, whereby, at least about 50%, and preferably at least about 75%,
5 at least about 90%, or at least about 95% of the nucleotide residue positions of each of the portions are occupied by the same nucleotide residue. More preferably, all nucleotide residue positions of each of the portions are occupied by the same nucleotide residue.

The term “immune cell” refers to cells that play a role in the immune response. Immune cells are of hematopoietic origin, and include lymphocytes, such as B cells and T
10 cells; natural killer cells; myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

The term “immune checkpoint” refers to a group of molecules on the cell surface of CD4+ and/or CD8+ T cells that fine-tune immune responses by down-modulating or inhibiting an anti-tumor immune response. Immune checkpoint proteins are well-known in
15 the art and include, without limitation, CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, GITR, 4-1BB, OX-40, BTLA, SIRPalpha (CD47), CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, and A2aR (see, for example, WO 2012/177624). The term further encompasses biologically active protein
20 fragment, as well as nucleic acids encoding full-length immune checkpoint proteins and biologically active protein fragments thereof. In some embodiment, the term further encompasses any fragment according to homology descriptions provided herein. In one embodiment, the immune checkpoint is PD-1.

Immune checkpoints and their sequences are well-known in the art and
25 representative embodiments are described below. For example, the term “PD-1” refers to a member of the immunoglobulin gene superfamily that functions as a coinhibitory receptor having PD-L1 and PD-L2 as known ligands. PD-1 was previously identified using a subtraction cloning based approach to select for genes upregulated during TCR-induced activated T cell death. PD-1 is a member of the CD28/CTLA-4 family of molecules based
30 on its ability to bind to PD-L1. Like CTLA-4, PD-1 is rapidly induced on the surface of T-cells in response to anti-CD3 (Agata *et al.* 25 (1996) *Int. Immunol.* 8:765). In contrast to CTLA-4, however, PD-1 is also induced on the surface of B-cells (in response to anti-IgM).

PD-1 is also expressed on a subset of thymocytes and myeloid cells (Agata *et al.* (1996) *supra*; Nishimura *et al.* (1996) *Int. Immunol.* 8:773).

The nucleic acid and amino acid sequences of a representative human PD-1 biomarker is available to the public at the GenBank database under NM_005018.2 and NP_005009.2 and is shown in Table 1 (see also Ishida *et al.* (1992) 20 *EMBO J* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; U.S. Patent 5,698,520). PD-1 has an extracellular region containing immunoglobulin superfamily domain, a transmembrane domain, and an intracellular region including an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; and U.S. Patent 5,698,520) and an immunoreceptor tyrosine-based switch motif (ITSM). These features also define a larger family of polypeptides, called the immunoinhibitory receptors, which also includes gp49B, PIR-B, and the killer inhibitory receptors (KIRs) (Vivier and Daeron (1997) *Immunol. Today* 18:286). It is often assumed that the tyrosyl phosphorylated ITIM and ITSM motif of these receptors interacts with SH2-domain containing phosphatases, which leads to inhibitory signals. A subset of these immunoinhibitory receptors bind to MHC polypeptides, for example the KIRs, and CTLA4 binds to B7-1 and B7-2. It has been proposed that there is a phylogenetic relationship between the MHC and B7 genes (Henry *et al.* (1999) *Immunol. Today* 20(6):285-8). Nucleic acid and polypeptide sequences of PD-1 orthologs in organisms other than humans are well-known and include, for example, mouse PD-1 (NM_008798.2 and NP_032824.1), rat PD-1 (NM_001106927.1 and NP_001100397.1), dog PD-1 (XM_543338.3 and XP_543338.3), cow PD-1 (NM_001083506.1 and NP_001076975.1), and chicken PD-1 (XM_422723.3 and XP_422723.2).

PD-1 polypeptides are inhibitory receptors capable of transmitting an inhibitory signal to an immune cell to thereby inhibit immune cell effector function, or are capable of promoting costimulation (*e.g.*, by competitive inhibition) of immune cells, *e.g.*, when present in soluble, monomeric form. Preferred PD-1 family members share sequence identity with PD-1 and bind to one or more B7 family members, *e.g.*, B7-1, B7-2, PD-1 ligand, and/or other polypeptides on antigen presenting cells.

The term “PD-1 activity,” includes the ability of a PD-1 polypeptide to modulate an inhibitory signal in an activated immune cell, *e.g.*, by engaging a natural PD-1 ligand on an antigen presenting cell. Modulation of an inhibitory signal in an immune cell results in modulation of proliferation of, and/or cytokine secretion by, an immune cell. Thus, the

term “PD-1 activity” includes the ability of a PD-1 polypeptide to bind its natural ligand(s), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

The term “PD-1 ligand” refers to binding partners of the PD-1 receptor and includes both PD-L1 (Freeman *et al.* (2000) *J. Exp. Med.* 192:1027) and PD-L2 (Latchman *et al.* (2001) *Nat. Immunol.* 2:261). At least two types of human PD-1 ligand polypeptides exist. PD-1 ligand proteins comprise a signal sequence, and an IgV domain, an IgC domain, a transmembrane domain, and a short cytoplasmic tail. Both PD-L1 (See Freeman *et al.* (2000) *J. Exp. Med.* 192:1027 for sequence data) and PD-L2 (See Latchman *et al.* (2001) *Nat. Immunol.* 2:261 for sequence data) are members of the B7 family of polypeptides. Both PD-L1 and PD-L2 are expressed in placenta, spleen, lymph nodes, thymus, and heart. Only PD-L2 is expressed in pancreas, lung and liver, while only PD-L1 is expressed in fetal liver. Both PD-1 ligands are upregulated on activated monocytes and dendritic cells, although PD-L1 expression is broader. For example, PD-L1 is known to be constitutively expressed and upregulated to higher levels on murine hematopoietic cells (*e.g.*, T cells, B cells, macrophages, dendritic cells (DCs), and bone marrow-derived mast cells) and non-hematopoietic cells (*e.g.*, endothelial, epithelial, and muscle cells), whereas PD-L2 is inducibly expressed on DCs, macrophages, and bone marrow-derived mast cells (see Butte *et al.* (2007) *Immunity* 27:111).

PD-1 ligands comprise a family of polypeptides having certain conserved structural and functional features. The term “family” when used to refer to proteins or nucleic acid molecules, is intended to mean two or more proteins or nucleic acid molecules having a common structural domain or motif and having sufficient amino acid or nucleotide sequence homology, as defined herein. Such family members can be naturally or non-naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin, as well as other, distinct proteins of human origin or alternatively, can contain homologues of non-human origin. Members of a family may also have common functional characteristics. PD-1 ligands are members of the B7 family of polypeptides. The term “B7 family” or “B7 polypeptides” as used herein includes costimulatory polypeptides that share sequence homology with B7 polypeptides, *e.g.*, with B7-1, B7-2, B7h (Swallow *et al.* (1999) *Immunity* 11:423), and/or PD-1 ligands (*e.g.*, PD-L1 or PD-L2). For example, human B7-1 and B7-2 share approximately 26% amino acid sequence identity when compared using the BLAST program at NCBI with the

default parameters (Blosom62 matrix with gap penalties set at existence 11 and extension 1 (See the NCBI website). The term B7 family also includes variants of these polypeptides which are capable of modulating immune cell function. The B7 family of molecules share a number of conserved regions, including signal domains, IgV domains and the IgC domains. IgV domains and the IgC domains are art-recognized Ig superfamily member domains. These domains correspond to structural units that have distinct folding patterns called Ig folds. Ig folds are comprised of a sandwich of two β sheets, each consisting of anti-parallel β strands of 5-10 amino acids with a conserved disulfide bond between the two sheets in most, but not all, IgC domains of Ig, TCR, and MHC molecules share the same types of sequence patterns and are called the C1-set within the Ig superfamily. Other IgC domains fall within other sets. IgV domains also share sequence patterns and are called V set domains. IgV domains are longer than IgC domains and contain an additional pair of β strands.

Preferred B7 polypeptides are capable of providing costimulatory or inhibitory signals to immune cells to thereby promote or inhibit immune cell responses. For example, B7 family members that bind to costimulatory receptors increase T cell activation and proliferation, while B7 family members that bind to inhibitory receptors reduce costimulation. Moreover, the same B7 family member may increase or decrease T cell costimulation. For example, when bound to a costimulatory receptor, PD-1 ligand can induce costimulation of immune cells or can inhibit immune cell costimulation, *e.g.*, when present in soluble form. When bound to an inhibitory receptor, PD-1 ligand polypeptides can transmit an inhibitory signal to an immune cell. Preferred B7 family members include B7-1, B7-2, B7h, PD-L1 or PD-L2 and soluble fragments or derivatives thereof. In one embodiment, B7 family members bind to one or more receptors on an immune cell, *e.g.*, CTLA4, CD28, ICOS, PD-1 and/or other receptors, and, depending on the receptor, have the ability to transmit an inhibitory signal or a costimulatory signal to an immune cell, preferably a T cell.

Modulation of a costimulatory signal results in modulation of effector function of an immune cell. Thus, the term “PD-1 ligand activity” includes the ability of a PD-1 ligand polypeptide to bind its natural receptor(s) (*e.g.* PD-1 or B7-1), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

The term “PD-L1” refers to a specific PD-1 ligand. Two forms of human PD-L1 molecules have been identified. One form is a naturally occurring PD-L1 soluble polypeptide, *i.e.*, having a short hydrophilic domain and no transmembrane domain, and is referred to herein as PD-L1S (shown in Table 1 as SEQ ID NO: 4). The second form is a cell-associated polypeptide, *i.e.*, having a transmembrane and cytoplasmic domain, referred to herein as PD-L1M (shown in SEQ ID NO: 6). The nucleic acid and amino acid sequences of representative human PD-L1 biomarkers regarding PD-L1M are also available to the public at the GenBank database under NM_014143.3 and NP_054862.1. PD-L1 proteins comprise a signal sequence, and an IgV domain and an IgC domain. The signal sequence of SEQ ID NO: 4 is shown from about amino acid 1 to about amino acid 18. The signal sequence of SEQ ID NO: 6 is shown from about amino acid 1 to about amino acid 18. The IgV domain of SEQ ID NO: 4 is shown from about amino acid 19 to about amino acid 134 and the IgV domain of SEQ ID NO: 6 is shown from about amino acid 19 to about amino acid 134. The IgC domain of SEQ ID NO: 4 is shown from about amino acid 135 to about amino acid 227 and the IgC domain of SEQ ID NO: 6 is shown from about amino acid 135 to about amino acid 227. The hydrophilic tail of the PD-L1 exemplified in SEQ ID NO: 4 comprises a hydrophilic tail shown from about amino acid 228 to about amino acid 245. The PD-L1 polypeptide exemplified in SEQ ID NO: 6 comprises a transmembrane domain shown from about amino acids 239 to about amino acid 259 of SEQ ID NO: 6 and a cytoplasmic domain shown from about 30 amino acid 260 to about amino acid 290 of SEQ ID NO: 6. In addition, nucleic acid and polypeptide sequences of PD-L1 orthologs in organisms other than humans are well-known and include, for example, mouse PD-L1 (NM_021893.3 and NP_068693.1), rat PD-L1 (NM_001191954.1 and NP_001178883.1), dog PD-L1 (XM_541302.3 and XP_541302.3), cow PD-L1 (NM_001163412.1 and NP_001156884.1), and chicken PD-L1 (XM_424811.3 and XP_424811.3).

The term “PD-L2” refers to another specific PD-1 ligand. PD-L2 is a B7 family member expressed on various APCs, including dendritic cells, macrophages and bone-marrow derived mast cells (Zhong *et al.* (2007) Eur. J. Immunol. 37:2405). APC-expressed PD-L2 is able to both inhibit T cell activation through ligation of PD-1 and costimulate T cell activation, through a PD-1 independent mechanism (Shin *et al.* (2005) J. Exp. Med. 201:1531). In addition, ligation of dendritic cell-expressed PD-L2 results in enhanced dendritic cell cytokine expression and survival (Radhakrishnan *et al.* (2003) J. Immunol.

37:1827; Nguyen *et al.* (2002) J. Exp. Med. 196:1393). The nucleic acid and amino acid sequences of representative human PD-L2 biomarkers (*e.g.*, SEQ ID NOs: 7 and 8) are well-known in the art and are also available to the public at the GenBank database under NM_025239.3 and NP_079515.2. PD-L2 proteins are characterized by common structural elements. In some embodiments, PD-L2 proteins include at least one or more of the following domains: a signal peptide domain, a transmembrane domain, an IgV domain, an IgC domain, an extracellular domain, a transmembrane domain, and a cytoplasmic domain. For example, amino acids 1-19 of SEQ ID NO: 8 comprises a signal sequence. As used herein, a “signal sequence” or “signal peptide” serves to direct a polypeptide containing such a sequence to a lipid bilayer, and is cleaved in secreted and membrane bound polypeptides and includes a peptide containing about 15 or more amino acids which occurs at the N-terminus of secretory and membrane bound polypeptides and which contains a large number of hydrophobic amino acid residues. For example, a signal sequence contains at least about 10-30 amino acid residues, preferably about 15- 25 amino acid residues, more preferably about 18-20 amino acid residues, and even more preferably about 19 amino acid residues, and has at least about 35-65%, preferably about 38-50%, and more preferably about 40-45% hydrophobic amino acid residues (*e.g.*, valine, leucine, isoleucine or phenylalanine). In another embodiment, amino acid residues 220-243 of the native human PD-L2 polypeptide and amino acid residues 201-243 of the mature polypeptide comprise a transmembrane domain. As used herein, the term “transmembrane domain” includes an amino acid sequence of about 15 amino acid residues in length which spans the plasma membrane. More preferably, a transmembrane domain includes about at least 20, 25, 30, 35, 40, or 45 amino acid residues and spans the plasma membrane. Transmembrane domains are rich in hydrophobic residues, and typically have an alpha-helical structure. In a preferred embodiment, at least 50%, 60%, 70%, 80%, 90%, 95% or more of the amino acids of a transmembrane domain are hydrophobic, *e.g.*, leucines, isoleucines, tyrosines, or tryptophans. Transmembrane domains are described in, for example, Zagotta, W. N. *et al.* (1996) Annu. Rev. Neurosci. 19: 235-263. In still another embodiment, amino acid residues 20-120 of the native human PD-L2 polypeptide and amino acid residues 1-101 of the mature polypeptide comprise an IgV domain. Amino acid residues 121- 219 of the native human PD-L2 polypeptide and amino acid residues 102-200 of the mature polypeptide comprise an IgC domain. As used herein, IgV and IgC domains are recognized in the art as Ig superfamily member domains. These domains correspond to structural units

that have distinct folding patterns called Ig folds. Ig folds are comprised of a sandwich of two β sheets, each consisting of antiparallel (3 strands of 5-10 amino acids with a conserved disulfide bond between the two sheets in most, but not all, domains. IgC domains of Ig, TCR, and MHC molecules share the same types of sequence patterns and are called the C1 set within the Ig superfamily. Other IgC domains fall within other sets. IgV domains also share sequence patterns and are called V set domains. IgV domains are longer than C-domains and form an additional pair of strands. In yet another embodiment, amino acid residues 1-219 of the native human PD-L2 polypeptide and amino acid residues 1-200 of the mature polypeptide comprise an extracellular domain. As used herein, the term “extracellular domain” represents the N-terminal amino acids which extend as a tail from the surface of a cell. An extracellular domain of the present invention includes an IgV domain and an IgC domain, and may include a signal peptide domain. In still another embodiment, amino acid residues 244-273 of the native human PD-L2 polypeptide and amino acid residues 225-273 of the mature polypeptide comprise a cytoplasmic domain. As used herein, the term “cytoplasmic domain” represents the C-terminal amino acids which extend as a tail into the cytoplasm of a cell. In addition, nucleic acid and polypeptide sequences of PD-L2 orthologs in organisms other than humans are well-known and include, for example, mouse PD-L2 (NM_021396.2 and NP_067371.1), rat PD-L2 (NM_001107582.2 and NP_001101052.2), dog PD-L2 (XM_847012.2 and XP_852105.2), cow PD-L2 (XM_586846.5 and XP_586846.3), and chimpanzee PD-L2 (XM_001140776.2 and XP_001140776.1).

The term “PD-L2 activity,” “biological activity of PD-L2,” or “functional activity of PD-L2,” refers to an activity exerted by a PD-L2 protein, polypeptide or nucleic acid molecule on a PD-L2-responsive cell or tissue, or on a PD-L2 polypeptide binding partner, as determined *in vivo*, or *in vitro*, according to standard techniques. In one embodiment, a PD-L2 activity is a direct activity, such as an association with a PD-L2 binding partner. As used herein, a “target molecule” or “binding partner” is a molecule with which a PD-L2 polypeptide binds or interacts in nature, such that PD-L2-mediated function is achieved. In an exemplary embodiment, a PD-L2 target molecule is the receptor RGMb. Alternatively, a PD-L2 activity is an indirect activity, such as a cellular signaling activity mediated by interaction of the PD-L2 polypeptide with its natural binding partner (*i.e.*, physiologically relevant interacting macromolecule involved in an immune function or other biologically relevant function), *e.g.*, RGMb. The biological activities of PD-L2 are described herein.

For example, the PD-L2 polypeptides of the present invention can have one or more of the following activities: 1) bind to and/or modulate the activity of the receptor RGMb, PD-1, or other PD-L2 natural binding partners, 2) modulate intra-or intercellular signaling, 3) modulate activation of immune cells, *e.g.*, T lymphocytes, and 4) modulate the immune response of an organism, *e.g.*, a mouse or human organism.

The term “TIM-3” refers to a type I cell-surface glycoprotein that comprises an N-terminal immunoglobulin (Ig)-like domain, a mucin domain with O-linked glycosylations and with N-linked glycosylations close to the membrane, a single transmembrane domain, and a cytoplasmic region with tyrosine phosphorylation motif(s) (see, for example, U.S. Pat. Publ. 2013/0156774). TIM-3 is a member of the T cell/transmembrane, immunoglobulin, and mucin (TIM) gene family. Nucleic acid and polypeptide sequences of human TIM-3 are well-known in the art and are publicly available, for example, as described in NM_032782.4 and NP_116171.3. The term, as described above for useful markers such as PD-L1 and PD-1, encompasses any naturally occurring allelic, splice variants, and processed forms thereof. Typically, TIM-3 refers to human TIM-3 and can include truncated forms or fragments of the TIM-3 polypeptide. In addition, nucleic acid and polypeptide sequences of TIM-3 orthologs in organisms other than humans are well-known and include, for example, mouse TIM-3 (NM_134250.2 and NP_599011.2), chimpanzee TIM-3 (XM_518059.4 and XP_518059.3), dog TIM-3 (NM_001254715.1 and NP_001241644.1), cow TIM-3 (NM_001077105.2 and NP_001070573.1), and rat TIM-3 (NM_001100762.1 and NP_001094232.1). In addition, neutralizing anti-TIM-3 antibodies are well-known in the art (see, at least U.S. Pat. Publ. 2013/0183688, Ngiow *et al.* (2011) *Cancer Res.* 71:3540-3551; and antibody 344823 from R&D Biosystems, as well as clones 2C23, 5D12, 2E2, 4A4, and IG5, which are all published and thus publicly available).

TIM-3 was originally identified as a mouse Th1-specific cell surface protein that was expressed after several rounds of *in vitro* Th1 differentiation, and was later shown to also be expressed on Th17 cells. In humans, TIM-3 is expressed on a subset of activated CD4⁺ T cells, on differentiated Th1 cells, on some CD8⁺ T cells, and at lower levels on Th17 cells (Hastings *et al.* (2009) *Eur. J. Immunol.* 39:2492-2501). TIM-3 is also expressed on cells of the innate immune system including mouse mast cells, subpopulations of macrophages and dendritic cells (DCs), NK and NKT cells, human monocytes, human dendritic cells, and on murine primary bronchial epithelial cell lines. TIM-3 expression is regulated by the transcription factor T-bet. TIM-3 can generate an inhibitory signal

resulting in apoptosis of Th1 and Tc1 cells, and can mediate phagocytosis of apoptotic cells and cross-presentation of antigen. Polymorphisms in TIM-1 and TIM-3 can reciprocally regulate the direction of T-cell responses (Freeman *et al.* (2010) *Immunol. Rev.* 235:172-89).

5 TIM-3 has several known ligands, including galectin-9, phosphatidylserine, and HMGB1. For example, galectin-9 is an S-type lectin with two distinct carbohydrate recognition domains joined by a long flexible linker, and has an enhanced affinity for larger poly-N-acetyllactosamine-containing structures. Galectin-9 does not have a signal sequence and is localized in the cytoplasm. However, it can be secreted and exerts its
10 function by binding to glycoproteins on the target cell surface via their carbohydrate chains (Freeman *et al.* (2010) *Immunol. Rev.* 235:172-89). Engagement of TIM-3 by galectin-9 leads to Th1 cell death and a consequent decline in IFN-gamma production. When given *in vivo*, galectin-9 had beneficial effects in several murine disease models, including an EAE model, a mouse model of arthritis, in cardiac and skin allograft transplant models, and
15 contact hypersensitivity and psoriatic models (Freeman *et al.* (2010) *Immunol. Rev.* 235:172-89). Residues important for TIM-3 binding to galectin-9 include TIM-3(44), TIM-3(74), and TIM-3(100), which undergo N- and/or O-glycosylation. It is also known that TIM-3 mediates T-cell dysfunction associated with chronic viral infections (Golden-Mason *et al.* (2009) *J. Virol.* 83:9122-9130; Jones *et al.* (2008) *J. Exp. Med.* 205:2763-2779) and
20 increases HIV-1-specific T cell responses when blocked *ex vivo* (Golden-Mason *et al.* (2009) *J. Virol.* 83:9122-9130). In addition, in chronic HCV infection, TIM-3 expression was increased on CD4⁺ and CD8⁺ T cells, specifically HCV-specific CD8⁺ cytotoxic T cells (CTLs) in chronic HCV infection and treatment with a blocking monoclonal antibody to TIM-3 reversed HCV-specific T cell exhaustion (Jones *et al.* (2008) *J. Exp. Med.*
25 205:2763-2779).

 The term “LAG-3,” also known as CD223, refers to a member of the immunoglobulin supergene family and is structurally and genetically related to CD4 (see, U.S. Pat. Publ. 2011/0150892). LAG-3 is generally known as a membrane protein encoded by a gene located on the distal part of the short arm of chromosome 12, near the CD4 gene,
30 suggesting that the LAG-3 gene may have evolved through gene duplication (Triebel *et al.* (1990) *J. Exp. Med.* 171:1393-1405). However, secreted forms of the protein are known (*e.g.*, for human and mouse TIM-3). Nucleic acid and polypeptide sequences of human

LAG-3 are well-known in the art and are publicly available, for example, as described in NM_002286.5 and NP_002277.4.

The term encompasses any naturally occurring allelic, splice variants, and processed forms thereof. Typically, LAG-3 refers to human LAG-3 and can include truncated forms or fragments of the LAG-3 polypeptide. In addition, nucleic acid and polypeptide sequences of LAG-3 orthologs in organisms other than humans are well-known and include, for example, mouse LAG-3 (NM_008479.2 and NP_032505.1), chimpanzee LAG-3 (XM_508966.4 and XP_508966.2), monkey LAG-3 (XM_001108923.2 and XP_001108923.1), cow LAG-3 (NM_00124949.1 and NP_001232878.1), rat LAG-3 (NM_212513.2 and NP_997678.2), and chicken LAG-3 (XM_416510.3, XP_416510.2, XM_004938117.1, and XP_004938174.1). In addition, neutralizing anti-LAG-3 antibodies are well-known in the art (see, at least U.S. Pat. Publs. 2011/0150892 and 2010/0233183; Macon-Lemaitre and Triebel (2005) *Immunology* 115:170-178; Drake *et al.* (2006) *J. Clin. Oncol.* 24:2573; Richter *et al.* (2010) *Int. Immunol.* 22:13-23).

LAG-3 is not expressed on resting peripheral blood lymphocytes but is expressed on activated T cells and NK cells and has a number of functions (see, U.S. Pat. Publ. 2011/0150892). Similar to CD4, LAG-3 has been demonstrated to interact with MHC Class II molecules but, unlike CD4, LAG-3 does not interact with the human immunodeficiency virus gp120 protein (Baixeras *et al.* (1992) *J. Exp. Med.* 176:327-337). Studies using a soluble LAG-3 immunoglobulin fusion protein (sLAG-3Ig) demonstrated direct and specific binding of LAG-3 to MHC class II on the cell surface (Huard *et al.* (1996) *Eur. J. Immunol.* 26:1180-1186). In *in vitro* studies of antigen-specific T cell responses, the addition of anti-LAG-3 antibodies led to increased T cell proliferation and higher expression of activation antigens such as CD25, supporting a role for the LAG-3/MHC class II interaction in down-regulating antigen-dependent stimulation of CD4⁺ T lymphocytes (Huard *et al.* (1994) *Eur. J. Immunol.* 24:3216-3221). The intra-cytoplasmic region of LAG-3 has been demonstrated to interact with a protein termed LAP, which is thought to be a signal transduction molecule involved in the downregulation of the CD3/TCR activation pathway (Iouzalet *et al.* (2001) *Eur. J. Immunol.* 31:2885-2891). Furthermore, CD4⁺CD25⁺ regulatory T cells (T_{reg}) have been shown to express LAG-3 upon activation and antibodies to LAG-3 inhibit suppression by induced regulatory T cells, both *in vitro* and *in vivo*, suggesting that LAG-3 contributes to the suppressor activity of regulatory T cells (Huang *et al.* (2004) *Immunity* 21:503-513). Still further, LAG-3 has

been shown to negatively regulate T cell homeostasis by regulatory T cells in both T cell-dependent and independent mechanisms (Workman and Vignali (2005) *J. Immunol.* 174:688-695).

In certain circumstances, LAG-3 also has been shown to have immunostimulatory effects. For example, LAG-3 transfected tumor cells transplanted into syngeneic mice showed marked growth reduction or complete regression as compared to untransfected tumor cells, suggesting that LAG-3 expression on the tumor cells stimulated an anti-tumor response by triggering antigen presenting cells via MHC class II molecules (Prigent *et al.* (1999) *Eur. J. Immunol.* 29:3867-3876). Additionally, soluble LAG-3 Ig fusion protein has been shown to stimulate both humoral and cellular immune responses when administered to mice together with an antigen, indicating that soluble LAG-3Ig can function as a vaccine adjuvant (El Mir and Triebel (2000) *J. Immunol.* 164:5583-5589). Furthermore, soluble human LAG-3Ig has been shown to amplify the *in vitro* generation of type I tumor-specific immunity (Casati *et al.* (2006) *Cancer Res.* 66:4450-4460). The functional activity of LAG-3 is reviewed further in Triebel (2003) *Trends Immunol.* 24:619-622.

“Anti-immune checkpoint therapy” refers to the use of agents that inhibit immune checkpoint nucleic acids and/or proteins. Inhibition of one or more immune checkpoints can block or otherwise neutralize inhibitory signaling to thereby upregulate an immune response in order to more efficaciously treat cancer. Exemplary agents useful for inhibiting immune checkpoints include antibodies, small molecules, peptides, peptidomimetics, natural ligands, and derivatives of natural ligands, that can either bind and/or inactivate or inhibit immune checkpoint proteins, or fragments thereof; as well as RNA interference, antisense, nucleic acid aptamers, etc. that can downregulate the expression and/or activity of immune checkpoint nucleic acids, or fragments thereof. Exemplary agents for upregulating an immune response include antibodies against one or more immune checkpoint proteins block the interaction between the proteins and its natural receptor(s); a non-activating form of one or more immune checkpoint proteins (*e.g.*, a dominant negative polypeptide); small molecules or peptides that block the interaction between one or more immune checkpoint proteins and its natural receptor(s); fusion proteins (*e.g.* the extracellular portion of an immune checkpoint inhibition protein fused to the Fc portion of an antibody or immunoglobulin) that bind to its natural receptor(s); nucleic acid molecules that block immune checkpoint nucleic acid transcription or translation; and the like. Such agents can directly block the interaction between the one or more immune checkpoints and

its natural receptor(s) (e.g., antibodies) to prevent inhibitory signaling and upregulate an immune response. Alternatively, agents can indirectly block the interaction between one or more immune checkpoint proteins and its natural receptor(s) to prevent inhibitory signaling and upregulate an immune response. For example, a soluble version of an immune

5 checkpoint protein ligand such as a stabilized extracellular domain can binding to its receptor to indirectly reduce the effective concentration of the receptor to bind to an appropriate ligand. In one embodiment, anti-PD-1 antibodies, anti-PD-L1 antibodies, and/or anti-PD-L2 antibodies, either alone or in combination, are used to inhibit immune checkpoints. These embodiments are also applicable to specific therapy against particular

10 immune checkpoints, such as the PD-1 pathway (e.g., anti-PD-1 pathway therapy, otherwise known as PD-1 pathway inhibitor therapy).

Chemokine, chemokine receptor, and their sequences are well-known in the art and representative embodiments are described below. For example, the term “CX3CR1” refers to a member of gene superfamily that functions as a receptor binding to seven-

15 transmembrane GPCR ligands (rhodopsin family), such as CX3CL1. CX3CR1 was previously identified as a human beta chemokine receptor-like gene (Combadiere *et al.*, 1995 *DNA and Cell Biology* 14:673-680). Expression of CX3CR1 is associated with at least lymphocytes (Imai *et al.*, 1997 *Cell* 91:521-30) and neurons (Meucci *et al.* 2000 *PNAS* 97:8075-8080). Studies have highlighted the role of CX3CR1 in various tumors, such as

20 gliomas, neuroblastoma, prostate cancer, breast cancer, pancreatic ductal carcinoma, epithelial ovarian cancer, B-CLL, leukemia, B cell lymphoma, and renal cell carcinoma. It is also involved in migration of immune cells and tumor cells, such as in metastasis. CX3CR1 is also a co-receptor for HIV-1, and some variations in this gene lead to increased susceptibility to HIV-1 infection and rapid progression to AIDS (Becker 2007 *Virus Genes*

25 34:93-109). CX3CR1 variants have been described to modify the survival time and the progression rate of patients with amyotrophic lateral sclerosis (Lopez-Lopez *et al.*, 2014 *PLOS ONE* doi:10.1371/journal.pone.0096528) and dysplasia of the hip (Feldman *et al.*, 2014 *The Journal of Arthroplasty* 29 (9 Suppl.):238-241).

The nucleic acid and amino acid sequences of a representative human CX3CR1 is

30 available to the public at the GenBank database and is shown in Table 1. Human CX3CR1 isoforms include the longer isoform a (GenBank database numbers NM_001171174.1 and NP_001164645.1) and the shorter isoform b (NM_001171171.1 and NP_001164642.1, NM_001171172.1 and NP_001164643.1, and NM_001337.3 and NP_001328.1; all these

three variants differ in their 5'UTRs and coding sequences compared to isoform a, but they encode the same isoform b polypeptide). CX3CR1 has a seven-transmembrane receptor domain containing the amino acids 80-325 of NP_001164645. The human CX3CR1 gene is conserved in at least chimpanzee, rhesus monkey, dog, cow, mouse, rat, and chicken.

5 Nucleic acid and polypeptide sequences of CX3CR1 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*) CX3CR1 (NM_001146253.1 and NP_001139725.1), Rhesus monkey (*Macaca mulatta*) CX3CR1 (NM_001265871.1 and NP_001252800.1), dog CX3CR1 (NM_001284491.1 and NP_001271420.1), mouse CX3CR1 (NM_009987.4 and NP_034117.3), cattle CX3CR1
10 (NM_001102558.2 and NP_001096028.1), Norway rat *Rattus norvegicus*) CX3CR1 (NM_133534.1 and NP_598218.1), and chicken CX3CR1 (XM_418820.5 and XP_418820.1).

The term "CX3CR1 activity," includes the ability of a CX3CR1 polypeptide to bind and modulate a chemokine signal in a cancer cell or an activated immune cell, *e.g.*, by
15 engaging a natural CX3CR1 ligand (*e.g.*, CX3CL1) on a cell surface. Thus, the term "CX3CR1 activity" includes the ability of a CX3CR1 polypeptide to bind its natural ligand(s), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

The term "CX3CR1 ligand" refers to binding partners of the CX3CR1 receptor and
20 includes at least chemokines, such as CX3CL1. The term "CX3CL1" refers to a member of gene superfamily that functions as a receptor binding to CX3CR1. CX3CL1, also commonly known under the names fractalkine (in humans) and neurotactin (in mice), is a large cytokine protein and the only known member of the CX3C chemokine family. The polypeptide structure of CX3CL1 differs from the typical structure of other chemokines.
25 For example, the spacing of the characteristic N-terminal cysteines differs. There are three amino acids separating the initial pair of cysteines in CX3CL1, with none in CC chemokines and only one intervening amino acid in CXC chemokines. CX3CL1 is produced as a long protein (with 373-amino acid in humans) with an extended mucin-like stalk and a chemokine domain on top. The mucin-like stalk permits it to bind to cell
30 surfaces. However, a soluble (90 kD) version of this chemokine has also been observed. Soluble CX3CL1 potently chemoattracts T cells and monocytes, while the cell-bound chemokine promotes strong adhesion of leukocytes to activated endothelial cells, where it is primarily expressed (Bazan *et al.*, 1997 *Nature* 385:640-644). CX3CL1 elicits its

adhesive and migratory functions by interacting with the chemokine receptor CX3CR1 (Imai *et al.*, 1997). Its gene is located on human chromosome 16 along with some CC chemokines known as CCL17 and CCL22 (Bazan *et al.*, 1997; Nomiya *et al.*, 1998 *Cytogenet. Cell Genet.* 81:10-11). CX3CL1 is found commonly throughout the brain, particularly in neural cells, and its receptor is known to be present on microglial cells. It has also been found to be essential for microglial cell migration (Maciejewski-Lenoir *et al.*, 1999 *J. Immunol.* 163:1628-1635). CX3CL1 is also up-regulated in the hippocampus during a brief temporal window following spatial learning, the purpose of which may be to regulate glutamate-mediated neurotransmission tone. This indicates a possible role for the chemokine in the protective plasticity process of synaptic scaling (Sheridan *et al.*, 2014 *Front Cell Neurosci.* 8:233). CX3CL1 functions as a chemokine, as well as an adhesion molecule. CX3CL1 is synthesized as a transmembrane protein but can also be released as a soluble protein through proteolytic cleavage, unlike other chemokines that are secreted. The role of CX3CR1-CX3CL1 signaling pathway has been indicated in many inflammation and neurodegenerative diseases, such as allergic asthma and rhinitis, rheumatoid arthritis, atherosclerotic disease, renal diseases, chronic liver disease, age-related macular denegeration, Crohn's disease, Alzheimer's disease, Parkinson's disease, HIV infection, B cell lymphomas, and cancers (such as gliomas, neuroblastoma (NB), prostate cancer, pancreatic ductal adenocarcinoma (PDAC), epithelial ovarian carcinoma (EOC), breast cancer, colorectal cancer, hepatocellular carcinoma, gastric adenocarcinoma, B-chronic lymphocytic leukemia (B-CLL), etc.) (see, for example, Ferretti *et al.*, 2014 *Mediators of Inflammation* (Hindawi Publishing Corporation) Vol. 2014, Article ID: 480941).

The nucleic acid and amino acid sequences of a representative human CX3CL1 is available to the public at the GenBank database and is shown in Table 1. Human CX3CL1 isoforms include the longer isoform 1 precursor (GenBank database numbers NM_002996.4 and NP_002987.1) and the shorter isoform 2 (NM_001304392.1 and NP_001291321.1). Isoform 2, which lacks an alternate exon in the 5' end and initiates translation at an alternate start codon compared to isoform 1, is shorter at the N-terminus compared to isoform 1. CX3CL1 has a signal peptide containing the amino acids 1-24 of NP_002987, a chemokine CX3C domain containing the amino acids 25-100 of NP_002987, a Mucin-like stalk containing the amino acids 101-341 of NP_002987, and a transmembrane region containing the amino acids 342-362 of NP_002987. Potential glycosylation sites and glycosaminoglycan (GAG) binding sites on CX3CL1 (*e.g.*,

NP_002987) are also well-known in the art (*e.g.*, see GeneBank database for NP_002987). The CX3CL1 gene is conserved in at least chimpanzee, rhesus monkey, dog, cow, mouse, and rat. Nucleic acid and polypeptide sequences of CX3CL1 orthologs in organisms other than humans are well-known and include, for example, chimpanzee (*Pan troglodytes*)

- 5 CX3CL1 (XM_016929898.1 and XP_016785387.1, or XM_510989.5 and XP_510989.2), rhesus monkey (*Macaca mulatta*) CX3CL1 (NM_001032881.1 and NP_001028053.1), dog CX3CL1 (NM_001284456.1 and NP_001271385.1), mouse CX3CL1 (NM_009142.3 and NP_033168.2), cattle CX3CR1 (XM_015475804.1 and XP_015331290.1; XM_002694885.4 and XP_002694931.1; XM_015475803.1 and XP_015331289.1;
- 10 XM_015467344.1 and XP_015322830.1; XM_595523.8 and XP_595523.4; or XM_015467343.1 and XP_015322829.1), and rat (*Rattus norvegicus*) CX3CL1 (NM_134455.1 and NP_604450.1).

The term “CX3CL1 activity” includes the ability of a CX3CL1 polypeptide to bind its natural receptor(s) (*e.g.* CX3CR1), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

The term “immune response” includes T cell mediated and/or B cell mediated immune responses. Exemplary immune responses include T cell responses, *e.g.*, cytokine production and cellular cytotoxicity. In addition, the term immune response includes immune responses that are indirectly effected by T cell activation, *e.g.*, antibody production (humoral responses) and activation of cytokine responsive cells, *e.g.*, macrophages.

The term “immunotherapeutic agent” can include any molecule, peptide, antibody or other agent which can stimulate a host immune system to generate an immune response to a tumor or cancer in the subject. Various immunotherapeutic agents are useful in the compositions and methods described herein.

25 The term “inhibit” includes the decrease, limitation, or blockage, of, for example a particular action, function, or interaction. In some embodiments, cancer is “inhibited” if at least one symptom of the cancer is alleviated, terminated, slowed, or prevented. As used herein, cancer is also “inhibited” if recurrence or metastasis of the cancer is reduced, slowed, delayed, or prevented.

30 The term “interaction”, when referring to an interaction between two molecules, refers to the physical contact (*e.g.*, binding) of the molecules with one another. Generally, such an interaction results in an activity (which produces a biological effect) of one or both of said molecules.

An “isolated protein” refers to a protein that is substantially free of other proteins, cellular material, separation medium, and culture medium when isolated from cells or produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. An “isolated” or “purified” protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the antibody, polypeptide, peptide or fusion protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language “substantially free of cellular material” includes preparations of a biomarker polypeptide or fragment thereof, in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language “substantially free of cellular material” includes preparations of a biomarker protein or fragment thereof, having less than about 30% (by dry weight) of non-biomarker protein (also referred to herein as a “contaminating protein”), more preferably less than about 20% of non-biomarker protein, still more preferably less than about 10% of non-biomarker protein, and most preferably less than about 5% non-biomarker protein. When antibody, polypeptide, peptide or fusion protein or fragment thereof, *e.g.*, a biologically active fragment thereof, is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

As used herein, the term “isotype” refers to the antibody class (*e.g.*, IgM, IgG1, IgG2C, IgG2, or IgG4 and the like) that is encoded by heavy chain constant region genes.

As used herein, the term “K_D” is intended to refer to the dissociation equilibrium constant of a particular antibody-antigen interaction. The binding affinity of antibodies of the disclosed invention may be measured or determined by standard antibody-antigen assays, for example, competitive assays, saturation assays, or standard immunoassays such as ELISA or RIA.

A “kit” is any manufacture (*e.g.* a package or container) comprising at least one reagent, *e.g.* a probe or small molecule, for specifically detecting and/or affecting the expression of a marker of the present invention. The kit may be promoted, distributed, or sold as a unit for performing the methods of the present invention. The kit may comprise one or more reagents necessary to express a composition useful in the methods of the present invention. In certain embodiments, the kit may further comprise a reference

standard, *e.g.*, a nucleic acid encoding a protein that does not affect or regulate signaling pathways controlling cell growth, division, migration, survival or apoptosis. One skilled in the art can envision many such control proteins, including, but not limited to, common molecular tags (*e.g.*, green fluorescent protein and beta-galactosidase), proteins not
 5 classified in any of pathway encompassing cell growth, division, migration, survival or apoptosis by GeneOntology reference, or ubiquitous housekeeping proteins. Reagents in the kit may be provided in individual containers or as mixtures of two or more reagents in a single container. In addition, instructional materials which describe the use of the compositions within the kit can be included.

10 The term “neoadjuvant therapy” refers to a treatment given before the primary treatment. Examples of neoadjuvant therapy can include chemotherapy, radiation therapy, and hormone therapy. For example, in treating breast cancer, neoadjuvant therapy can allows patients with large breast cancer to undergo breast-conserving surgery.

The “normal” level of expression of a biomarker is the level of expression of the
 15 biomarker in cells of a subject, *e.g.*, a human patient, not afflicted with a cancer. An “over-expression” or “significantly higher level of expression” of a biomarker refers to an expression level in a test sample that is greater than the standard error of the assay employed to assess expression, and is preferably at least 10%, and more preferably 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5, 5,
 20 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times or more higher than the expression activity or level of the biomarker in a control sample (*e.g.*, sample from a healthy subject not having the biomarker associated disease) and preferably, the average expression level of the biomarker in several control samples. A “significantly lower level of expression” of a biomarker refers to an expression level in a test sample that
 25 is at least 10%, and more preferably 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times or more lower than the expression level of the biomarker in a control sample (*e.g.*, sample from a healthy subject not having the biomarker associated disease) and preferably, the average expression level of the biomarker in several
 30 control samples.

An “over-expression” or “significantly higher level of expression” of a biomarker refers to an expression level in a test sample that is greater than the standard error of the assay employed to assess expression, and is preferably at least 10%, and more preferably

1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times or more higher than the expression activity or level of the biomarker in a control sample (e.g., sample from a healthy subject not having the biomarker associated disease) and preferably, the average expression level of the biomarker in several control samples. A “significantly lower level of expression” of a biomarker refers to an expression level in a test sample that is at least 10%, and more preferably 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times or more lower than the expression level of the biomarker in a control sample (e.g., sample from a healthy subject not having the biomarker associated disease) and preferably, the average expression level of the biomarker in several control samples.

The term “pre-determined” biomarker amount and/or activity measurement(s) may be a biomarker amount and/or activity measurement(s) used to, by way of example only, evaluate a subject that may be selected for a particular treatment, evaluate a response to a treatment such as CX3CR1/immune checkpoint combination inhibitor therapy, and/or evaluate the disease state. A pre-determined biomarker amount and/or activity measurement(s) may be determined in populations of patients with or without cancer. The pre-determined biomarker amount and/or activity measurement(s) can be a single number, equally applicable to every patient, or the pre-determined biomarker amount and/or activity measurement(s) can vary according to specific subpopulations of patients. Age, weight, height, and other factors of a subject may affect the pre-determined biomarker amount and/or activity measurement(s) of the individual. Furthermore, the pre-determined biomarker amount and/or activity can be determined for each subject individually. In one embodiment, the amounts determined and/or compared in a method described herein are based on absolute measurements. In another embodiment, the amounts determined and/or compared in a method described herein are based on relative measurements, such as ratios (e.g., serum biomarker normalized to the expression of housekeeping or otherwise generally constant biomarker). The pre-determined biomarker amount and/or activity measurement(s) can be any suitable standard. For example, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from the same or a different human for whom a patient selection is being assessed. In one embodiment, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from a previous

assessment of the same patient. In such a manner, the progress of the selection of the patient can be monitored over time. In addition, the control can be obtained from an assessment of another human or multiple humans, *e.g.*, selected groups of humans, if the subject is a human. In such a manner, the extent of the selection of the human for whom selection is being assessed can be compared to suitable other humans, *e.g.*, other humans who are in a similar situation to the human of interest, such as those suffering from similar or the same condition(s) and/or of the same ethnic group.

The term “predictive” includes the use of a biomarker nucleic acid and/or protein status, *e.g.*, over- or under- activity, emergence, expression, growth, remission, recurrence or resistance of tumors before, during or after therapy, for determining the likelihood of response of a cancer to CX3CR1/immune checkpoint combination inhibitor treatment (*e.g.*, treatment with a combination of a CX3CR1 inhibitor and an immune checkpoint inhibitor, such as an inhibitory antibody against PD-1, PD-L1, and/or PD-L2). Such predictive use of the biomarker may be confirmed by, *e.g.*, (1) increased or decreased copy number (*e.g.*, by FISH, FISH plus SKY, single-molecule sequencing, *e.g.*, as described in the art at least at J. Biotechnol., 86:289-301, or qPCR), overexpression or underexpression of a biomarker nucleic acid (*e.g.*, by ISH, Northern Blot, or qPCR), increased or decreased biomarker protein (*e.g.*, by IHC), or increased or decreased activity, *e.g.*, in more than about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, or more of assayed human cancers types or cancer samples; (2) its absolute or relatively modulated presence or absence in a biological sample, *e.g.*, a sample containing tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, or bone marrow, from a subject, *e.g.* a human, afflicted with cancer; (3) its absolute or relatively modulated presence or absence in clinical subset of patients with cancer (*e.g.*, those responding to a particular CX3CR1/immune checkpoint combination inhibitor therapy or those developing resistance thereto).

The term “pre-malignant lesions” as described herein refers to a lesion that, while not cancerous, has potential for becoming cancerous. It also includes the term “pre-malignant disorders” or “potentially malignant disorders.” In particular this refers to a benign, morphologically and/or histologically altered tissue that has a greater than normal risk of malignant transformation, and a disease or a patient's habit that does not necessarily alter the clinical appearance of local tissue but is associated with a greater than normal risk of precancerous lesion or cancer development in that tissue (leukoplakia, erythroplakia,

erytroleukoplakia lichen planus (lichenoid reaction) and any lesion or an area which histological examination showed atypia of cells or dysplasia. In one embodiment, a metaplasia is a pre-malignant lesion.

5 The terms “prevent,” “preventing,” “prevention,” “prophylactic treatment,” and the like refer to reducing the probability of developing a disease, disorder, or condition in a subject, who does not have, but is at risk of or susceptible to developing a disease, disorder, or condition.

10 The term “probe” refers to any molecule which is capable of selectively binding to a specifically intended target molecule, for example, a nucleotide transcript or protein encoded by or corresponding to a biomarker nucleic acid. Probes can be either synthesized by one skilled in the art, or derived from appropriate biological preparations. For purposes of detection of the target molecule, probes may be specifically designed to be labeled, as described herein. Examples of molecules that can be utilized as probes include, but are not limited to, RNA, DNA, proteins, antibodies, and organic molecules.

15 The term “prognosis” includes a prediction of the probable course and outcome of cancer or the likelihood of recovery from the disease. In some embodiments, the use of statistical algorithms provides a prognosis of cancer in an individual. For example, the prognosis can be surgery, development of a clinical subtype of cancer (*e.g.*, solid tumors, such as esophageal cancer and gastric cancer), development of one or more clinical factors, or recovery from the disease.

20 The term “response to anti-cancer therapy” or “response to anti-CX3CR1/immune checkpoint therapy” relates to any response of the hyperproliferative disorder (*e.g.*, cancer) to an anti-cancer agent such as an CX3CR1/immune checkpoint agent, preferably to a change in tumor mass and/or volume after initiation of neoadjuvant or adjuvant therapy.

25 Hyperproliferative disorder response may be assessed, for example for efficacy or in a neoadjuvant or adjuvant situation, where the size of a tumor after systemic intervention can be compared to the initial size and dimensions as measured by CT, PET, mammogram, ultrasound or palpation. Responses may also be assessed by caliper measurement or pathological examination of the tumor after biopsy or surgical resection. Response may be recorded in a quantitative fashion like percentage change in tumor volume or in a

30 qualitative fashion like “pathological complete response” (pCR), “clinical complete remission” (cCR), “clinical partial remission” (cPR), “clinical stable disease” (cSD), “clinical progressive disease” (cPD) or other qualitative criteria. Assessment of

hyperproliferative disorder response may be done early after the onset of neoadjuvant or adjuvant therapy, *e.g.*, after a few hours, days, weeks or preferably after a few months. A typical endpoint for response assessment is upon termination of neoadjuvant chemotherapy or upon surgical removal of residual tumor cells and/or the tumor bed. This is typically

5 three months after initiation of neoadjuvant therapy. In some embodiments, clinical efficacy of the therapeutic treatments described herein may be determined by measuring the clinical benefit rate (CBR). The clinical benefit rate is measured by determining the sum of the percentage of patients who are in complete remission (CR), the number of patients who are in partial remission (PR) and the number of patients having stable disease (SD) at a time

10 point at least 6 months out from the end of therapy. The shorthand for this formula is $CBR = CR + PR + SD$ over 6 months. In some embodiments, the CBR for a particular cancer therapeutic regimen is at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or more. Additional criteria for evaluating the response to cancer therapies are related to “survival,” which includes all of the following: survival until

15 mortality, also known as overall survival (wherein said mortality may be either irrespective of cause or tumor related); “recurrence-free survival” (wherein the term recurrence shall include both localized and distant recurrence); metastasis free survival; disease free survival (wherein the term disease shall include cancer and diseases associated therewith). The length of said survival may be calculated by reference to a defined start point (*e.g.*, time of

20 diagnosis or start of treatment) and end point (*e.g.*, death, recurrence or metastasis). In addition, criteria for efficacy of treatment can be expanded to include response to chemotherapy, probability of survival, probability of metastasis within a given time period, and probability of tumor recurrence. For example, in order to determine appropriate threshold values, a particular cancer therapeutic regimen can be administered to a

25 population of subjects and the outcome can be correlated to biomarker measurements that were determined prior to administration of any cancer therapy. The outcome measurement may be pathologic response to therapy given in the neoadjuvant setting. Alternatively, outcome measures, such as overall survival and disease-free survival can be monitored over a period of time for subjects following cancer therapy for which biomarker measurement

30 values are known. In certain embodiments, the doses administered are standard doses known in the art for cancer therapeutic agents. The period of time for which subjects are monitored can vary. For example, subjects may be monitored for at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, or 60 months. Biomarker measurement threshold

values that correlate to outcome of a cancer therapy can be determined using well-known methods in the art, such as those described in the Examples section.

The term “resistance” refers to an acquired or natural resistance of a cancer sample or a mammal to a cancer therapy (*i.e.*, being nonresponsive to or having reduced or limited response to the therapeutic treatment), such as having a reduced response to a therapeutic treatment by 25% or more, for example, 30%, 40%, 50%, 60%, 70%, 80%, or more, to 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold or more. The reduction in response can be measured by comparing with the same cancer sample or mammal before the resistance is acquired, or by comparing with a different cancer sample or a mammal that is known to have no resistance to the therapeutic treatment. A typical acquired resistance to chemotherapy is called “multidrug resistance.” The multidrug resistance can be mediated by P-glycoprotein or can be mediated by other mechanisms, or it can occur when a mammal is infected with a multi-drug-resistant microorganism or a combination of microorganisms. The determination of resistance to a therapeutic treatment is routine in the art and within the skill of an ordinarily skilled clinician, for example, can be measured by cell proliferative assays and cell death assays as described herein as “sensitizing.” In some embodiments, the term “reverses resistance” means that the use of a second agent in combination with a primary cancer therapy (*e.g.*, chemotherapeutic or radiation therapy) is able to produce a significant decrease in tumor volume at a level of statistical significance (*e.g.*, $p < 0.05$) when compared to tumor volume of untreated tumor in the circumstance where the primary cancer therapy (*e.g.*, chemotherapeutic or radiation therapy) alone is unable to produce a statistically significant decrease in tumor volume compared to tumor volume of untreated tumor. This generally applies to tumor volume measurements made at a time when the untreated tumor is growing log rhythmically.

The terms “response” or “responsiveness” refers to an anti-cancer response, *e.g.* in the sense of reduction of tumor size or inhibiting tumor growth. The terms can also refer to an improved prognosis, for example, as reflected by an increased time to recurrence, which is the period to first recurrence censoring for second primary cancer as a first event or death without evidence of recurrence, or an increased overall survival, which is the period from treatment to death from any cause. To respond or to have a response means there is a beneficial endpoint attained when exposed to a stimulus. Alternatively, a negative or detrimental symptom is minimized, mitigated or attenuated on exposure to a stimulus. It will be appreciated that evaluating the likelihood that a tumor or subject will exhibit a

favorable response is equivalent to evaluating the likelihood that the tumor or subject will not exhibit favorable response (*i.e.*, will exhibit a lack of response or be non-responsive).

An “RNA interfering agent” as used herein, is defined as any agent which interferes with or inhibits expression of a target biomarker gene by RNA interference (RNAi). Such RNA interfering agents include, but are not limited to, nucleic acid molecules including RNA molecules which are homologous to the target biomarker gene of the present invention, or a fragment thereof, short interfering RNA (siRNA), and small molecules which interfere with or inhibit expression of a target biomarker nucleic acid by RNA interference (RNAi).

“RNA interference (RNAi)” is an evolutionally conserved process whereby the expression or introduction of RNA of a sequence that is identical or highly similar to a target biomarker nucleic acid results in the sequence specific degradation or specific post-transcriptional gene silencing (PTGS) of messenger RNA (mRNA) transcribed from that targeted gene (*see* Coburn and Cullen (2002) *J. Virol.* 76:9225), thereby inhibiting expression of the target biomarker nucleic acid. In one embodiment, the RNA is double stranded RNA (dsRNA). This process has been described in plants, invertebrates, and mammalian cells. In nature, RNAi is initiated by the dsRNA-specific endonuclease Dicer, which promotes processive cleavage of long dsRNA into double-stranded fragments termed siRNAs. siRNAs are incorporated into a protein complex that recognizes and cleaves target mRNAs. RNAi can also be initiated by introducing nucleic acid molecules, *e.g.*, synthetic siRNAs or RNA interfering agents, to inhibit or silence the expression of target biomarker nucleic acids. As used herein, “inhibition of target biomarker nucleic acid expression” or “inhibition of marker gene expression” includes any decrease in expression or protein activity or level of the target biomarker nucleic acid or protein encoded by the target biomarker nucleic acid. The decrease may be of at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more as compared to the expression of a target biomarker nucleic acid or the activity or level of the protein encoded by a target biomarker nucleic acid which has not been targeted by an RNA interfering agent.

The term “sample” used for detecting or determining the presence or level of at least one biomarker is typically brain tissue, cerebrospinal fluid, whole blood, plasma, serum, saliva, urine, stool (*e.g.*, feces), tears, and any other bodily fluid (*e.g.*, as described above under the definition of “body fluids”), or a tissue sample (*e.g.*, biopsy) such as a small intestine, colon sample, or surgical resection tissue. In certain instances, the method of the

present invention further comprises obtaining the sample from the individual prior to detecting or determining the presence or level of at least one marker in the sample.

The term “sensitize” means to alter cancer cells or tumor cells in a way that allows for more effective treatment of the associated cancer with a cancer therapy (*e.g.*, anti-immune checkpoint, chemotherapeutic, and/or radiation therapy). In some embodiments, normal cells are not affected to an extent that causes the normal cells to be unduly injured by the anti-immune checkpoint therapy. An increased sensitivity or a reduced sensitivity to a therapeutic treatment is measured according to a known method in the art for the particular treatment and methods described herein below, including, but not limited to, cell proliferative assays (Tanigawa N, Kern D H, Kikasa Y, Morton D L, Cancer Res 1982; 42: 2159-2164), cell death assays (Weisenthal L M, Shoemaker R H, Marsden J A, Dill P L, Baker J A, Moran E M, Cancer Res 1984; 94: 161-173; Weisenthal L M, Lippman M E, Cancer Treat Rep 1985; 69: 615-632; Weisenthal L M, In: Kaspers G J L, Pieters R, Twentyman P R, Weisenthal L M, Veerman A J P, eds. Drug Resistance in Leukemia and Lymphoma. Langhorne, P A: Harwood Academic Publishers, 1993: 415-432; Weisenthal L M, Contrib Gynecol Obstet 1994; 19: 82-90). The sensitivity or resistance may also be measured in animal by measuring the tumor size reduction over a period of time, for example, 6 month for human and 4-6 weeks for mouse. A composition or a method sensitizes response to a therapeutic treatment if the increase in treatment sensitivity or the reduction in resistance is 25% or more, for example, 30%, 40%, 50%, 60%, 70%, 80%, or more, to 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold or more, compared to treatment sensitivity or resistance in the absence of such composition or method. The determination of sensitivity or resistance to a therapeutic treatment is routine in the art and within the skill of an ordinarily skilled clinician. It is to be understood that any method described herein for enhancing the efficacy of a cancer therapy can be equally applied to methods for sensitizing hyperproliferative or otherwise cancerous cells (*e.g.*, resistant cells) to the cancer therapy.

“Short interfering RNA” (siRNA), also referred to herein as “small interfering RNA” is defined as an agent which functions to inhibit expression of a target biomarker nucleic acid, *e.g.*, by RNAi. An siRNA may be chemically synthesized, may be produced by *in vitro* transcription, or may be produced within a host cell. In one embodiment, siRNA is a double stranded RNA (dsRNA) molecule of about 15 to about 40 nucleotides in length, preferably about 15 to about 28 nucleotides, more preferably about 19 to about 25

nucleotides in length, and more preferably about 19, 20, 21, or 22 nucleotides in length, and may contain a 3' and/or 5' overhang on each strand having a length of about 0, 1, 2, 3, 4, or 5 nucleotides. The length of the overhang is independent between the two strands, *i.e.*, the length of the overhang on one strand is not dependent on the length of the overhang on the second strand. Preferably the siRNA is capable of promoting RNA interference through degradation or specific post-transcriptional gene silencing (PTGS) of the target messenger RNA (mRNA).

In another embodiment, an siRNA is a small hairpin (also called stem loop) RNA (shRNA). In one embodiment, these shRNAs are composed of a short (*e.g.*, 19-25 nucleotide) antisense strand, followed by a 5-9 nucleotide loop, and the analogous sense strand. Alternatively, the sense strand may precede the nucleotide loop structure and the antisense strand may follow. These shRNAs may be contained in plasmids, retroviruses, and lentiviruses and expressed from, for example, the pol III U6 promoter, or another promoter (*see, e.g.*, Stewart, *et al.* (2003) *RNA* Apr;9(4):493-501 incorporated by reference herein).

RNA interfering agents, *e.g.*, siRNA molecules, may be administered to a patient having or at risk for having cancer, to inhibit expression of a biomarker gene which is overexpressed in cancer and thereby treat, prevent, or inhibit cancer in the subject.

The term “small molecule” is a term of the art and includes molecules that are less than about 1000 molecular weight or less than about 500 molecular weight. In one embodiment, small molecules do not exclusively comprise peptide bonds. In another embodiment, small molecules are not oligomeric. Exemplary small molecule compounds which can be screened for activity include, but are not limited to, peptides, peptidomimetics, nucleic acids, carbohydrates, small organic molecules (*e.g.*, polyketides) (Cane *et al.* (1998) *Science* 282:63), and natural product extract libraries. In another embodiment, the compounds are small, organic non-peptidic compounds. In a further embodiment, a small molecule is not biosynthetic.

The term “specific binding” refers to antibody binding to a predetermined antigen. Typically, the antibody binds with an affinity (K_D) of approximately less than 10^{-7} M, such as approximately less than 10^{-8} M, 10^{-9} M or 10^{-10} M or even lower when determined by surface plasmon resonance (SPR) technology in a BIAcore® assay instrument using an antigen of interest as the analyte and the antibody as the ligand, and binds to the predetermined antigen with an affinity that is at least 1.1-, 1.2-, 1.3-, 1.4-, 1.5-, 1.6-, 1.7-,

1.8-, 1.9-, 2.0-, 2.5-, 3.0-, 3.5-, 4.0-, 4.5-, 5.0-, 6.0-, 7.0-, 8.0-, 9.0-, or 10.0-fold or greater than its affinity for binding to a non-specific antigen (*e.g.*, BSA, casein) other than the predetermined antigen or a closely-related antigen. The phrases “an antibody recognizing an antigen” and “an antibody specific for an antigen” are used interchangeably herein with
5 the term “an antibody which binds specifically to an antigen.” Selective binding is a relative term referring to the ability of an antibody to discriminate the binding of one antigen over another.

The term “subject” refers to any healthy animal, mammal or human, or any animal, mammal or human afflicted with a cancer, *e.g.*, brain, lung, ovarian, pancreatic, liver,
10 breast, prostate, and/or colorectal cancers, melanoma, multiple myeloma, and the like. The term “subject” is interchangeable with “patient.”

The term “survival” includes all of the following: survival until mortality, also known as overall survival (wherein said mortality may be either irrespective of cause or tumor related); “recurrence-free survival” (wherein the term recurrence shall include both
15 localized and distant recurrence); metastasis free survival; disease free survival (wherein the term disease shall include cancer and diseases associated therewith). The length of said survival may be calculated by reference to a defined start point (*e.g.* time of diagnosis or start of treatment) and end point (*e.g.* death, recurrence or metastasis). In addition, criteria for efficacy of treatment can be expanded to include response to chemotherapy, probability
20 of survival, probability of metastasis within a given time period, and probability of tumor recurrence.

The term “synergistic effect” refers to the combined effect of two or more anti-cancer agents (*e.g.*, CX3CR1/immune checkpoint inhibitors) can be greater than the sum of the separate effects of the anticancer agents alone.

25 The term “T cell” includes CD4⁺ T cells and CD8⁺ T cells. The term T cell also includes both T helper 1 type T cells and T helper 2 type T cells. The term “antigen presenting cell” includes professional antigen presenting cells (*e.g.*, B lymphocytes, monocytes, dendritic cells, Langerhans cells), as well as other antigen presenting cells (*e.g.*, keratinocytes, endothelial cells, astrocytes, fibroblasts, and oligodendrocytes).

30 The term “therapeutic effect” refers to a local or systemic effect in animals, particularly mammals, and more particularly humans, caused by a pharmacologically active substance. The term thus means any substance intended for use in the diagnosis, cure, mitigation, treatment or prevention of disease or in the enhancement of desirable physical

or mental development and conditions in an animal or human. The phrase “therapeutically-effective amount” means that amount of such a substance that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. In certain embodiments, a therapeutically effective amount of a compound will depend on its

5 therapeutic index, solubility, and the like. For example, certain compounds discovered by the methods of the present invention may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

The terms “therapeutically-effective amount” and “effective amount” as used herein means that amount of a compound, material, or composition comprising a compound of the

10 present invention which is effective for producing some desired therapeutic effect in at least a sub-population of cells in an animal at a reasonable benefit/risk ratio applicable to any medical treatment. Toxicity and therapeutic efficacy of subject compounds may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ and the ED₅₀. Compositions that exhibit large therapeutic

15 indices are preferred. In some embodiments, the LD₅₀ (lethal dosage) can be measured and can be, for example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more reduced for the agent relative to no administration of the agent. Similarly, the ED₅₀ (*i.e.*, the concentration which achieves a half-maximal inhibition of symptoms) can be measured and can be, for

20 example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more increased for the agent relative to no administration of the agent. Also, Similarly, the IC₅₀ (*i.e.*, the concentration which achieves half-maximal cytotoxic or cytostatic effect on cancer cells) can be measured and can be, for example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%,

25 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more increased for the agent relative to no administration of the agent. In some embodiments, cancer cell growth in an assay can be inhibited by at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or even 100%. In another embodiment, at least about a 10% , 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%,

30 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or even 100% decrease in a solid malignancy can be achieved.

A “transcribed polynucleotide” or “nucleotide transcript” is a polynucleotide (*e.g.* an mRNA, hnRNA, a cDNA, or an analog of such RNA or cDNA) which is complementary

to or homologous with all or a portion of a mature mRNA made by transcription of a biomarker nucleic acid and normal post-transcriptional processing (*e.g.* splicing), if any, of the RNA transcript, and reverse transcription of the RNA transcript.

As used herein, the term “unresponsiveness” includes refractivity of cancer cells to therapy or refractivity of therapeutic cells, such as immune cells, to stimulation, *e.g.*, stimulation via an activating receptor or a cytokine. Unresponsiveness can occur, *e.g.*, because of exposure to immunosuppressants or exposure to high doses of antigen. As used herein, the term “anergy” or “tolerance” includes refractivity to activating receptor-mediated stimulation. Such refractivity is generally antigen-specific and persists after exposure to the tolerizing antigen has ceased. For example, anergy in T cells (as opposed to unresponsiveness) is characterized by lack of cytokine production, *e.g.*, IL-2. T cell anergy occurs when T cells are exposed to antigen and receive a first signal (a T cell receptor or CD-3 mediated signal) in the absence of a second signal (a costimulatory signal). Under these conditions, reexposure of the cells to the same antigen (even if reexposure occurs in the presence of a costimulatory polypeptide) results in failure to produce cytokines and, thus, failure to proliferate. Anergic T cells can, however, proliferate if cultured with cytokines (*e.g.*, IL-2). For example, T cell anergy can also be observed by the lack of IL-2 production by T lymphocytes as measured by ELISA or by a proliferation assay using an indicator cell line. Alternatively, a reporter gene construct can be used. For example, anergic T cells fail to initiate IL-2 gene transcription induced by a heterologous promoter under the control of the 5' IL-2 gene enhancer or by a multimer of the AP1 sequence that can be found within the enhancer (Kang *et al.* (1992) *Science* 257:1134).

There is a known and definite correspondence between the amino acid sequence of a particular protein and the nucleotide sequences that can code for the protein, as defined by the genetic code (shown below). Likewise, there is a known and definite correspondence between the nucleotide sequence of a particular nucleic acid and the amino acid sequence encoded by that nucleic acid, as defined by the genetic code.

GENETIC CODE

Alanine (Ala, A)	GCA, GCC, GCG, GCT
Arginine (Arg, R)	AGA, ACG, CGA, CGC, CGG, CGT
Asparagine (Asn, N)	AAC, AAT
Aspartic acid (Asp, D)	GAC, GAT
Cysteine (Cys, C)	TGC, TGT

	Glutamic acid (Glu, E)	GAA, GAG
	Glutamine (Gln, Q)	CAA, CAG
	Glycine (Gly, G)	GGA, GGC, GGG, GGT
	Histidine (His, H)	CAC, CAT
5	Isoleucine (Ile, I)	ATA, ATC, ATT
	Leucine (Leu, L)	CTA, CTC, CTG, CTT, TTA, TTG
	Lysine (Lys, K)	AAA, AAG
	Methionine (Met, M)	ATG
	Phenylalanine (Phe, F)	TTC, TTT
10	Proline (Pro, P)	CCA, CCC, CCG, CCT
	Serine (Ser, S)	AGC, AGT, TCA, TCC, TCG, TCT
	Threonine (Thr, T)	ACA, ACC, ACG, ACT
	Tryptophan (Trp, W)	TGG
	Tyrosine (Tyr, Y)	TAC, TAT
15	Valine (Val, V)	GTA, GTC, GTG, GTT
	Termination signal (end)	TAA, TAG, TGA

An important and well-known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet may be employed (illustrated above). Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they result in the production of the same amino acid sequence in all organisms (although certain organisms may translate some sequences more efficiently than they do others). Moreover, occasionally, a methylated variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such methylations do not affect the coding relationship between the trinucleotide codon and the corresponding amino acid.

In view of the foregoing, the nucleotide sequence of a DNA or RNA encoding a biomarker nucleic acid (or any portion thereof) can be used to derive the polypeptide amino acid sequence, using the genetic code to translate the DNA or RNA into an amino acid sequence. Likewise, for polypeptide amino acid sequence, corresponding nucleotide sequences that can encode the polypeptide can be deduced from the genetic code (which, because of its redundancy, will produce multiple nucleic acid sequences for any given amino acid sequence). Thus, description and/or disclosure herein of a nucleotide sequence

which encodes a polypeptide should be considered to also include description and/or disclosure of the amino acid sequence encoded by the nucleotide sequence. Similarly, description and/or disclosure of a polypeptide amino acid sequence herein should be considered to also include description and/or disclosure of all possible nucleotide sequences that can encode the amino acid sequence.

Finally, nucleic acid and amino acid sequence information for the loci and biomarkers of the present invention (*e.g.*, biomarkers listed in Tables 1 and 2) are well-known in the art and readily available on publicly available databases, such as the National Center for Biotechnology Information (NCBI). For example, exemplary nucleic acid and amino acid sequences derived from publicly available sequence databases are provided below and include, for example, PCT Publ. WO 2014/022759, which is incorporated herein in its entirety by this reference.

Table 1

SEQ ID NO: 1 Human CX3CR1 isoform a cDNA Acid Sequence
(NM_001171174)

1	ggggcgggcc	gtgcttacca	ggccgtggac	ttaaaccagg	atgagagaac	ccctggagggc
61	gtttaagttg	gcagacttgg	atttcaggaa	gagctctctg	gcttctgggt	ggagaatggc
121	cagtggggcc	ttcaccatgg	atcagttccc	tgaatcagtg	acagaaaact	ttgagtacga
181	tgatttggct	gaggcctgtt	atattgggga	catcgtggtc	tttgggactg	tgttcctgtc
241	catattctac	tccgtcatct	ttgccattgg	cctggtggga	aatttgttgg	tagtgtttgc
301	cctcaccaac	agcaagaagc	ccaagagtgt	caccgacatt	tacctcctga	acctggcctt
361	gtctgatctg	ctgtttgtag	ccactttgcc	cttctggact	cactatttga	taaatgaaaa
421	gggcctccac	aatgccatgt	gcaaattcac	taccgccttc	ttcttcatcg	gcttttttgg
481	aagcatattc	ttcatcaccg	tcatacagcat	tgataggtac	ctggccatcg	tcctggccgc
541	caactccatg	aacaaccgga	ccgtgcagca	tggcgtcacc	atcagcctag	gcgtctgggc
601	agcagccatt	ttggtggcag	caccccagtt	catgttcaca	aagcagaaag	aaaatgaatg
661	ccttggtgac	taccccgagg	tcctccagga	aatctggccc	gtgctccgca	atgtggaaac
721	aaattttctt	ggcttcctac	tccccctgct	cattatgagt	tattgctact	tcagaatcat
781	ccagacgtg	ttttcctgca	agaaccacaa	gaaagccaaa	gccattaaac	tgatccttct
841	ggtggctatc	gtgtttttcc	tcttctggac	accctacaac	gttatgattt	tcctggagac
901	gcttaagctc	tatgacttct	ttcccagttg	tgacatgagg	aaggatctga	ggctggccct
961	cagtgtgact	gagacggttg	catttagcca	ttgttgccctg	aatcctctca	tctatgcatt
1021	tgctgggggag	aagttcagaa	gataccttta	ccacctgtat	gggaaatgcc	tggctgtcct
1081	gtgtggggcgc	tcagtccacg	ttgatattctc	ctcatctgaa	tcacaaagga	gcaggcatgg
1141	aagtgttctg	agcagcaatt	ttacttacca	cacgagtgat	ggagatgcat	tgctccttct
1201	ctgaagggaa	tcccaaagcc	ttgtgtctac	agagaacctg	gagttcctga	acctgatgct
1261	gactagttag	gaaagatttt	tgttgttatt	tcttacaggc	acaaaatgat	ggaccaaatg
1321	cacacaaaac	aaccctagag	tgttgttgag	aattgtgctc	aaaatttgaa	gaatgaacaa
1381	attgaactct	ttgaatgaca	aagagtagac	atttctctta	ctgcaaagt	catcagaact
1441	ttttgggttg	cagatgacaa	aaattccaact	cagactagtt	tagttaaagt	agggtggtga
1501	atattgttca	tattgtggca	caagcaaaag	ggtgtctgag	ccctcaaagt	gaggggaaac
1561	cagggcctga	gccaaagctag	aattccctct	ctctgactct	caaactcttt	agtcattata
1621	gatccccccag	actttacatg	acacagcttt	atcaccagag	agggactgac	acctatgttt
1681	ctctggcccc	aagggcacaaa	ttcccaggga	agtgtctgta	taggccaagt	ttgtatcagg
1741	tgcccatccc	tggaggtgct	tggtatccat	ggggaaggga	tatataagat	ggaagcttcc
1801	agtccaatct	catggagaag	cagaaataca	tattttccaag	aagttggatg	ggtgggtact
1861	attctgatta	cacaaaacaa	atgccacaca	tcacccttac	catgtgcctg	atccagcctc
1921	tccctgatt	acaccagcct	cgtcttcatt	aagccctctt	ccatcatgtc	cccaaacctg

1981 caaggggctcc ccactgccta ctgcatcgag tcaaaactca aatgcttggc ttctcatacg
 2041 tccaccatgg ggtcctacca atagattccc cattgcctcc tccttcccaa aggactccac
 2101 ccatacctatc agcctgtctc ttccatatga cctcatgcat ctccacctgc tcccaggcca
 2161 gtaagggaaa tagaaaaacc ctgcccccaa ataagaaggg atggattcca accccaactc
 5 2221 cagtagcttg ggacaaatca agcttcagtt tcctgggtctg tagaagaggg ataaggtacc
 2281 tttcacatag agatcatcct ttccagcatg aggaactagc caccaactct tgcaggtctc
 2341 aacccttttg tctgcctctt agactttctgc tttccacacc tggcactgct gtgctgtgcc
 2401 caagttgttg tgctgacaaa gcttggaaga gcctgcaggt gctgctgctg ggcatagccc
 2461 agacacagaa gaggctgggt cttacgatgg caccagtgga gcactcccaa gtctacagag
 10 2521 tgatagcctt ccgtaaccca actctcctgg actgccttga atatcccctc ccagtcacct
 2581 tgtggcaagc ccctgcccac ctgggaaaat accccatcat tcatgctact gccaacctgg
 2641 ggagccaggg ctatggggagc agcttttttt tccccctag aaacgttttg aacaatctaa
 2701 aagtttaaag ctcgaaaaca attgtaataa tgctaaagaa aaagtcattc aatctaacca
 2761 catcaatatt gtcattcctg tattcacccg tccagacctt gttcacactc tcacatgttt
 15 2821 agagttgcaa tcgtaatgta cagatggttt tataatctga tttgttttcc tcttaacgtt
 2881 agaccacaaa tagtgctcgc tttctatgta gtttggtaat tatcatttta gaagactcta
 2941 ccagactgtg tattcattga agtcagatgt ggtaactgtt aaattgctgt gtatctgata
 3001 gctctttggc agtctatatg tttgtataat gaatgagaga ataagtcatg ttccttcaag
 3061 atcatgtacc ccaatttact tgccattact caattgataa acatttaact tgtttccaat
 20 3121 gtttagcaaa tacatatattt atagaacttc caaaaaaaaa

SEQ ID NO: 2 Human CX3CR1 isoform a Amino Acid Sequence
(NP_001164645.1)

1 mrepleafkl adldfrkssl asgwrmasga ftmdqfpesv tenfeyddla eacyigdivv
 25 61 fgtvflsify svifaiglvq nllvvfalt n skkpksvtdi yllnlalsdl lfvatlpfwt
 121 hylinekgllh namckfttaf ffigffgsif fitvisidry laiivlaansm nnrtvqhgv
 181 islgvwaaai lvaapqfmft kqkenecldg ypevlqeiwp vlrvetnfl gfillpllims
 241 ycyfriiqtl fscknhkkak aiklillvvi vfflfwtpyn vmifletlkl ydffpscdmr
 301 kdrlrlalsvt etvafshccl nplyafage kfrrylyhly gkclavlcgr svhvdffsse
 30 361 sqrsrhgsvl ssnftyhtsd gdallll

SEQ ID NO: 3 Human CX3CR1 isoform b variant 1 cDNA Acid Sequence
(NM_001171171)

1 gcttttgtcc tgtggccttc tggccaaagg cagggaaagg tggccaaaca ctgagaccaa
 35 61 gaacaaagaa agaaaactgc tgggtggactt cttccaccat gagcaggcca ccaagcccgc
 121 agcactgcac tgcagccccc agctctgtcc tgggggttggg ggaggtgagg aggggcaagg
 181 tggggagcac acagagcacc cgctgtcctc ggaacaccac agcgactaga ggccttcacc
 241 atggatcagt tccctgaatc agtgacagaa aactttgagt acgatgattt ggctgaggcc
 301 tgttatatatt gggacatcgt ggtctttggg actgtgttcc tgtccatatt ctactccgtc
 40 361 atctttgcca ttggcctggg gggaaatttg ttggtagtgt ttgccctcac caacagcaag
 421 aagcccaaga gtgtcacoga catttacctc ctgaacctgg ccttgtctga tctgctgttt
 481 gtagccactt tgcccttctg gactcactat ttgataaatg aaaagggcct ccacaatgcc
 541 atgtgcaa at tcaactaccgc cttcttcttc atcggctttt ttggaagcat attcttcata
 601 accgtcatca gcattgatag gtacctggcc atcgtcctgg ccgccaaact catgaacaac
 45 661 cggaccgtgc agcatggcgt caccatcagc ctaggcgtct gggcagcagc cattttggtg
 721 gcagcaccac agttcatgtt cacaagcag aaagaaaatg aatgccttgg tgactacccc
 781 gaggtcctcc aggaaatctg gcccggtgctc cgcaatgtgg aaacaaattt tcttggett
 841 ctactcccc tgctcattat gagttattgc tacttcagaa tcatccagac gctgttttcc
 901 tgcaagaacc acaagaaagc caaagccatt aaactgatcc ttctgggtgt catcgtgttt
 50 961 ttctcttctt ggacacccta caacgttatg attttcctgg agacgcttaa gctctatgac
 1021 ttctttccca gttgtgacat gaggaaggat ctgaggctgg ccctcagtgt gactgagacg
 1081 gttgcattta gccattgttg cctgaatcct ctcatctatg catttgctgg ggagaagttc
 1141 agaagatacc tttaccacct gtatgggaaa tgcttggtgt tctgtgtgg gcgctcagtc
 1201 cacgttgatt tctcctcatc tgaatcacia aggagcaggc atggaagtgt tctgagcagc
 55 1261 aattttactt accacacgag tgatggagat gcattgctcc ttctctgaag ggaatcccaa
 1321 agccttgtgt ctacagagaa cctggagtct ctgaacctga tgctgactag tgaggaaaga
 1381 tttttgttgt tatttcttac aggcacaaaa tgatggaccc aatgcacaca aaacaaccct
 1441 agagtgttgt tgagaattgt gctcaaaatt tgaagaatga acaaattgaa ctctttgaat
 1501 gacaaagagt agacatttct cttactgcaa atgtcatcag aactttttgg tttgcagatg
 60 1561 acaaaaattc aactcagact agtttagtta aatgagggtg gtgaatattg ttcattattg

1621 ggcacaagca aaagggtgtc tgagccctca aagtgagggg aaaccagggc ctgagccaag
 1681 ctagaattcc ctctctctga ctctcaaatc ttttagtcat tatagatccc ccgacttta
 1741 catgacacag ctttatcacc agagagggac tgacacccat gtttctctgg cccaagggc
 1801 aaaattccca gggaagtgtc ctgataggcc aagtttgtat caggtgccca tccttggaag
 5 1861 gtgctgttat ccatggggaa gggatatata agatggaagc ttccagtcca atctcatgga
 1921 gaagcagaaa tacatatctt caagaagttg gatgggtggg tactattctg attacacaaa
 1981 acaaatgcc aacatcaccc ttaccatgtg cctgatccag cctctccctt gattacacca
 2041 gcctcgtctt cattaagccc tcttccatca tgtcccaaaa cctgcaaggg ctccccactg
 2101 cctactgcat cgagtcaaaa ctcaaatgct tggcttctca tacgtccacc atgggggtcct
 10 2161 accaatagat tccccattgc ctctccttc ccaaaggact ccaccatcc tatcagcctg
 2221 tctcttccat atgacctcat gcatctccac ctgctcccag gccagtaagg gaaatagaaa
 2281 aaccctgccc ccaataaaga agggatggat tccaacccca actccagtag cttgggacaa
 2341 atcaagcttc agtttctctg tctgtagaag agggataagg tacctttcac atagagatca
 2401 tcctttccag catgaggaac tagccaccaa ctcttgacag tctcaacctt tttgtctgcc
 15 2461 tcttagactt ctgctttcca cacctggcac tgctgtgctg tgcccaagtt gtgggtgctga
 2521 caaagcttgg aagagcctgc aggtgctgct gcgtggcata gccagacac agaagaggct
 2581 gggtcttacg atggcaccca gtgagcactc ccaagtctac agagtgatag ccttccgtaa
 2641 ccaactctc ctggactgcc ttgaatatcc cctccagtc accttggtgc aagcccctgc
 2701 ccatctggga aaatacccca tcattcatgc tactggcaac ctggggagtc agggctatgg
 20 2761 gagcagcttt tttttccccc ctagaaacgt ttggaacaat ctaaaagttt aaagctcgaa
 2821 aacaattgta ataattgcta agaaaaagtc atccaatcta accacatcaa tattgtcatt
 2881 cctgtattca cccgtccaga ccttggtcac actctcacat gtttagagtt gcaatcgtaa
 2941 tgtacagatg gttttataat ctgatttgtt ttctcttaa cgtagacca caaatagtgc
 3001 tcgctttcta tgtagtgttg taattatcat tttagaagac tctaccagac tgtgtattca
 25 3061 ttgaagtcag atgtggtaac tgttaaattg ctgtgtatct gatagctctt tggcagtcta
 3121 tatgtttgta taatgaatga gagaataagt catgttcctt caagatcatg taccccaatt
 3181 tacttgccat tactcaattg ataaacattt aacttgtttc caatgtttag caaatacata
 3241 ttttatagaa cttccaaaaa aaaa

30 **SEQ ID NO: 4 Human CX3CR1 isoform b variant 2 cDNA Acid Sequence**
(NM 001171172.1)

1 actttgagga gaagagctgt gtgtgcccca gtgtggtcca gtgagtactc tgggtccct
 61 ctctgtgggca gggaagctga gggcccatat agctctccca gcttctctga ggctccccat
 121 taatgagagc tgactgtgct gtgctttgct gactgcaggg cctgctccct gccccccacc
 35 181 tccagggttg ggcttcacc atggatcagt tccctgaatc agtgacagaa aactttgagt
 241 acgatgattt ggctgaggcc tgttatattg gggacatcgt ggtctttggg actgtgttcc
 301 tgtccatatt ctactcgtc atctttgcca ttggcctggt gggaaatttg ttggtagtgt
 361 ttgccctcac caacagcaag aagcccaaga gtgtcaccga catttacctc ctgaacctgg
 421 ccttgtctga tctgtgtgtt gtagccactt tgcccttctg gactcactat ttgataaatg
 40 481 aaaagggcct ccacaatgcc atgtgcaaat tcaactaccg cttcttcttc atcggtttt
 541 ttggaagcat attcttcatc accgtcatca gcattgatag gtacctggcc atcgctctg
 601 ccgccaactc catgaacaac cggaccgtgc agcatggcgt caccatcagc ctaggcgtct
 661 gggcagcagc cattttggtg gcagcacccc agttcatgtt cacaaagcag aaagaaaatg
 721 aatgccttgg tgactacccc gaggtcctcc aggaaatctg gccctgtctc cgcaatgtgg
 45 781 aaacaaattt tcttggtctc ctactcccc tgctcattat gattatttgc tacttcagaa
 841 tcatccagac gctgttttcc tgcaagaacc acaagaaagc caaagccatt aaactgatcc
 901 ttctggtggt catcgtgttt ttctctctct ggacacccta caacgttatg attttcctgg
 961 agacgcttaa gctctatgac ttctttccca gttgtgacat gaggaaggat ctgaggctgg
 1021 ccctcagtgt gactgagacg gttgcattta gccattgttg cctgaatcct ctcatctatg
 50 1081 catttgctgg ggagaagttc agaagatacc ttaccacct gtatgggaaa tgcttgctg
 1141 tcctgtgtgg gcgctcagtc cacgttgatt tctcctcatc tgaatcacia aggagcaggc
 1201 atggaagtgt tctgagcagc aattttactt accacacgag tgatggagat gcattgtctc
 1261 ttctctgaag ggaatcccaa agccttgtgt ctacagagaa cctggagtcc ctgaacctga
 1321 tgctgactag tgaggaaaga tttttgttgt tatttcttac aggcacaaaa tgatggacct
 55 1381 aatgcacaca aaacaacct agagtgttgt tgagaattgt gctcaaaatt tgaagaatga
 1441 acaaattgaa ctctttgaat gacaaagagt agacatttct ctactgcaa atgtcatcag
 1501 aactttttgg tttgcagatg acaaaaattc aactcagact agtttagtta aatgagggtg
 1561 gtgaatattg ttcatattgt ggcacaagca aaagggtgtc tgagccctca aagtgagggg
 1621 aaaccagggc ctgagccaag ctagaattcc ctctctctga ctctcaaatc ttttagtcat
 60 1681 tatagatccc ccgacttta catgacacag ctttatcacc agagagggac tgacacccat

	1741	gtttctctg	ccccaaagggc	aaaattccca	gggaagtgt	ctgataggcc	aagtttgtat
	1801	caggtgccc	tccctggaag	gtgctgttat	ccatggggaa	gggatata	agatggaagc
	1861	ttccagtcca	atctcatgga	gaagcagaaa	tacataatttc	caagaagttg	gatgggtggg
	1921	tactattctg	attacacaaa	acaaatgcc	cacatcaccc	ttaccatgtg	cctgatccag
5	1981	cctctccct	gattacacca	gcctcgtctt	cattaagccc	tcttccatca	tgtccccaaa
	2041	cctgcaaggg	ctccccactg	cctactgcat	cgagtcaaaa	ctcaaagtgt	tggcttctca
	2101	tacgtccacc	atgggggtcct	accaatagat	tccccattgc	ctcctccttc	ccaaaggact
	2161	ccacccatcc	tatcagcctg	tctcttccat	atgacctcat	gcatctccac	ctgctcccag
10	2221	gccagtaagg	gaaatagaaa	aaccctgccc	ccaaataaga	agggatggat	tccaaccca
	2281	actccagtag	cttgggacaa	atcaagcttc	agtttcctg	tctgtagaag	agggataagg
	2341	tacctttcac	atagagatca	tcctttccag	catgaggaac	tagccaccaa	ctcttgagg
	2401	tctcaaccct	tttgtctgcc	tcttagactt	ctgctttcca	cacctggcac	tgtgtgtctg
	2461	tgcccaagtt	gtgggtgctga	caaagcttgg	aagagcctgc	aggtgctgct	gcgtggcata
	2521	gccagacac	agaagaggct	ggttcttacg	atggcaccca	gtgagcactc	ccaagtctac
15	2581	agagtgatag	ccttccgtaa	cccaactctc	ctggactgcc	ttgaatatcc	cctcccagtc
	2641	accttggtggc	aagcccctgc	ccatctggga	aaatacccca	tcattcatgc	tactgccaac
	2701	ctggggagcc	agggctatgg	gagcagcttt	tttttcccc	ctagaaacgt	ttggaacaat
	2761	ctaaaagttt	aaagctcgaa	aacaattgta	ataatgctaa	agaaaaagtc	atccaactca
	2821	accacatcaa	tattgtcatt	cctgtattca	ccgtccaga	ccttggtcac	actctcacat
20	2881	gttttagagtt	gcaatcgtaa	tgtcacagatg	gttttataat	ctgatttgtt	ttcctcttaa
	2941	cgtttagacca	caaatagtgc	tcgctttcta	tgtagtgttg	taattatcat	tttagaagac
	3001	tctaccagac	tgtgtattca	ttgaagtcag	atgtggtaac	tgttaaattg	ctgtgtatct
	3061	gatagctctt	tggcagtcta	tatgtttgta	taatgaatga	gagaataagt	catgttctct
	3121	caagatcatg	tacccaatt	tacttgccat	tactcaattg	ataaacattt	aacttgtttc
25	3181	caatgttttag	caaatacata	ttttatagaa	cttccaaaaa	aaaa	

SEQ ID NO: 5 Human CX3CR1 isoform b variant 3 cDNA Acid Sequence
(NM_001337.3)

	1	gaaatactcg	tctctggtaa	agtctgagca	ggacagggtg	gctgactggc	agatccagag
30	61	gttcccttgg	cagtccacgc	caggccttca	ccatggatca	gttccctgaa	tcagtgcacag
	121	aaaactttga	gtacgatgat	ttggctgagg	cctgttatat	tggggacatc	gtgggtctttg
	181	ggactgtgtt	cctgtccata	ttctactccg	tcactcttgc	cattggcctg	gtgggaaatt
	241	tgttggtagt	gtttgccctc	accaacagca	agaagcccaa	gagtgtcacc	gacatttacc
	301	tcctgaacct	ggccttgtct	gatctgctgt	ttgtagccac	tttgcccttc	tggactcact
35	361	atltgataaa	tgaaaagggc	ctccacaatg	ccatgtgcaa	attcactacc	gccttcttct
	421	tcacggtctt	ttttggaagc	atattcttca	tcaccgtcat	cagcattgat	aggtacctgg
	481	ccatcgctct	ggccgccaac	tcctgaaca	accggaccgt	gcagcatggc	gtcaccatca
	541	gcctaggcgt	ctgggcagca	gccattttgg	tggcagcacc	ccagttcatg	ttcaciaaagc
	601	agaaagaaaa	tgaatgcctt	ggtgactacc	ccgaggtcct	ccaggaaatc	tggcccgtgc
40	661	tcgcgaatgt	ggaaacaaat	tttcttggct	tcctactccc	cctgctcatt	atgagttatt
	721	gtactttcag	aatcatccag	acgctgtttt	cctgcaagaa	ccacaagaaa	gccaaagcca
	781	ttaaactgat	ccttctgggtg	gtcatcgtgt	ttttcctctt	ctggacaccc	tacaacgtta
	841	tgattttcct	ggagacgctt	aagctctatg	acttctttcc	cagttgtgac	atgaggaagg
	901	atctgaggct	ggccctcagt	gtgactgaga	cggttgcat	tagccattgt	tgcctgaatc
45	961	ctctcatcta	tgcatttgc	ggggagaagt	tcagaagata	cctttaccac	ctgtatggga
	1021	aatgcctggc	tgtcctgtgt	gggcgtcag	tccacgttga	tttctcctca	tctgaatcac
	1081	aaaggagcag	gcatggaagt	gttctgagca	gcaattttac	ttaccacacg	agtgatggag
	1141	atgcattgct	ccttctctga	agggaatccc	aaagccttgt	gtctacagag	aacctggagt
	1201	tcctgaacct	gatgctgact	agtgaggaaa	gatttttgtt	gttatttctt	acaggcacaa
50	1261	aatgatggac	ccaatgcaca	caaaacaacc	ctagagtgtt	gttgagaatt	gtgctcaaaa
	1321	tttgaagaat	gaacaaattg	aactctttga	atgacaaaga	gtagacattt	ctcttactgc
	1381	aaatgtcatc	agaacttttt	ggtttgacga	tgacaaaaat	tcaactcaga	ctagttagt
	1441	taaatgaggg	tggatgaat	tgttcataat	gtggcacaag	caaaaggggtg	tctgagccct
	1501	caaagtgagg	ggaaaccagg	gcctgagcca	agctagaatt	ccctctctct	gactctcaaa
55	1561	tcttttagtc	attatagatc	cccagactt	tacatgacac	agctttatca	ccagagaggg
	1621	actgacaccc	atgtttctct	ggccccaagg	gcaaaattcc	cagggaagtg	ctctgatagg
	1681	ccaagtttgt	atcaggtgcc	catccctgga	aggtgctgtt	atccatgggg	aaggatata
	1741	taagatggaa	gcttccagtc	caatctcatg	gagaagcaga	aatacatatt	tccaagaagt
	1801	tggatgggtg	ggtactattc	tgattacaca	aaacaaatgc	cacacatcac	ccttaccatg
60	1861	tgcctgatcc	agcctctccc	ctgattacac	cagcctcgtc	ttcattaagc	cctcttccat

1921 catgtcccca aacctgcaag ggctccccac tgcctactgc atcgagtcaa aactcaaagt
 1981 cttggcttct catacgcca ccatgggggc ctaccaatag attccccatt gcctcctcct
 2041 tcccaaagga ctccacccat cctatcagcc tgtctcttcc atatgacctc atgcatctcc
 2101 acctgctccc aggccagtaa gggaaataga aaaaccctgc ccccaaataa gaagggatgg
 5 2161 attccaaccc caactccagt agcttgggac aaatcaagct tcagtttctt ggtctgtaga
 2221 agagggataa ggtacctttc acatagagat catcctttcc agcatgagga actagccacc
 2281 aactcttgca ggtctcaacc cttttgtctg cctcttagac ttctgctttc cacacctggc
 2341 actgctgtgc tgtgccaag ttgtggtgct gacaaagctt ggaagagcct gcaggtgctg
 2401 ctgctgggca tagccagac acagaagagg ctggttctta cgatggcacc cagtgcacac
 10 2461 tcccaagtct acagagtgat agccttccgt aaccctaactc tcctggactg ccttgaatat
 2521 cccctcccag tcacctgtg gcaagcccct gccatctgg gaaaataccc catcattcat
 2581 gctactgcca acctggggag ccagggctat gggagcagct ttttttccc ccctagaaac
 2641 gtttggaaca atctaaaagt ttaaagctcg aaaacaattg taataatgct aaagaaaaag
 2701 tcatccaatc taaccacatc aatattgtca ttctgtatt caccgtcca gacctgttc
 15 2761 aactctcac atgttttagag ttgcaatcgt aatgtacaga tggttttata atctgatttg
 2821 ttttctctt aacgttagac cacaatagt gctcgctttc tatgtagttt ggtaattatc
 2881 attttagaag actctaccag actgtgtatt cattgaagtc agatgtgga actgttaaat
 2941 tgctgtgtat ctgatatgctc ttggcagtc tataatggtt tataatgaat gagagaataa
 3001 gtcagtgttc ttcaagatca tgtaccccaa ttacttgcc attactaat tgataaacat
 20 3061 ttaacttggt tccaatggtt agcaaatata tattttatag aacttcca

SEQ ID NO: 6 Human CX3CR1 isoform b Amino Acid Sequence
(NP_001164643.1, NP_001164642.1, or NP_001328)

1 mdqfpesvte nfeyddlaea cyigdivvfg tvflsifysv ifaiglvgnl lvvfaltnsk
 25 61 kpksvtdiyl lnllalsdllf vatlpfwthy linekglna mckfttafff igffgsiffi
 121 tvisidryla ivlaansmn rtvqhgvts lgvwaaailv aapqfmftkq kenecldgyp
 181 evlqeiwpvl rnvetnflgf llpllimsyf yfrieqtlfs cknhkkakai klillvvivf
 241 flfwtpynvm ifletlklyd ffpscdmrkd lrlalsvtet vafshcclnp liyafagekf
 301 rrylyhlygk clavlclgrsv hvdfsssesq rsrhgsvlss nftyhtsdgd allll

SEQ ID NO: 7 Mouse CX3CR1 cDNA Acid Sequence (NM_009987)

1 tagagagggga agcacttgcc tctggtggag tctgctgag actgggtgag tgactggcac
 61 ttcttgcaga agttcccttc ccatctgctc aggacctcac catgtccacc tccttccctg
 121 aactggatct agagaatttt gagtatgacg attctgctga ggctgttat ttgggcgaca
 35 181 ttgtggcctt tggaaaccatc ttctgtccg tcttctacgc cctcgcttc acgttcggtc
 241 tgggtgggaaa tctgttggtg gtcctcgctc tcaccaacag ccggaagccc aagagcatca
 301 ctgacatcta cctcctgaac ctggccttga gcgacctgct ctttgtggcc acctgacct
 361 tctggactca ctacctcatc agccatgagg gcctccacaa tgccatgtgc aagctcacga
 421 ctgccttctt cttcattggc ttctttgggg gcatattctt catcacgctc atcagcatcg
 40 481 accggtacct tgccatgctc ctggccgcca actccatgaa caaccggaca gtgcagcacg
 541 gtgtcaccat tagtctgggc gtctgggcgg cggccatctt agtggcgta cccagttca
 601 tgttcacaaa gagaaaggac aacgagtgtc tgggtgacta ccccgaggct ctgcaggaaa
 661 tgtggcccggt gctccgcaac tcggaagtca acatcctggg cttcgccctg cccttgctta
 721 tcatgagctt ttgctacttc cgcacatccc agacgctgtt ttctgcaag aatcgcaaga
 45 781 aggcagagac cgtcagactc atcctcctgg tggcttttgc cttcttctc ttctggacac
 841 catacaacat catgattttc ctggagactc tcaagttcta caacttctc ccagttgtg
 901 acatgaagag ggacctgagg ttggccctca gtgtgacgga gacagtggcg ttccagccact
 961 gttgcctcaa cccctttatc tacgcctttg ccgggggaaaa gttcagaaga tacctgggac
 1021 acctgtatag gaagtgcctg gccgtcctgt gcggtcatcc tgtccacacc ggcttctcgc
 50 1081 cagagtccca gaggagcagg caggacagca ttctgagcag ttctactcac tacacaagcg
 1141 agggagatgg gtctctcctg ctctgaaggg gtctccccga ccctagctcc actaggaacc
 1201 cagagtctt gcacagatt tccttgcgcg tccccctgca tcttatgtgc aagaatatg
 1261 gaccagatgc ctgcaaacca accccgtggg gtttttttga aaaatttatg ttcaatgtgt
 1321 gaaaaacaca cgtatctctt actgcaaatg ttgaacattg gggcttactg gtgacaaaaa
 55 1381 ttctaaccag attagtgcga ttacaaaggg gtttggtgag tcctgggtgc atgatcatgt
 1441 gataaaggac aactaagtcc tcagactgag tggaaaccaa ggcttggctc caatgtcccc
 1501 tctctgacct tcagatcctt catagtgcga gatcatccag gttctatcat cagagaagga
 1561 ccacatctct ctgatttcaa aattggtatt cctaggggaa acctccgttg gccgagtgtg
 1621 tcgggtgtcc attccttgac taggtggtgt tattgaaata gaagggatac ctaagatgct
 60 1681 gttggaatct caagggttagc gggtgagaga gacacatctc tcagaagctg ggggggtggga

35

1 mstsfpeldl enfeyddsae acylgdivaf gtiflsvfya lvftfglvgn llv

40

50

	aac cag acg gac aag ctg gcc gcc ttc ccc gag gac cgc agc cag ccc	291
	Asn Gln Thr Asp Lys Leu Ala Ala Phe Pro Glu Asp Arg Ser Gln Pro	
	75 80 85	
5	ggc cag gac tgc cgc ttc cgt gtc aca caa ctg ccc aac ggg cgt gac	339
	Gly Gln Asp Cys Arg Phe Arg Val Thr Gln Leu Pro Asn Gly Arg Asp	
	90 95 100 105	
	ttc cac atg agc gtg gtc agg gcc cgg cgc aat gac agc ggc acc tac	387
	Phe His Met Ser Val Val Arg Ala Arg Arg Asn Asp Ser Gly Thr Tyr	
	110 115 120	
10	ctc tgt ggg gcc atc tcc ctg gcc ccc aag gcg cag atc aaa gag agc	435
	Leu Cys Gly Ala Ile Ser Leu Ala Pro Lys Ala Gln Ile Lys Glu Ser	
	125 130 135	
	ctg cgg gca gag ctc agg gtg aca gag aga agg gca gaa gtg ccc aca	483
	Leu Arg Ala Glu Leu Arg Val Thr Glu Arg Arg Ala Glu Val Pro Thr	
15	140 145 150	
	gcc cac ccc agc ccc tca ccc agg tca gcc ggc cag ttc caa acc ctg	531
	Ala His Pro Ser Pro Ser Pro Arg Ser Ala Gly Gln Phe Gln Thr Leu	
	155 160 165	
20	gtg gtt ggt gtc gtg ggc ggc ctg ctg ggc agc ctg gtg ctg cta gtc	579
	Val Val Gly Val Val Gly Gly Leu Leu Gly Ser Leu Val Leu Leu Val	
	170 175 180 185	
	tgg gtc ctg gcc gtc atc tgc tcc cgg gcc gca cga ggg aca ata gga	627
	Trp Val Leu Ala Val Ile Cys Ser Arg Ala Ala Arg Gly Thr Ile Gly	
	190 195 200	
25	gcc agg cgc acc ggc cag ccc ctg aag gag gac ccc tca gcc gtg cct	675
	Ala Arg Arg Thr Gly Gln Pro Leu Lys Glu Asp Pro Ser Ala Val Pro	
	205 210 215	
	gtg ttc tct gtg gac tat ggg gag ctg gat ttc cag tgg cga gag aag	723
	Val Phe Ser Val Asp Tyr Gly Glu Leu Asp Phe Gln Trp Arg Glu Lys	
30	220 225 230	
	acc ccg gag ccc ccc gtg ccc tgt gtc cct gag cag acg gag tat gcc	771
	Thr Pro Glu Pro Pro Val Pro Cys Val Pro Glu Gln Thr Glu Tyr Ala	
	235 240 245	
35	acc att gtc ttt cct agc gga atg ggc acc tca tcc ccc gcc cgc agg	819
	Thr Ile Val Phe Pro Ser Gly Met Gly Thr Ser Ser Pro Ala Arg Arg	
	250 255 260 265	
	ggc tca gct gac ggc cct cgg agt gcc cag cca ctg agg cct gag gat	867
	Gly Ser Ala Asp Gly Pro Arg Ser Ala Gln Pro Leu Arg Pro Glu Asp	
	270 275 280	
40	gga cac tgc tct tgg ccc ctc tgaccggcctt ccttggccac cagtgttctg cag	921
	Gly His Cys Ser Trp Pro Leu	
	285	

SEQ ID NO: 10 Human PD-1 Amino Acid Sequence

45	Met Gln Ile Pro Gln Ala Pro Trp Pro Val Val Trp Ala Val Leu Gln	
	1 5 10 15	
	Leu Gly Trp Arg Pro Gly Trp Phe Leu Asp Ser Pro Asp Arg Pro Trp	
	20 25 30	
	Asn Pro Pro Thr Phe Ser Pro Ala Leu Leu Val Val Thr Glu Gly Asp	
50	35 40 45	
	Asn Ala Thr Phe Thr Cys Ser Phe Ser Asn Thr Ser Glu Ser Phe Val	
	50 55 60	
	Leu Asn Trp Tyr Arg Met Ser Pro Ser Asn Gln Thr Asp Lys Leu Ala	
	65 70 75 80	
55	Ala Phe Pro Glu Asp Arg Ser Gln Pro Gly Gln Asp Cys Arg Phe Arg	
	85 90 95	
	Val Thr Gln Leu Pro Asn Gly Arg Asp Phe His Met Ser Val Val Arg	
	100 105 110	
	Ala Arg Arg Asn Asp Ser Gly Thr Tyr Leu Cys Gly Ala Ile Ser Leu	
60	115 120 125	
	Ala Pro Lys Ala Gln Ile Lys Glu Ser Leu Arg Ala Glu Leu Arg Val	

		130				135				140					
		Thr	Glu	Arg	Arg	Ala	Glu	Val	Pro	Thr	Ala	His	Pro	Ser	Pro
		145					150					155			160
5		Arg	Ser	Ala	Gly	Gln	Phe	Gln	Thr	Leu	Val	Val	Gly	Val	Gly
					165						170				175
		Leu	Leu	Gly	Ser	Leu	Val	Leu	Leu	Val	Trp	Val	Leu	Ala	Val
					180						185				190
		Ser	Arg	Ala	Ala	Arg	Gly	Thr	Ile	Gly	Ala	Arg	Arg	Thr	Gly
					195						200				205
10		Leu	Lys	Glu	Asp	Pro	Ser	Ala	Val	Pro	Val	Phe	Ser	Val	Asp
					210										220
		Glu	Leu	Asp	Phe	Gln	Trp	Arg	Glu	Lys	Thr	Pro	Glu	Pro	Pro
															240
		Cys	Val	Pro	Glu	Gln	Thr	Glu	Tyr	Ala	Thr	Ile	Val	Phe	Pro
15															255
		Met	Gly	Thr	Ser	Ser	Pro	Ala	Arg	Arg	Gly	Ser	Ala	Asp	Gly
															270
		Ser	Ala	Gln	Pro	Leu	Arg	Pro	Glu	Asp	Gly	His	Cys	Ser	Trp
					275										285
20															

SEQ ID NO: 11 Human PD-L1S cDNA Acid Sequence

		gcttccccgag gctccgcacc agccgcgctt ctgtccgcct gcagggcatt ccagaaag	58
		atg agg ata ttt gct gtc ttt ata ttc atg acc tac tgg cat ttg ctg	106
25		Met Arg Ile Phe Ala Val Phe Ile Phe Met Thr Tyr Trp His Leu Leu	
	1	5 10 15	
		aac gca ttt act gtc acg gtt ccc aag gac cta tat gtg gta gag tat	154
		Asn Ala Phe Thr Val Thr Val Pro Lys Asp Leu Tyr Val Val Glu Tyr	
		20 25 30	
30		ggg agc aat atg aca att gaa tgc aaa ttc cca gta gaa aaa caa tta	202
		Gly Ser Asn Met Thr Ile Glu Cys Lys Phe Pro Val Glu Lys Gln Leu	
		35 40 45	
		gac ctg gct gca cta att gtc tat tgg gaa atg gag gat aag aac att	250
		Asp Leu Ala Ala Leu Ile Val Tyr Trp Glu Met Glu Asp Lys Asn Ile	
		50 55 60	
35		att caa ttt gtg cat gga gag gaa gac ctg aag gtt cag cat agt agc	298
		Ile Gln Phe Val His Gly Glu Glu Asp Leu Lys Val Gln His Ser Ser	
		65 70 75 80	
		tac aga cag agg gcc cgg ctg ttg aag gac cag ctc tcc ctg gga aat	346
40		Tyr Arg Gln Arg Ala Arg Leu Leu Lys Asp Gln Leu Ser Leu Gly Asn	
		85 90 95	
		gct gca ctt cag atc aca gat gtg aaa ttg cag gat gca ggg gtg tac	394
		Ala Ala Leu Gln Ile Thr Asp Val Lys Leu Gln Asp Ala Gly Val Tyr	
		100 105 110	
45		cgc tgc atg atc agc tat ggt ggt gcc gac tac aag cga att act gtg	442
		Arg Cys Met Ile Ser Tyr Gly Gly Ala Asp Tyr Lys Arg Ile Thr Val	
		115 120 125	
		aaa gtc aat gcc cca tac aac aaa atc aac caa aga att ttg gtt gtg	490
		Lys Val Asn Ala Pro Tyr Asn Lys Ile Asn Gln Arg Ile Leu Val Val	
		130 135 140	
50		gat cca gtc acc tct gaa cat gaa ctg aca tgt cag gct gag ggc tac	538
		Asp Pro Val Thr Ser Glu His Glu Leu Thr Cys Gln Ala Glu Gly Tyr	
		145 150 155 160	
		ccc aag gcc gaa gtc atc tgg aca agc agt gac cat caa gtc ctg agt	586
55		Pro Lys Ala Glu Val Ile Trp Thr Ser Ser Asp His Gln Val Leu Ser	
		165 170 175	
		ggg aag acc acc acc acc aat tcc aag aga gag gag aag ctt ttc aat	634
		Gly Lys Thr Thr Thr Thr Asn Ser Lys Arg Glu Glu Lys Leu Phe Asn	
		180 185 190	
60		gtg acc agc aca ctg aga atc aac aca aca act aat gag att ttc tac	682
		Val Thr Ser Thr Leu Arg Ile Asn Thr Thr Thr Asn Glu Ile Phe Tyr	

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          195          200          205
tgc act ttt agg aga tta gat cct gag gaa aac cat aca gct gaa ttg 730
Cys Thr Phe Arg Arg Leu Asp Pro Glu Glu Asn His Thr Ala Glu Leu
          210          215          220
5  gtc atc cca ggt aat att ctg aat gtg tcc att aaa ata tgt cta aca 778
Val Ile Pro Gly Asn Ile Leu Asn Val Ser Ile Lys Ile Cys Leu Thr
          225          230          235          240
ctg tcc cct agc acc tagcatgatg tctgcctatc atagtcattc agtgattgtt 833
Leu Ser Pro Ser Thr
          245
10 gaataaatga atgaatgaat aacactatgt ttacaaaata taticctaatt cctcacctcc 893
attcatccaa accatattgt tacttaataa acattcagca gatattttatg gaataaaaaa 953
aaaaaaaaa aaaaa 968

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15 **SEQ ID NO: 12 Human PD-L1S Amino Acid Sequence**

```

Met Arg Ile Phe Ala Val Phe Ile Phe Met Thr Tyr Trp His Leu Leu
 1          5          10          15
Asn Ala Phe Thr Val Thr Val Pro Lys Asp Leu Tyr Val Val Glu Tyr
          20          25          30
20 Gly Ser Asn Met Thr Ile Glu Cys Lys Phe Pro Val Glu Lys Gln Leu
          35          40          45
Asp Leu Ala Ala Leu Ile Val Tyr Trp Glu Met Glu Asp Lys Asn Ile
          50          55          60
Ile Gln Phe Val His Gly Glu Glu Asp Leu Lys Val Gln His Ser Ser
25 65          70          75          80
Tyr Arg Gln Arg Ala Arg Leu Leu Lys Asp Gln Leu Ser Leu Gly Asn
          85          90          95
Ala Ala Leu Gln Ile Thr Asp Val Lys Leu Gln Asp Ala Gly Val Tyr
          100          105          110
30 Arg Cys Met Ile Ser Tyr Gly Gly Ala Asp Tyr Lys Arg Ile Thr Val
          115          120          125
Lys Val Asn Ala Pro Tyr Asn Lys Ile Asn Gln Arg Ile Leu Val Val
          130          135          140
Asp Pro Val Thr Ser Glu His Glu Leu Thr Cys Gln Ala Glu Gly Tyr
35 145          150          155          160
Pro Lys Ala Glu Val Ile Trp Thr Ser Ser Asp His Gln Val Leu Ser
          165          170          175
Gly Lys Thr Thr Thr Thr Asn Ser Lys Arg Glu Glu Lys Leu Phe Asn
          180          185          190
40 Val Thr Ser Thr Leu Arg Ile Asn Thr Thr Thr Asn Glu Ile Phe Tyr
          195          200          205
Cys Thr Phe Arg Arg Leu Asp Pro Glu Glu Asn His Thr Ala Glu Leu
          210          215          220
Val Ile Pro Gly Asn Ile Leu Asn Val Ser Ile Lys Ile Cys Leu Thr
45 225          230          235          240
Leu Ser Pro Ser Thr
          245

```

SEQ ID NO: 13 Human PD-L1M cDNA Acid Sequence

```

50 cgaggctccg caccagccgc gcttctgtcc gcctgcaggg cattccagaa agatgagg 58
Met Arg
1
ata ttt gct gtc ttt ata ttc atg acc tac tgg cat ttg ctg aac gca 106
Ile Phe Ala Val Phe Ile Phe Met Thr Tyr Trp His Leu Leu Asn Ala
5 5          10          15
55 ttt act gtc acg gtt ccc aag gac cta tat gtg gta gag tat ggt agc 154
Phe Thr Val Thr Val Pro Lys Asp Leu Tyr Val Val Glu Tyr Gly Ser
          20          25          30
aat atg aca att gaa tgc aaa ttc cca gta gaa aaa caa tta gac ctg 202

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	Asn	Met	Thr	Ile	Glu	Cys	Lys	Phe	Pro	Val	Glu	Lys	Gln	Leu	Asp	Leu	
	35					40					45					50	
	gct	gca	cta	att	gtc	tat	tgg	gaa	atg	gag	gat	aag	aac	att	att	caa	250
5	Ala	Ala	Leu	Ile	Val	Tyr	Trp	Glu	Met	Glu	Asp	Lys	Asn	Ile	Ile	Gln	
					55					60					65		
	ttt	gtg	cat	gga	gag	gaa	gac	ctg	aag	gtt	cag	cat	agt	agc	tac	aga	298
	Phe	Val	His	Gly	Glu	Glu	Asp	Leu	Lys	Val	Gln	His	Ser	Ser	Tyr	Arg	
					70				75					80			
10	cag	agg	gcc	cgg	ctg	ttg	aag	gac	cag	ctc	tcc	ctg	gga	aat	gct	gca	346
	Gln	Arg	Ala	Arg	Leu	Leu	Lys	Asp	Gln	Leu	Ser	Leu	Gly	Asn	Ala	Ala	
					85				90					95			
	ctt	cag	atc	aca	gat	gtg	aaa	ttg	cag	gat	gca	ggg	gtg	tac	cgc	tgc	394
	Leu	Gln	Ile	Thr	Asp	Val	Lys	Leu	Gln	Asp	Ala	Gly	Val	Tyr	Arg	Cys	
					100			105						110			
15	atg	atc	agc	tat	ggg	ggg	gcc	gac	tac	aag	cga	att	act	gtg	aaa	gtc	442
	Met	Ile	Ser	Tyr	Gly	Gly	Ala	Asp	Tyr	Lys	Arg	Ile	Thr	Val	Lys	Val	
					115			120						125		130	
	aat	gcc	cca	tac	aac	aaa	atc	aac	caa	aga	att	ttg	gtt	gtg	gat	cca	490
20	Asn	Ala	Pro	Tyr	Asn	Lys	Ile	Asn	Gln	Arg	Ile	Leu	Val	Val	Asp	Pro	
					135				140					145			
	gtc	acc	tct	gaa	cat	gaa	ctg	aca	tgt	cag	gct	gag	ggc	tac	ccc	aag	538
	Val	Thr	Ser	Glu	His	Glu	Leu	Thr	Cys	Gln	Ala	Glu	Gly	Tyr	Pro	Lys	
					150				155					160			
25	gcc	gaa	gtc	atc	tgg	aca	agc	agt	gac	cat	caa	gtc	ctg	agt	ggg	aag	586
	Ala	Glu	Val	Ile	Trp	Thr	Ser	Ser	Asp	His	Gln	Val	Leu	Ser	Gly	Lys	
					165			170						175			
	acc	acc	acc	acc	aat	tcc	aag	aga	gag	gag	aag	ctt	ttc	aat	gtg	acc	634
	Thr	Thr	Thr	Thr	Asn	Ser	Lys	Arg	Glu	Glu	Lys	Leu	Phe	Asn	Val	Thr	
					180			185						190			
30	agc	aca	ctg	aga	atc	aac	aca	aca	act	aat	gag	att	ttc	tac	tgc	act	682
	Ser	Thr	Leu	Arg	Ile	Asn	Thr	Thr	Thr	Asn	Glu	Ile	Phe	Tyr	Cys	Thr	
					195			200						205		210	
	ttt	agg	aga	tta	gat	cct	gag	gaa	aac	cat	aca	gct	gaa	ttg	gtc	atc	730
35	Phe	Arg	Arg	Leu	Asp	Pro	Glu	Glu	Asn	His	Thr	Ala	Glu	Leu	Val	Ile	
					215					220				225			
	cca	gaa	cta	cct	ctg	gca	cat	cct	cca	aat	gaa	agg	act	cac	ttg	gta	778
	Pro	Glu	Leu	Pro	Leu	Ala	His	Pro	Pro	Asn	Glu	Arg	Thr	His	Leu	Val	
					230				235					240			
40	att	ctg	gga	gcc	atc	tta	tta	tgc	ctt	ggg	gta	gca	ctg	aca	ttc	atc	826
	Ile	Leu	Gly	Ala	Ile	Leu	Leu	Cys	Leu	Gly	Val	Ala	Leu	Thr	Phe	Ile	
					245			250						255			
	ttc	cgt	tta	aga	aaa	ggg	aga	atg	atg	gat	gtg	aaa	aaa	tgt	ggc	atc	874
	Phe	Arg	Leu	Arg	Lys	Gly	Arg	Met	Met	Asp	Val	Lys	Lys	Cys	Gly	Ile	
					260			265						270			
45	caa	gat	aca	aac	tca	aag	aag	caa	agt	gat	aca	cat	ttg	gag	gag	acg	922
	Gln	Asp	Thr	Asn	Ser	Lys	Lys	Gln	Ser	Asp	Thr	His	Leu	Glu	Glu	Thr	
					275			280						285		290	
	taatccagca	ttggaacttc	tgatcttcaa	gcagggattc	tcaacctgtg	gtttaggggt	982										
	tcacggtggc	tgagcgtgac	aagaggaagg	aatgggccc	tggtgatgcag	gcaatgtggg	1042										
50	acttaaaagg	ccaagcact	gaaaatggaa	cctggcgaaa	gcagaggagg	agaatgaaga	1102										
	aagatggagt	caaacaggga	gcctggaggg	agaccttgat	actttcaaat	gcctgagggg	1162										
	ctcatcgacg	cctgtgacag	ggagaaagga	tacttctgaa	caaggagcct	ccaagcaaat	1222										
	catccattgc	tcacccctagc	aagacgggtt	gagaatccct	aatttgaggg	tcagttccctg	1282										
	cagaagtgcc	ctttgcctcc	actcaatgcc	tcaatttggt	ttctgcatga	ctgagagtct	1342										
55	cagtgttgga	acgggacagt	atztatgtat	gagtttttcc	tattttatttt	gagtcgtgta	1402										
	gggtcttcttg	tcattgtgagt	gtggttgtga	atgattttctt	ttgaagatat	attgtagtag	1462										
	atgtttacaat	tttgtcgcca	aactaaactt	gctgcttaat	gatttgctca	catctagtaa	1522										
	aacatggagt	atttgtaaaa	aaaaaaaaaa	a			1553										

SEQ ID NO: 14 Human PD-L1M Amino Acid Sequence

	Met	Arg	Ile	Phe	Ala	Val	Phe	Ile	Phe	Met	Thr	Tyr	Trp	His	Leu	Leu	
	1				5					10					15		
5	Asn	Ala	Phe	Thr	Val	Thr	Val	Pro	Lys	Asp	Leu	Tyr	Val	Val	Glu	Tyr	
				20					25					30			
	Gly	Ser	Asn	Met	Thr	Ile	Glu	Cys	Lys	Phe	Pro	Val	Glu	Lys	Gln	Leu	
				35				40					45				
	Asp	Leu	Ala	Ala	Leu	Ile	Val	Tyr	Trp	Glu	Met	Glu	Asp	Lys	Asn	Ile	
		50					55					60					
10	Ile	Gln	Phe	Val	His	Gly	Glu	Glu	Asp	Leu	Lys	Val	Gln	His	Ser	Ser	
	65					70					75					80	
	Tyr	Arg	Gln	Arg	Ala	Arg	Leu	Leu	Lys	Asp	Gln	Leu	Ser	Leu	Gly	Asn	
					85					90					95		
15	Ala	Ala	Leu	Gln	Ile	Thr	Asp	Val	Lys	Leu	Gln	Asp	Ala	Gly	Val	Tyr	
				100					105					110			
	Arg	Cys	Met	Ile	Ser	Tyr	Gly	Gly	Ala	Asp	Tyr	Lys	Arg	Ile	Thr	Val	
			115					120					125				
	Lys	Val	Asn	Ala	Pro	Tyr	Asn	Lys	Ile	Asn	Gln	Arg	Ile	Leu	Val	Val	
		130					135					140					
20	Asp	Pro	Val	Thr	Ser	Glu	His	Glu	Leu	Thr	Cys	Gln	Ala	Glu	Gly	Tyr	
						150					155					160	
	Pro	Lys	Ala	Glu	Val	Ile	Trp	Thr	Ser	Ser	Asp	His	Gln	Val	Leu	Ser	
					165					170					175		
25	Gly	Lys	Thr	Thr	Thr	Thr	Asn	Ser	Lys	Arg	Glu	Glu	Lys	Leu	Phe	Asn	
				180					185					190			
	Val	Thr	Ser	Thr	Leu	Arg	Ile	Asn	Thr	Thr	Thr	Asn	Glu	Ile	Phe	Tyr	
			195					200					205				
	Cys	Thr	Phe	Arg	Arg	Leu	Asp	Pro	Glu	Glu	Asn	His	Thr	Ala	Glu	Leu	
		210					215					220					
30	Val	Ile	Pro	Glu	Leu	Pro	Leu	Ala	His	Pro	Pro	Asn	Glu	Arg	Thr	His	
						230					235					240	
	Leu	Val	Ile	Leu	Gly	Ala	Ile	Leu	Leu	Cys	Leu	Gly	Val	Ala	Leu	Thr	
					245					250					255		
35	Phe	Ile	Phe	Arg	Leu	Arg	Lys	Gly	Arg	Met	Met	Asp	Val	Lys	Lys	Cys	
				260				265					270				
	Gly	Ile	Gln	Asp	Thr	Asn	Ser	Lys	Lys	Gln	Ser	Asp	Thr	His	Leu	Glu	
			275					280					285				
	Glu	Thr															
		290															

40 SEQ ID NO: 15 Human PD-L2 cDNA Acid Sequence

	atg	atc	ttc	ctc	ctg	cta	atg	ttg	agc	ctg	gaa	ttg	cag	ctt	cac	cag	48
	Met	Ile	Phe	Leu	Leu	Leu	Met	Leu	Ser	Leu	Glu	Leu	Gln	Leu	His	Gln	
	1				5					10					15		
45	ata	gca	gct	tta	ttc	aca	gtg	aca	gtc	cct	aag	gaa	ctg	tac	ata	ata	96
	Ile	Ala	Ala	Leu	Phe	Thr	Val	Thr	Val	Pro	Lys	Glu	Leu	Tyr	Ile	Ile	
				20					25					30			
	gag	cat	ggc	agc	aat	gtg	acc	ctg	gaa	tgc	aac	ttt	gac	act	gga	agt	144
	Glu	His	Gly	Ser	Asn	Val	Thr	Leu	Glu	Cys	Asn	Phe	Asp	Thr	Gly	Ser	
			35					40					45				
50	cat	gtg	aac	ctt	gga	gca	ata	aca	gcc	agt	ttg	caa	aag	gtg	gaa	aat	192
	His	Val	Asn	Leu	Gly	Ala	Ile	Thr	Ala	Ser	Leu	Gln	Lys	Val	Glu	Asn	
		50					55					60					
	gat	aca	tcc	cca	cac	cgt	gaa	aga	gcc	act	ttg	ctg	gag	gag	cag	ctg	240
	Asp	Thr	Ser	Pro	His	Arg	Glu	Arg	Ala	Thr	Leu	Leu	Glu	Glu	Gln	Leu	
55						70					75					80	
	ccc	cta	ggg	aag	gcc	tcg	ttc	cac	ata	cct	caa	gtc	caa	gtg	agg	gac	288
	Pro	Leu	Gly	Lys	Ala	Ser	Phe	His	Ile	Pro	Gln	Val	Gln	Val	Arg	Asp	
					85					90					95		
	gaa	gga	cag	tac	caa	tgc	ata	atc	atc	tat	ggg	gtc	gcc	tgg	gac	tac	336

	Glu	Gly	Gln	Tyr	Gln	Cys	Ile	Ile	Ile	Tyr	Gly	Val	Ala	Trp	Asp	Tyr	
			100					105						110			
	aag	tac	ctg	act	ctg	aaa	gtc	aaa	gct	tcc	tac	agg	aaa	ata	aac	act	384
5	Lys	Tyr	Leu	Thr	Leu	Lys	Val	Lys	Ala	Ser	Tyr	Arg	Lys	Ile	Asn	Thr	
			115					120						125			
	cac	atc	cta	aag	gtt	cca	gaa	aca	gat	gag	gta	gag	ctc	acc	tgc	cag	432
	His	Ile	Leu	Lys	Val	Pro	Glu	Thr	Asp	Glu	Val	Glu	Leu	Thr	Cys	Gln	
			130					135						140			
10	gct	aca	ggg	tat	cct	ctg	gca	gaa	gta	tcc	tgg	cca	aac	gtc	agc	gtt	480
	Ala	Thr	Gly	Tyr	Pro	Leu	Ala	Glu	Val	Ser	Trp	Pro	Asn	Val	Ser	Val	
			145					150						155		160	
	cct	gcc	aac	acc	agc	cac	tcc	agg	acc	cct	gaa	ggc	ctc	tac	cag	gtc	528
	Pro	Ala	Asn	Thr	Ser	His	Ser	Arg	Thr	Pro	Glu	Gly	Leu	Tyr	Gln	Val	
					165					170					175		
15	acc	agt	gtt	ctg	cgc	cta	aag	cca	ccc	cct	ggc	aga	aac	ttc	agc	tgt	576
	Thr	Ser	Val	Leu	Arg	Leu	Lys	Pro	Pro	Pro	Gly	Arg	Asn	Phe	Ser	Cys	
					180					185					190		
	gtg	ttc	tgg	aat	act	cac	gtg	agg	gaa	ctt	act	ttg	gcc	agc	att	gac	624
20	Val	Phe	Trp	Asn	Thr	His	Val	Arg	Glu	Leu	Thr	Leu	Ala	Ser	Ile	Asp	
			195					200						205			
	ctt	caa	agt	cag	atg	gaa	ccc	agg	acc	cat	cca	act	tgg	ctg	ctt	cac	672
	Leu	Gln	Ser	Gln	Met	Glu	Pro	Arg	Thr	His	Pro	Thr	Trp	Leu	Leu	His	
			210					215						220			
25	att	ttc	atc	ccc	tcc	tgc	atc	att	gct	ttc	att	ttc	ata	gcc	aca	gtg	720
	Ile	Phe	Ile	Pro	Ser	Cys	Ile	Ile	Ala	Phe	Ile	Phe	Ile	Ala	Thr	Val	
						230				235						240	
	ata	gcc	cta	aga	aaa	caa	ctc	tgt	caa	aag	ctg	tat	tct	tca	aaa	gac	768
	Ile	Ala	Leu	Arg	Lys	Gln	Leu	Cys	Gln	Lys	Leu	Tyr	Ser	Ser	Lys	Asp	
					245					250					255		
30	aca	aca	aaa	aga	cct	gtc	acc	aca	aca	aag	agg	gaa	gtg	aac	agt	gct	816
	Thr	Thr	Lys	Arg	Pro	Val	Thr	Thr	Thr	Lys	Arg	Glu	Val	Asn	Ser	Ala	
				260						265					270		
	atc																819
	Ile																
35																	

SEQ ID NO: 16 Human PD-L2 Amino Acid Sequence

	Met	Ile	Phe	Leu	Leu	Leu	Met	Leu	Ser	Leu	Glu	Leu	Gln	Leu	His	Gln	
	1			5						10					15		
40	Ile	Ala	Ala	Leu	Phe	Thr	Val	Thr	Val	Pro	Lys	Glu	Leu	Tyr	Ile	Ile	
				20					25					30			
	Glu	His	Gly	Ser	Asn	Val	Thr	Leu	Glu	Cys	Asn	Phe	Asp	Thr	Gly	Ser	
			35					40					45				
	His	Val	Asn	Leu	Gly	Ala	Ile	Thr	Ala	Ser	Leu	Gln	Lys	Val	Glu	Asn	
			50				55					60					
45	Asp	Thr	Ser	Pro	His	Arg	Glu	Arg	Ala	Thr	Leu	Leu	Glu	Glu	Gln	Leu	
						70					75					80	
	Pro	Leu	Gly	Lys	Ala	Ser	Phe	His	Ile	Pro	Gln	Val	Gln	Val	Arg	Asp	
					85					90					95		
50	Glu	Gly	Gln	Tyr	Gln	Cys	Ile	Ile	Ile	Tyr	Gly	Val	Ala	Trp	Asp	Tyr	
			100						105					110			
	Lys	Tyr	Leu	Thr	Leu	Lys	Val	Lys	Ala	Ser	Tyr	Arg	Lys	Ile	Asn	Thr	
			115					120						125			
	His	Ile	Leu	Lys	Val	Pro	Glu	Thr	Asp	Glu	Val	Glu	Leu	Thr	Cys	Gln	
			130					135					140				
55	Ala	Thr	Gly	Tyr	Pro	Leu	Ala	Glu	Val	Ser	Trp	Pro	Asn	Val	Ser	Val	
						150					155					160	
	Pro	Ala	Asn	Thr	Ser	His	Ser	Arg	Thr	Pro	Glu	Gly	Leu	Tyr	Gln	Val	
					165					170					175		
	Thr	Ser	Val	Leu	Arg	Leu	Lys	Pro	Pro	Pro	Gly	Arg	Asn	Phe	Ser	Cys	
60				180					185					190			
	Val	Phe	Trp	Asn	Thr	His	Val	Arg	Glu	Leu	Thr	Leu	Ala	Ser	Ile	Asp	

		195					200					205				
	Leu	Gln	Ser	Gln	Met	Glu	Pro	Arg	Thr	His	Pro	Thr	Trp	Leu	Leu	His
		210					215					220				
	Ile	Phe	Ile	Pro	Ser	Cys	Ile	Ile	Ala	Phe	Ile	Phe	Ile	Ala	Thr	Val
5	225					230					235					240
	Ile	Ala	Leu	Arg	Lys	Gln	Leu	Cys	Gln	Lys	Leu	Tyr	Ser	Ser	Lys	Asp
					245					250					255	
	Thr	Thr	Lys	Arg	Pro	Val	Thr	Thr	Thr	Lys	Arg	Glu	Val	Asn	Ser	Ala
				260					265					270		
10	Ile															

SEQ ID NO: 17 Human TIM-3 cDNA Sequence

1 atgtttttcac atcttccctt tgactgtgtc ctgctgctgc tgctgctact acttacaagg
61 tcctcagaag tggaatacag agcggagggtc ggtcagaatg cctatctgcc ctgcttctac
15 121 accccagccg cccaggggaa cctcgtgccc gtctgctggg gcaaaggagc ctgtcctgtg
181 tttgaatgtg gcaacgtggg gctcaggact gatgaaagg atgtgaatta ttggacatcc
241 agatactggc taaatgggga tttccgcaaa ggagatgtgt cctgaccat agagaatgtg
301 actctagcag acagtgggat ctactgctgc cggatccaaa tcccaggcat aatgaatgat
361 gaaaaattta acctgaagtt ggcatcaaaa ccagccaagg tcaccctgc accgactcgg
20 421 cagagagact tactgcagc ctttccaagg atgcttacca ccaggggaca tggcccagca
481 gagacacaga cactggggag cctccctgat ataaatctaa cacaaatc cacttggcc
541 aatgagttac gggactctag attggccaat gacttacggg actctggagc aaccatcaga
601 ataggcatct acatcggagc agggatctgt gctgggctgg ctctggctct tatcttcggc
661 gctttaattt tcaaatggta ttctcatagc aaagagaaga tacagaattt aagcctcatc
25 721 tctttggcca acctccctcc ctcaggattg gcaaatgcag tagcagaggg aattcgctca
781 gaagaaaaca tctataccat tgaagagaac gtatatgaag tggaggagcc caatgagtat
841 tattgctatg tcagcagcag gcagcaaccc tcacaacctt tgggttgctg ctttgcaatg
901 ccatag

SEQ ID NO: 18 Human TIM-3 Amino Acid Sequence

1 mfshlpfdcv lllllllltr sseveyraev gqnaylpcfy tpaapgnlvp vcwkggacpv
61 fecgnvvlrt derdvnwts rywlngdfrk gdvsltienv tladsgiycc riqipgimnd
121 ekfnlklvik pakvtpaptr qrdftaafpr mlttrghgpa etqtlgslpd inltqistla
181 nelrdsrlan dlrdsгатir igiyigagic aglallalifg alifkwysks kekiqnslsi
35 241 slanlppsgl anavaegirs eeniytieen vyeveepney ycyvssrqpp sqplgcrfam
301 p

SEQ ID NO: 19 Mouse TIM-3 cDNA Sequence

1 atgtttttcag gtcttaccct caactgtgtc ctgctgctgc tgcaactact acttgcaagg
40 61 tcattggaaa atgcttatgt gtttgagggt ggtaagaatg cctatctgcc ctgcagttac
121 actctatcta cacctggggc acttgctgct atgtgctggg gcaagggatt ctgtccttgg
181 tcacagtgtg ccaacgagtt gctcagaact gatgaaagaa atgtgacata tcagaaatcc
241 agcagatacc agctaaaggc cgatctcaac aaaggagacg tgtctctgat cataaagaat
301 gtgactctgg atgacatgg gacctactgc tgcaggatac agttccctgg tcttatgaat
45 361 gataaaaaat tagaactgaa attagacatc aaagcagcca aggtcactcc agctcagact
421 gccatgggg actctactac agcttctcca agaaccctaa ccacggagag aaatgggtca
481 gagacacaga cactgggtgac cctccataat aacaatggaa caaaaatttc cacatgggct

541 gatgaaatta aggactctgg agaaacgatc agaactgcta tccacattgg agtgggagtc
 601 tctgctgggt tgaccctggc acttatcatt ggtgtcttaa tccttaaagt gtattcctgt
 661 aagaaaaaga agttatcgag tttgagcctt attacactgg ccaacttgcc tccaggaggg
 721 ttggcaaatg caggagcagt caggattcgc tctgaggaaa atatctacac catcgaggag
 5 781 aacgtatatg aagtggagaa ttcaaatgag tactactgct acgtcaacag ccagcagcca
 841 tcctga

SEQ ID NO: 20 Mouse TIM-3 Amino Acid Sequence

1 mfsqgltnlcv llllqlllar slenayvfev gknaylpcsy tlstpgalvp mcwgkgfcpw
 10 61 sqctnellrt dernvtyqks sryqlkgdln kgdvsliikn vtlddhgtyc criqfpglmn
 121 dkklelkldi kaakvtpaqt ahgdsttasp rtltternngs etqtlvtlhn nngtkistwa
 181 deikdsgeti rtaihigvgv sagltlalii gvlilkwysc kkkklssls l itlanlppgg
 241 lanagavrir seeniytiee nvyevensne yycvnsqqp s

SEQ ID NO: 21 Human LAG-3 cDNA Sequence

1 atgtgggagg ctccagttcct gggcttgctg tttctgcagc cgctttgggt ggctccagtg
 61 aagcctctcc agccaggggc tgagggtccc gtggtgtggg cccaggaggg ggctcctgcc
 121 cagctccccct gcagccccac aatccccctc caggatctca gccttctgcg aagagcaggg
 181 gtcacttggc agcatcagcc agacagtggc ccgcccgcgt ccgcccccg ccacccccctg
 20 241 gcccccgcc ctcacccggc ggcgccctcc tctgggggc ccaggccccg ccgctacacg
 301 gtgctgagcg tgggtcccgg aggcctgcgc agcgggaggc tgccccctga gccccgcgtc
 361 cagctggatg agcgcggccg gcagcgcggg gacttctcgc tatggctgcg cccagccccg
 421 cgcgcggacg ccggcgagta ccgcgcgcgc gtgcacctca gggaccgcgc cctctcctgc
 481 cgcctccgtc tgcgcctggg ccaggcctcg atgactgcc accccccagg atctctcaga
 25 541 gcctccgact gggtcatttt gaactgctcc ttcagccgcc ctgaccgcc agcctctgtg
 601 cattggttcc ggaaccgggg ccaggggcca gtccctgtcc gggagtcccc ccatcaccac
 661 ttagcggaaa gcttcctctt cctgccccaa gtcagcccca tggactctgg gcctggggc
 721 tgcatcctca cctacagaga tggcttcaac gtctccatca tgtataacct cactgttctg
 781 ggtctggagc ccccaactcc cttgacagtg tacgctggag caggttccag ggtggggctg
 30 841 ccctgccgcc tgctgctgg tgtggggacc cggcttttcc tactgcca gtggactcct
 901 cctgggggag gccctgacct cctggtgact ggagacaatg gcgactttac ccttcgacta
 961 gaggatgtga gccaggccca ggctgggacc tacacctgcc atatccatct gcaggaacag
 1021 cagctcaatg ccaactgtac attggcaatc atcacagtga ctcccaaatc ctttgggtca
 1081 cctggatccc tggggaagct gctttgtgag gtgactccag tatctggaca agaacgcttt
 35 1141 gtgtggagct ctctggacac cccatcccag aggagtctt caggacctg gctggaggca
 1201 caggaggccc agctcctttc ccagccttgg caatgccagc tgtaccaggg ggagaggctt
 1261 cttggagcag cagtgtactt cacagagctg tctagcccag gtgcccacg ctctgggaga
 1321 gccccagggt ccctcccagc aggccacctc ctgctgtttc tcatccttgg tgccttttct
 1381 ctgctccttt tgggtgactgg agccttttgg tttcaccttt ggagaagaca gtggcgacca
 40 1441 agacgatttt ctgccttaga gcaagggatt caccctccgc aggctcagag caagatagag
 1501 gagctggagc aagaaccgga gccggagccg gagccggaac cggagccccg gcccgagccc
 1561 gagccggagc agctctga

SEQ ID NO: 22 Human LAG-3 Amino Acid Sequence

1 mweaqflgll flqplwvapv kplqpgaevp vvwagegapa qlpcstipl qdlsllrrag
 61 vtqhqpdsg ppaaapghpl apgphpaaps swgprprryt vlsvgpgglr sgrlplqprv
 121 qldergrqrg dfslwlrpar radageyraa vhlrdralsc rlrlrlgqas mtasppgslr
 5 181 asdwvilncs fsrpdrrpasv hwfrnrgqgr vpvresphhh laesflflpq vspmdsgpwg
 241 ciltyrdgfn vsimynltvl glepptpltv yagagsrvgl pcrlpagvgt rsfltakwtp
 301 pgggpdllvt gdngdftlrl edvsqaqagt ytchihlqeq qlnatvtlai itvtpksfgs
 361 pgslgkllce vtpvsgqerf vwssldtspq rsfsgpwlea qeaqlsqpw qcqlyqgerl
 421 lgaavyftel sspgaqrsg rpgalpaghl llflilgvls llllvtgafg fhllwrrqwrp
 10 481 rrfsaleggi hppqaqskie eleqepepep epepepepep epeql

SEQ ID NO: 23 Mouse LAG-3 cDNA Sequence

1 atgagggagg acctgctcct tggctttttg cttctgggac tgctttggga agctccagtt
 61 gtgtcttcag ggcctgggaa agagctcccc gtggtgtggg cccaggaggg agctcccgtc
 15 121 catcttcctt gcagcctcaa atcccccaac ctggatccta actttctacg aagaggaggg
 181 gttatctggc aacatcaacc agacagtggc caaccactc ccatcccggc ccttgacctt
 241 caccagggga tgccctcgcc tagacaaccc gcaccgggtc gctacacggt gctgagcgtg
 301 gctccaggag gcctgcgcag cgggaggcag cccctgcac cccacgtgca gctggaggag
 361 cgcggcctcc agcgcgggga cttctctctg tgggtgcgcc cagctctgcg caccgatgcg
 20 421 ggcgagtacc acgccaccgt gcgcctcccg aaccgcgccc tctcctgcag tctccgctg
 481 cgcgtcggcc aggcctcgat gattgctagt ccctcaggag tcctcaagct gtctgattgg
 541 gtctctttga actgctcctt cagccgtcct gaccgcccag tctctgtgca ctggttccag
 601 ggccagaacc gaggctcgt ctacaactca ccgcgtcatt ttttagctga aactttcctg
 661 ttactgcccc aagtcagccc cctggactct gggacctggg gctgtgtcct cacctacaga
 25 721 gatggcttca atgtctccat cacgtacaac ctcaaggttc tgggtctgga gcccgtagcc
 781 cctctgacag tgtacgtgc tgaaggttct aggggtggagc tgccctgtca tttgccccca
 841 ggagtgggga ccccttcttt gctcattgcc aagtggactc ctctggagg aggtcctgag
 901 ctccccgtgg ctggaaagag tggcaatttt acccttcacc ttgaggctgt gggctctggca
 961 caggctggga cctacacctg tagcatccat ctgcaggac agcagctcaa tgccactgtc
 30 1021 acgttggcgg tcatcacagt gactcccaa tcttctgggt tacctggctc ccgggggaag
 1081 ctgttgtgtg aggttaacccc ggcattctgga aaggaaagat ttgtgtggcg tcccctgaac
 1141 aatctgtcca ggagttgccc gggccctgtg ctggagattc aggaggccag gctccttgct
 1201 gagcgtatggc agtgtcagct gtacgagggc cagaggcttc ttggagcgac agtgtacgcc
 1261 gcagagtcta gctcaggcgc ccacagtgtc aggagaatct cagggtgacct taaaggaggc
 35 1321 catctcgttc tcgttctcat ccttggtgcc ctctccctgt tcttttgggt ggccggggcc
 1381 tttggctttc actggtggag aaaacagttg ctactgagaa gattttctgc cttagaacat
 1441 gggattcagc catttccggc tcagagggaag atagaggagc tggagcgaga actggagacg
 1501 gagatgggac aggagccgga gcccgagccg gagccacagc tggagccaga gccaggcag
 1561 ctctga

40

SEQ ID NO: 24 Mouse LAG-3 Amino Acid Sequence

1 mredlllgfl llgllweapv vssgpgkelp vvwagegapv hlpclkspn ldpnflrrgg
 61 viwqhqpdsq qptpipaldl hqgmprpqp apgrytvlsv apgglrsgrq plhphvqlee
 121 rglqrqdfsl wlrpalrtda geyhatvrlp nralscsrl rvqqasmias psgvlklsdw

181 vllncsfserp drpvsvhwf qgnrvpvyns prhflaetfl llpqvsplds gtwgcvltyr
 241 dgfnvsityn lkvlglepva pltvyaags rvelpchlp gvgtpsllia kwtppgggpe
 301 lpvagksgnf tlhleavgla qagtytcsih lggqqlnatv tlavitvtpk sfglpgsrgk
 361 llcevtpasg kerfvwrpln nlsrscpgpv leiqearlla erwqclyeg qrllgatvya
 5 421 aesssgahsa rrisgdlkkg hlvlvlilga lslfllvaga fgfhwwrkql llrrfsaleh
 481 giqpfpaqrk ieelerelet emgqepepep epqlepeprq l

* Included in Table 1 are RNA nucleic acid molecules (*e.g.*, thymines replaced with
 uredines), nucleic acid molecules encoding orthologs of the encoded proteins, as well as
 10 DNA or RNA nucleic acid sequences comprising a nucleic acid sequence having at least
 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%,
 95%, 96%, 97%, 98%, 99%, 99.5%, or more identity across their full length with the
 nucleic acid sequence of any SEQ ID NO listed in Table 1, or a portion thereof. Such
 nucleic acid molecules can have a function of the full-length nucleic acid as described
 15 further herein.

* Included in Table 1 are orthologs of the proteins, as well as polypeptide molecules
 comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%,
 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or more
 20 identity across their full length with an amino acid sequence of any SEQ ID NO listed in
 Table 1, or a portion thereof. Such polypeptides can have a function of the full-length
 polypeptide as described further herein.

Table 2 Anti-CX3CR1 Monoclonal Antibody Sequences

25 455.1C11 anti-CX3CR1 Variable Region Sequences

VH (Variable Heavy)

Amino Acid Sequence in FASTA format

30 > MHC2559HC.1

MELGLSWVFLVALLNGVQCQVQLVETGGGLVRPGNSLKLSCVTSGFTFSNYRMH
 WLRQFPGKRLEWIAVIAFKSDKFGANYAESVKGRFAISRDDSKNSVYLEMSRLRE
 EDTATYFCSRDGNPFDYWGGGTTTLTVSS

35 Nucleotide Sequence in FASTA format

> MHC2559HC.1

ATGGAGTTGGGACTGAGCTGGGTATTTCTTGTGGCTCTTTTGAACGGTGTCCA
 GTGTCAGGTGCAGCTTGTAGAGACCGGGGGAGGCTTGGTGAGGCCTGGAAA
 TTCTCTGAAACTCTCCTGTGTTACCTCGGGATTCACTTTCAGTAACTACCGGAT
 40 GCACTGGCTTCGCCAGTTTCCAGGGAAGAGGCTGGAGTGGATTGCTGTAATT
 GCATTCAAATCTGATAAATTTGGAGCAAATTATGCAGAGTCTGTGAAAGGCAG
 ATTCGCCATTTCAAGAGATGATTCAAAAAACAGTGTCTACCTAGAGATGAGCA

GATTAAGAGAGGAAGACACTGCCACCTATTTTTGTAGTAGAGATGGTAATGGG
CCCTTTGACTATTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

VL (Variable Light)

5 MHC2559LC.4

Amino Acid Sequence in FASTA format

> MHC2559LC.4

MDSQAQVLMLLLLWVSGTCGDIVMSQSPSSLTVSVGEKVTMSCKSSQSLLYSGN
QKSYLAWYQQKPGQSPRLLIYWASTRESGVPDRFTGSGSGTDFTLTISGVKAED
10 LAVYYCQRYYGYPRTFGGGTKLEIK

Nucleotide Sequence in FASTA format

> MHC2559LC.4

ATGGATTACAGGCCCCAGGTTCTTATGTTACTGCTGCTATGGGTATCTGGAAC
15 CTGTGGGGACATTGTGATGTCACAGTCACCGTCCTCCCTAACTGTGTCAGTTG
GAGAGAAGGTTACTATGAGCTGCAAGTCCAGTCAGAGCCTTTTATATAGTGGC
AATCAAAAGAGTTACTTGGCCTGGTACCAGCAGAAACCAGGGGCAGTCTCCCA
GACTGCTGATTTACTGGGCATCCACTAGGGAATCTGGGGTCCCTGATCGCTT
CACAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCGGTGTGAAG
20 GCTGAAGACCTGGCAGTTTATTACTGTCAGCGATATTATGGCTATCCTCGGAC
GTTCGGTGGAGGCACCAAACCTGGAAATCAAA

1C11 Heavy Chain

Region	Sequence Fragment	Residues	Length
Leader	MELGLSWFLVALLNGVQC	1 - 19	19
HFR1	QVQLVETGGGLVRPGNSLKLSCVTS	20 - 44	25
CDR-H1	GFTFSNY	45 - 51	7
HFR2	RMHWLRQFPGRLEWIAVI	52 - 70	19
CDR-H2	AFKSDKFG	71 - 78	8
HFR3	ANYAESVKGRFAISRDDSKNSVYLEMSRLREEDTATYFCSR	79 - 119	41
CDR-H3	DNGNPFDY	120 - 127	8
HFR4	WGQGTTLTVSS	128 - 138	11

1C11 Light Chain

Region	Sequence Fragment	Residues	Length
Leader	MDSQAQVLMLLLLWVSGTCG	1 - 20	20
LFR1	DIVMSQSPSSLTVSVGEKVTMSC	21 - 43	23
CDR-L1	KSSQSLLYSGNQKSYLA	44 - 60	17
LFR2	WYQQKPGQSPRLLIY	61 - 75	15
CDR-L2	WASTRES	76 - 82	7
LFR3	GVPDRFTGSGSGTDFTLTISGVKAEDLAVYYC	83 - 114	32
CDR-L3	QRYYGYPRT	115 - 123	9
LFR4	FGGGTKLEIK	124 - 133	10

25

II. Subjects

In one embodiment, the subject for whom predicted likelihood of efficacy of a CX3CR1 and an immune checkpoint inhibitor combination therapy is determined, is a mammal (*e.g.*, mouse, rat, primate, non-human mammal, domestic animal, such as a dog, cat, cow, horse, and the like), and is preferably a human. In another embodiment, the subject is an animal model of cancer. For example, the animal model can be an orthotopic xenograft animal model of a human-derived cancer.

In another embodiment of the methods of the present invention, the subject has not undergone treatment, such as chemotherapy, radiation therapy, targeted therapy, and/or anti-immune checkpoint therapy. In still another embodiment, the subject has undergone treatment, such as chemotherapy, radiation therapy, targeted therapy, and/or anti-immune checkpoint therapy.

In certain embodiments, the subject has had surgery to remove cancerous or precancerous tissue. In other embodiments, the cancerous tissue has not been removed, *e.g.*, the cancerous tissue may be located in an inoperable region of the body, such as in a tissue that is essential for life, or in a region where a surgical procedure would cause considerable risk of harm to the patient.

The methods of the present invention can be used to determine the responsiveness to CX3CR1 and immune checkpoint inhibitor combination therapies of many different cancers in subjects such as those described herein.

III. Sample Collection, Preparation and Separation

In some embodiments, biomarker amount and/or activity measurement(s) in a sample from a subject is compared to a predetermined control (standard) sample. The sample from the subject is typically from a diseased tissue, such as cancer cells or tissues. The control sample can be from the same subject or from a different subject. The control sample is typically a normal, non-diseased sample. However, in some embodiments, such as for staging of disease or for evaluating the efficacy of treatment, the control sample can be from a diseased tissue. The control sample can be a combination of samples from several different subjects. In some embodiments, the biomarker amount and/or activity measurement(s) from a subject is compared to a pre-determined level. This pre-determined level is typically obtained from normal samples. As described herein, a “pre-determined” biomarker amount and/or activity measurement(s) may be a biomarker amount and/or

activity measurement(s) used to, by way of example only, evaluate a subject that may be selected for treatment (*e.g.*, based on the number of genomic mutations and/or the number of genomic mutations causing non-functional proteins for DNA repair genes), evaluate a response to a CX3CR1 and an immune checkpoint combination inhibitor therapy, and/or
5 evaluate a response to a CX3CR1 and an immune checkpoint combination inhibitor therapy with one or more additional anti-cancer therapies. A pre-determined biomarker amount and/or activity measurement(s) may be determined in populations of patients with or without cancer. The pre-determined biomarker amount and/or activity measurement(s) can be a single number, equally applicable to every patient, or the pre-determined biomarker
10 amount and/or activity measurement(s) can vary according to specific subpopulations of patients. Age, weight, height, and other factors of a subject may affect the pre-determined biomarker amount and/or activity measurement(s) of the individual. Furthermore, the pre-determined biomarker amount and/or activity can be determined for each subject individually. In one embodiment, the amounts determined and/or compared in a method
15 described herein are based on absolute measurements.

In another embodiment, the amounts determined and/or compared in a method described herein are based on relative measurements, such as ratios (*e.g.*, biomarker copy numbers, level, and/or activity before a treatment vs. after a treatment, such biomarker measurements relative to a spiked or man-made control, such biomarker measurements
20 relative to the expression of a housekeeping gene, and the like). For example, the relative analysis can be based on the ratio of pre-treatment biomarker measurement as compared to post-treatment biomarker measurement. Pre-treatment biomarker measurement can be made at any time prior to initiation of anti-cancer therapy. Post-treatment biomarker measurement can be made at any time after initiation of anti-cancer therapy. In some
25 embodiments, post-treatment biomarker measurements are made 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 weeks or more after initiation of anti-cancer therapy, and even longer toward indefinitely for continued monitoring. Treatment can comprise anti-cancer therapy, such as a therapeutic regimen comprising one or more
CX3CR1/immune checkpoint combination inhibitors alone or in combination with other
30 anti-cancer agents, such as with immune checkpoint inhibitors.

The pre-determined biomarker amount and/or activity measurement(s) can be any suitable standard. For example, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from the same or a different human for whom a patient

selection is being assessed. In one embodiment, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from a previous assessment of the same patient. In such a manner, the progress of the selection of the patient can be monitored over time. In addition, the control can be obtained from an assessment of another human or multiple humans, *e.g.*, selected groups of humans, if the subject is a human. In such a manner, the extent of the selection of the human for whom selection is being assessed can be compared to suitable other humans, *e.g.*, other humans who are in a similar situation to the human of interest, such as those suffering from similar or the same condition(s) and/or of the same ethnic group.

In some embodiments of the present invention the change of biomarker amount and/or activity measurement(s) from the pre-determined level is about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 fold or greater, or any range in between, inclusive. Such cutoff values apply equally when the measurement is based on relative changes, such as based on the ratio of pre-treatment biomarker measurement as compared to post-treatment biomarker measurement.

Biological samples can be collected from a variety of sources from a patient including a body fluid sample, cell sample, or a tissue sample comprising nucleic acids and/or proteins. "Body fluids" refer to fluids that are excreted or secreted from the body as well as fluids that are normally not (*e.g.*, amniotic fluid, aqueous humor, bile, blood and blood plasma, cerebrospinal fluid, cerumen and earwax, cowper's fluid or pre-ejaculatory fluid, chyle, chyme, stool, female ejaculate, interstitial fluid, intracellular fluid, lymph, menses, breast milk, mucus, pleural fluid, pus, saliva, sebum, semen, serum, sweat, synovial fluid, tears, urine, vaginal lubrication, vitreous humor, vomit). In a preferred embodiment, the subject and/or control sample is selected from the group consisting of cells, cell lines, histological slides, paraffin embedded tissues, biopsies, whole blood, nipple aspirate, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow. In one embodiment, the sample is serum, plasma, or urine. In another embodiment, the sample is serum.

The samples can be collected from individuals repeatedly over a longitudinal period of time (*e.g.*, once or more on the order of days, weeks, months, annually, biannually, etc.). Obtaining numerous samples from an individual over a period of time can be used to verify results from earlier detections and/or to identify an alteration in biological pattern as a result of, for example, disease progression, drug treatment, *etc.* For example, subject samples can

be taken and monitored every month, every two months, or combinations of one, two, or three month intervals according to the present invention. In addition, the biomarker amount and/or activity measurements of the subject obtained over time can be conveniently compared with each other, as well as with those of normal controls during the monitoring period, thereby providing the subject's own values, as an internal, or personal, control for long-term monitoring.

Sample preparation and separation can involve any of the procedures, depending on the type of sample collected and/or analysis of biomarker measurement(s). Such procedures include, by way of example only, concentration, dilution, adjustment of pH, removal of high abundance polypeptides (*e.g.*, albumin, gamma globulin, and transferrin, etc.), addition of preservatives and calibrants, addition of protease inhibitors, addition of denaturants, desalting of samples, concentration of sample proteins, extraction and purification of lipids.

The sample preparation can also isolate molecules that are bound in non-covalent complexes to other protein (*e.g.*, carrier proteins). This process may isolate those molecules bound to a specific carrier protein (*e.g.*, albumin), or use a more general process, such as the release of bound molecules from all carrier proteins via protein denaturation, for example using an acid, followed by removal of the carrier proteins.

Removal of undesired proteins (*e.g.*, high abundance, uninformative, or undetectable proteins) from a sample can be achieved using high affinity reagents, high molecular weight filters, ultracentrifugation and/or electrodialysis. High affinity reagents include antibodies or other reagents (*e.g.*, aptamers) that selectively bind to high abundance proteins. Sample preparation could also include ion exchange chromatography, metal ion affinity chromatography, gel filtration, hydrophobic chromatography, chromatofocusing, adsorption chromatography, isoelectric focusing and related techniques. Molecular weight filters include membranes that separate molecules on the basis of size and molecular weight. Such filters may further employ reverse osmosis, nanofiltration, ultrafiltration and microfiltration.

Ultracentrifugation is a method for removing undesired polypeptides from a sample. Ultracentrifugation is the centrifugation of a sample at about 15,000-60,000 rpm while monitoring with an optical system the sedimentation (or lack thereof) of particles. Electrodialysis is a procedure which uses an electromembrane or semipermeable membrane in a process in which ions are transported through semi-permeable membranes from one

solution to another under the influence of a potential gradient. Since the membranes used in electrodialysis may have the ability to selectively transport ions having positive or negative charge, reject ions of the opposite charge, or to allow species to migrate through a semipermeable membrane based on size and charge, it renders electrodialysis useful for concentration, removal, or separation of electrolytes.

Separation and purification in the present invention may include any procedure known in the art, such as capillary electrophoresis (*e.g.*, in capillary or on-chip) or chromatography (*e.g.*, in capillary, column or on a chip). Electrophoresis is a method which can be used to separate ionic molecules under the influence of an electric field.

Electrophoresis can be conducted in a gel, capillary, or in a microchannel on a chip. Examples of gels used for electrophoresis include starch, acrylamide, polyethylene oxides, agarose, or combinations thereof. A gel can be modified by its cross-linking, addition of detergents, or denaturants, immobilization of enzymes or antibodies (affinity electrophoresis) or substrates (zymography) and incorporation of a pH gradient. Examples of capillaries used for electrophoresis include capillaries that interface with an electrospray.

Capillary electrophoresis (CE) is preferred for separating complex hydrophilic molecules and highly charged solutes. CE technology can also be implemented on microfluidic chips. Depending on the types of capillary and buffers used, CE can be further segmented into separation techniques such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), capillary isotachophoresis (cITP) and capillary electrochromatography (CEC). An embodiment to couple CE techniques to electrospray ionization involves the use of volatile solutions, for example, aqueous mixtures containing a volatile acid and/or base and an organic such as an alcohol or acetonitrile.

Capillary isotachophoresis (cITP) is a technique in which the analytes move through the capillary at a constant speed but are nevertheless separated by their respective mobilities. Capillary zone electrophoresis (CZE), also known as free-solution CE (FSCE), is based on differences in the electrophoretic mobility of the species, determined by the charge on the molecule, and the frictional resistance the molecule encounters during migration which is often directly proportional to the size of the molecule. Capillary isoelectric focusing (CIEF) allows weakly-ionizable amphoteric molecules, to be separated by electrophoresis in a pH gradient. CEC is a hybrid technique between traditional high performance liquid chromatography (HPLC) and CE.

Separation and purification techniques used in the present invention include any

chromatography procedures known in the art. Chromatography can be based on the differential adsorption and elution of certain analytes or partitioning of analytes between mobile and stationary phases. Different examples of chromatography include, but not limited to, liquid chromatography (LC), gas chromatography (GC), high performance liquid chromatography (HPLC), etc.

IV. Biomarker Nucleic Acids and Polypeptides

One aspect of the present invention pertains to the use of isolated nucleic acid molecules that correspond to biomarker nucleic acids that encode a biomarker polypeptide or a portion of such a polypeptide. As used herein, the term “nucleic acid molecule” is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An “isolated” nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. Preferably, an “isolated” nucleic acid molecule is free of sequences (preferably protein-encoding sequences) which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kB, 4 kB, 3 kB, 2 kB, 1 kB, 0.5 kB or 0.1 kB of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an “isolated” nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

A biomarker nucleic acid molecule of the present invention can be isolated using standard molecular biology techniques and the sequence information in the database records described herein. Using all or a portion of such nucleic acid sequences, nucleic acid molecules of the present invention can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook *et al.*, ed., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

A nucleic acid molecule of the present invention can be amplified using cDNA, mRNA, or genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid molecules so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

5 Furthermore, oligonucleotides corresponding to all or a portion of a nucleic acid molecule of the present invention can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

Moreover, a nucleic acid molecule of the present invention can comprise only a portion of a nucleic acid sequence, wherein the full length nucleic acid sequence comprises
10 a marker of the present invention or which encodes a polypeptide corresponding to a marker of the present invention. Such nucleic acid molecules can be used, for example, as a probe or primer. The probe/primer typically is used as one or more substantially purified oligonucleotides. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 7, preferably about 15, more
15 preferably about 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, or 400 or more consecutive nucleotides of a biomarker nucleic acid sequence. Probes based on the sequence of a biomarker nucleic acid molecule can be used to detect transcripts or genomic sequences corresponding to one or more markers of the present invention. The probe comprises a label group attached thereto, *e.g.*, a radioisotope, a fluorescent compound, an
20 enzyme, or an enzyme co-factor.

A biomarker nucleic acid molecules that differ, due to degeneracy of the genetic code, from the nucleotide sequence of nucleic acid molecules encoding a protein which corresponds to the biomarker, and thus encode the same protein, are also contemplated.

In addition, it will be appreciated by those skilled in the art that DNA sequence
25 polymorphisms that lead to changes in the amino acid sequence can exist within a population (*e.g.*, the human population). Such genetic polymorphisms can exist among individuals within a population due to natural allelic variation. An allele is one of a group of genes which occur alternatively at a given genetic locus. In addition, it will be appreciated that DNA polymorphisms that affect RNA expression levels can also exist that
30 may affect the overall expression level of that gene (*e.g.*, by affecting regulation or degradation).

The term “allele,” which is used interchangeably herein with “allelic variant,” refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position

on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for the gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene or allele. For example, biomarker alleles can differ from each other in a single nucleotide, or several nucleotides, and can include substitutions, deletions, and insertions of nucleotides. An allele of a gene can also be a form of a gene containing one or more mutations.

The term “allelic variant of a polymorphic region of gene” or “allelic variant”, used interchangeably herein, refers to an alternative form of a gene having one of several possible nucleotide sequences found in that region of the gene in the population. As used herein, allelic variant is meant to encompass functional allelic variants, non-functional allelic variants, SNPs, mutations and polymorphisms.

The term “single nucleotide polymorphism” (SNP) refers to a polymorphic site occupied by a single nucleotide, which is the site of variation between allelic sequences. The site is usually preceded by and followed by highly conserved sequences of the allele (*e.g.*, sequences that vary in less than 1/100 or 1/1000 members of a population). A SNP usually arises due to substitution of one nucleotide for another at the polymorphic site. SNPs can also arise from a deletion of a nucleotide or an insertion of a nucleotide relative to a reference allele. Typically the polymorphic site is occupied by a base other than the reference base. For example, where the reference allele contains the base “T” (thymidine) at the polymorphic site, the altered allele can contain a “C” (cytidine), “G” (guanine), or “A” (adenine) at the polymorphic site. SNP’s may occur in protein-coding nucleic acid sequences, in which case they may give rise to a defective or otherwise variant protein, or genetic disease. Such a SNP may alter the coding sequence of the gene and therefore specify another amino acid (a “missense” SNP) or a SNP may introduce a stop codon (a “nonsense” SNP). When a SNP does not alter the amino acid sequence of a protein, the SNP is called “silent.” SNP’s may also occur in noncoding regions of the nucleotide sequence. This may result in defective protein expression, *e.g.*, as a result of alternative splicing, or it may have no effect on the function of the protein.

As used herein, the terms “gene” and “recombinant gene” refer to nucleic acid molecules comprising an open reading frame encoding a polypeptide corresponding to a marker of the present invention. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of a given gene. Alternative alleles can be identified by sequencing the gene of interest in a number of different individuals. This can be readily

carried out by using hybridization probes to identify the same genetic locus in a variety of individuals. Any and all such nucleotide variations and resulting amino acid polymorphisms or variations that are the result of natural allelic variation and that do not alter the functional activity are intended to be within the scope of the present invention.

5 In another embodiment, a biomarker nucleic acid molecule is at least 7, 15, 20, 25, 30, 40, 60, 80, 100, 150, 200, 250, 300, 350, 400, 450, 550, 650, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2200, 2400, 2600, 2800, 3000, 3500, 4000, 4500, or more nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule corresponding to a marker of the present invention or to a nucleic
10 acid molecule encoding a protein corresponding to a marker of the present invention. As used herein, the term “hybridizes under stringent conditions” is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% (65%, 70%, 75%, 80%, preferably 85%) identical to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be
15 found in sections 6.3.1-6.3.6 of *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989). A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 50-65°C.

In addition to naturally-occurring allelic variants of a nucleic acid molecule of the
20 present invention that can exist in the population, the skilled artisan will further appreciate that sequence changes can be introduced by mutation thereby leading to changes in the amino acid sequence of the encoded protein, without altering the biological activity of the protein encoded thereby. For example, one can make nucleotide substitutions leading to amino acid substitutions at “non-essential” amino acid residues. A “non-essential” amino
25 acid residue is a residue that can be altered from the wild-type sequence without altering the biological activity, whereas an “essential” amino acid residue is required for biological activity. For example, amino acid residues that are not conserved or only semi-conserved among homologs of various species may be non-essential for activity and thus would be likely targets for alteration. Alternatively, amino acid residues that are conserved among
30 the homologs of various species (*e.g.*, murine and human) may be essential for activity and thus would not be likely targets for alteration.

Accordingly, another aspect of the present invention pertains to nucleic acid molecules encoding a polypeptide of the present invention that contain changes in amino

acid residues that are not essential for activity. Such polypeptides differ in amino acid sequence from the naturally-occurring proteins which correspond to the markers of the present invention, yet retain biological activity. In one embodiment, a biomarker protein has an amino acid sequence that is at least about 40% identical, 50%, 60%, 70%, 75%,
5 80%, 83%, 85%, 87.5%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or identical to the amino acid sequence of a biomarker protein described herein.

An isolated nucleic acid molecule encoding a variant protein can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of nucleic acids of the present invention, such that one or more amino acid residue
10 substitutions, additions, or deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar
15 side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), non-polar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine,
20 tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed
25 recombinantly and the activity of the protein can be determined.

In some embodiments, the present invention further contemplates the use of anti-biomarker antisense nucleic acid molecules, *i.e.*, molecules which are complementary to a sense nucleic acid of the present invention, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule corresponding to a marker of the present invention or
30 complementary to an mRNA sequence corresponding to a marker of the present invention. Accordingly, an antisense nucleic acid molecule of the present invention can hydrogen bond to (*i.e.* anneal with) a sense nucleic acid of the present invention. The antisense nucleic acid can be complementary to an entire coding strand, or to only a portion thereof,

e.g., all or part of the protein coding region (or open reading frame). An antisense nucleic acid molecule can also be antisense to all or part of a non-coding region of the coding strand of a nucleotide sequence encoding a polypeptide of the present invention. The non-coding regions (“5' and 3' untranslated regions”) are the 5' and 3' sequences which flank the coding region and are not translated into amino acids.

An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides in length. An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (*v*), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (*v*), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)*w*, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been sub-cloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the present invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a polypeptide corresponding to a selected marker of the present invention to thereby inhibit expression of the marker, *e.g.*, by

inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. Examples of a route of administration of antisense nucleic acid molecules of the present invention includes direct injection at a tissue site or infusion of the antisense nucleic acid into a blood- or bone marrow-associated body fluid. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

An antisense nucleic acid molecule of the present invention can be an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual α -units, the strands run parallel to each other (Gaultier *et al.*, 1987, *Nucleic Acids Res.* 15:6625-6641).

The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.*, 1987, *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.*, 1987, *FEBS Lett.* 215:327-330).

The present invention also encompasses ribozymes. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes as described in Haselhoff and Gerlach, 1988, *Nature* 334:585-591) can be used to catalytically cleave mRNA transcripts to thereby inhibit translation of the protein encoded by the mRNA. A ribozyme having specificity for a nucleic acid molecule encoding a polypeptide corresponding to a marker of the present invention can be designed based upon the nucleotide sequence of a cDNA corresponding to the marker. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved (see Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S.

Patent No. 5,116,742). Alternatively, an mRNA encoding a polypeptide of the present invention can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules (see, *e.g.*, Bartel and Szostak, 1993, *Science* 261:1411-1418).

The present invention also encompasses nucleic acid molecules which form triple
5 helical structures. For example, expression of a biomarker protein can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the gene encoding the polypeptide (*e.g.*, the promoter and/or enhancer) to form triple helical structures that prevent transcription of the gene in target cells. See generally Helene (1991) *Anticancer Drug Des.* 6(6):569-84; Helene (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and
10 Maher (1992) *Bioassays* 14(12):807-15.

In various embodiments, the nucleic acid molecules of the present invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to generate peptide
15 nucleic acid molecules (see Hyrup *et al.*, 1996, *Bioorganic & Medicinal Chemistry* 4(1): 5-23). As used herein, the terms “peptide nucleic acids” or “PNAs” refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA
20 under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup *et al.* (1996), *supra*; Perry-O'Keefe *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:14670-675.

PNAs can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene
25 expression by, *e.g.*, inducing transcription or translation arrest or inhibiting replication. PNAs can also be used, *e.g.*, in the analysis of single base pair mutations in a gene by, *e.g.*, PNA directed PCR clamping; as artificial restriction enzymes when used in combination with other enzymes, *e.g.*, S1 nucleases (Hyrup (1996), *supra*; or as probes or primers for DNA sequence and hybridization (Hyrup, 1996, *supra*; Perry-O'Keefe *et al.*, 1996, *Proc.*
30 *Natl. Acad. Sci. USA* 93:14670-675).

In another embodiment, PNAs can be modified, *e.g.*, to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery

known in the art. For example, PNA-DNA chimeras can be generated which can combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, *e.g.*, RNASE H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup, 1996, *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup (1996), *supra*, and Finn *et al.* (1996) *Nucleic Acids Res.* 24(17):3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs. Compounds such as 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite can be used as a link between the PNA and the 5' end of DNA (Mag *et al.*, 1989, *Nucleic Acids Res.* 17:5973-88). PNA monomers are then coupled in a step-wise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn *et al.*, 1996, *Nucleic Acids Res.* 24(17):3357-63). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser *et al.*, 1975, *Bioorganic Med. Chem. Lett.* 5:1119-11124).

In other embodiments, the oligonucleotide can include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:6553-6556; Lemaitre *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. WO 88/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (see, *e.g.*, Krol *et al.*, 1988, *Bio/Techniques* 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, *Pharm. Res.* 5:539-549). To this end, the oligonucleotide can be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, *etc.*

Another aspect of the present invention pertains to the use of biomarker proteins and biologically active portions thereof. In one embodiment, the native polypeptide corresponding to a marker can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, polypeptides corresponding to a marker of the present invention are produced by recombinant DNA techniques. Alternative to recombinant expression, a polypeptide

corresponding to a marker of the present invention can be synthesized chemically using standard peptide synthesis techniques.

An “isolated” or “purified” protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The language “substantially free of cellular material” includes preparations of protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of heterologous protein (also referred to herein as a “contaminating protein”). When the protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, 10%, or 5% of the volume of the protein preparation. When the protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, *i.e.*, it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of the protein have less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or compounds other than the polypeptide of interest.

Biologically active portions of a biomarker polypeptide include polypeptides comprising amino acid sequences sufficiently identical to or derived from a biomarker protein amino acid sequence described herein, but which includes fewer amino acids than the full length protein, and exhibit at least one activity of the corresponding full-length protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the corresponding protein. A biologically active portion of a protein of the present invention can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length. Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of the native form of a polypeptide of the present invention.

Preferred polypeptides have an amino acid sequence of a biomarker protein encoded by a nucleic acid molecule described herein. Other useful proteins are substantially identical (*e.g.*, at least about 40%, preferably 50%, 60%, 70%, 75%, 80%, 83%, 85%, 88%,

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) to one of these sequences and retain the functional activity of the protein of the corresponding naturally-occurring protein yet differ in amino acid sequence due to natural allelic variation or mutagenesis.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical positions/total # of positions (*e.g.*, overlapping positions) × 100). In one embodiment the two sequences are the same length.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the present invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the present invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, (1988) *Comput Appl Biosci*, 4:11-7. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is

part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Yet another useful algorithm for identifying regions of local sequence similarity and alignment is the FASTA algorithm as
5 described in Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444-2448. When using the FASTA algorithm for comparing nucleotide or amino acid sequences, a PAM120 weight residue table can, for example, be used with a k -tuple value of 2.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent
10 identity, only exact matches are counted.

The present invention also provides chimeric or fusion proteins corresponding to a biomarker protein. As used herein, a “chimeric protein” or “fusion protein” comprises all or part (preferably a biologically active part) of a polypeptide corresponding to a marker of the present invention operably linked to a heterologous polypeptide (*i.e.*, a polypeptide
15 other than the polypeptide corresponding to the marker). Within the fusion protein, the term “operably linked” is intended to indicate that the polypeptide of the present invention and the heterologous polypeptide are fused in-frame to each other. The heterologous polypeptide can be fused to the amino-terminus or the carboxyl-terminus of the polypeptide of the present invention.

20 One useful fusion protein is a GST fusion protein in which a polypeptide corresponding to a marker of the present invention is fused to the carboxyl terminus of GST sequences. Such fusion proteins can facilitate the purification of a recombinant polypeptide of the present invention.

In another embodiment, the fusion protein contains a heterologous signal sequence, immunoglobulin fusion protein, toxin, or other useful protein sequence. Chimeric and
25 fusion proteins of the present invention can be produced by standard recombinant DNA techniques. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary
30 overhangs between two consecutive gene fragments which can subsequently be annealed and re-amplified to generate a chimeric gene sequence (see, *e.g.*, Ausubel *et al.*, *supra*). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A nucleic acid encoding a polypeptide of the present

invention can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the polypeptide of the present invention.

5 A signal sequence can be used to facilitate secretion and isolation of the secreted protein or other proteins of interest. Signal sequences are typically characterized by a core of hydrophobic amino acids which are generally cleaved from the mature protein during secretion in one or more cleavage events. Such signal peptides contain processing sites that allow cleavage of the signal sequence from the mature proteins as they pass through the secretory pathway. Thus, the present invention pertains to the described polypeptides having a signal sequence, as well as to polypeptides from which the signal sequence has been proteolytically cleaved (*i.e.*, the cleavage products). In one embodiment, a nucleic acid sequence encoding a signal sequence can be operably linked in an expression vector to a protein of interest, such as a protein which is ordinarily not secreted or is otherwise difficult to isolate. The signal sequence directs secretion of the protein, such as from a eukaryotic host into which the expression vector is transformed, and the signal sequence is subsequently or concurrently cleaved. The protein can then be readily purified from the extracellular medium by art recognized methods. Alternatively, the signal sequence can be linked to the protein of interest using a sequence which facilitates purification, such as with a GST domain.

20 The present invention also pertains to variants of the biomarker polypeptides described herein. Such variants have an altered amino acid sequence which can function as either agonists (mimetics) or as antagonists. Variants can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation. An agonist can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of the protein. An antagonist of a protein can inhibit one or more of the activities of the naturally occurring form of the protein by, for example, competitively binding to a downstream or upstream member of a cellular signaling cascade which includes the protein of interest. Thus, specific biological effects can be elicited by treatment with a variant of limited function. Treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein can have fewer side effects in a subject relative to treatment with the naturally occurring form of the protein.

30 Variants of a biomarker protein which function as either agonists (mimetics) or as antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of the protein of the present invention for agonist or antagonist activity.

In one embodiment, a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential protein sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display). There are a variety of methods which can be used to produce libraries of potential variants of the polypeptides of the present invention from a degenerate oligonucleotide sequence. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, 1983, *Tetrahedron* 39:3; Itakura *et al.*, 1984, *Annu. Rev. Biochem.* 53:323; Itakura *et al.*, 1984, *Science* 198:1056; Ike *et al.*, 1983 *Nucleic Acid Res.* 11:477).

In addition, libraries of fragments of the coding sequence of a polypeptide corresponding to a marker of the present invention can be used to generate a variegated population of polypeptides for screening and subsequent selection of variants. For example, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of the coding sequence of interest with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes amino terminal and internal fragments of various sizes of the protein of interest.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. The most widely used techniques, which are amenable to high throughput analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants of a protein of the present

invention (Arkin and Yourvan, 1992, *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delgrave *et al.*, 1993, *Protein Engineering* 6(3):327- 331).

An isolated polypeptide or a fragment thereof (or a nucleic acid encoding such a polypeptide) corresponding to one or more biomarkers of the invention, including the biomarkers listed in Table 1 or fragments thereof, can be used as an immunogen to generate antibodies that bind to said immunogen, using standard techniques for polyclonal and monoclonal antibody preparation according to well-known methods in the art. An antigenic peptide comprises at least 8 amino acid residues and encompasses an epitope present in the respective full length molecule such that an antibody raised against the peptide forms a specific immune complex with the respective full length molecule. Preferably, the antigenic peptide comprises at least 10 amino acid residues. In one embodiment such epitopes can be specific for a given polypeptide molecule from one species, such as mouse or human (*i.e.*, an antigenic peptide that spans a region of the polypeptide molecule that is not conserved across species is used as immunogen; such non conserved residues can be determined using an alignment such as that provided herein).

In one embodiment, an antibody binds substantially specifically to an immune checkpoint, such as PD-1, and inhibits or blocks its immunoinhibitory function, such as by interrupting its interaction with a binding partner of the immune checkpoint, such as PD-L1 and/or PD-L2 binding partners of PD-1. In one embodiment, an antibody binds substantially specifically to CX3CR1 and inhibits or blocks its biological function, such as by interrupting its interaction with a ligand like CX3CL1. In another embodiment, an antibody binds substantially specifically to a CX3CR1 binding partner, such as CX3CL1, and inhibits or blocks its biological function, such as by interrupting its interaction to CX3CR1.

For example, a polypeptide immunogen typically is used to prepare antibodies by immunizing a suitable subject (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. A preferred animal is a mouse deficient in the desired target antigen. For example, a PD-1 knockout mouse if the desired antibody is an anti-PD-1 antibody, may be used. This results in a wider spectrum of antibody recognition possibilities as antibodies reactive to common mouse and human epitopes are not removed by tolerance mechanisms. An appropriate immunogenic preparation can contain, for example, a recombinantly expressed or chemically synthesized molecule or fragment thereof to which the immune response is to be generated. The preparation can further include an adjuvant, such as

Freund's complete or incomplete adjuvant, or similar immunostimulatory agent.

Immunization of a suitable subject with an immunogenic preparation induces a polyclonal antibody response to the antigenic peptide contained therein.

5 Polyclonal antibodies can be prepared as described above by immunizing a suitable subject with a polypeptide immunogen. The polypeptide antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized polypeptide. If desired, the antibody directed against the antigen can be isolated from the mammal (*e.g.*, from the blood) and further purified by well-known techniques, such as protein A chromatography, to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique (originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci.* 76:2927-31; Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well-known (see generally Kenneth, R. H. in 20 *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); Lerner, E. A. (1981) *Yale J. Biol. Med.* 54:387-402; Gefter, M. L. *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with an immunogen as described above, and the culture supernatants of the 25 resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to the polypeptide antigen, preferably specifically. In some embodiments, the immunization is performed in a cell or animal host that has a knockout of a target antigen of interest (*e.g.*, does not produce the antigen prior to immunization).

Any of the many well-known protocols used for fusing lymphocytes and 30 immortalized cell lines can be applied for the purpose of generating a monoclonal antibody against one or more biomarkers of the invention, including the biomarkers listed in Table 1, or a fragment thereof (see, *e.g.*, Galfre, G. *et al.* (1977) *Nature* 266:55052; Gefter *et al.* (1977) *supra*; Lerner (1981) *supra*; Kenneth (1980) *supra*). Moreover, the ordinary skilled

worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, MD. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind a given polypeptide, *e.g.*, using a standard ELISA assay.

As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal specific for one of the above described polypeptides can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with the appropriate polypeptide to thereby isolate immunoglobulin library members that bind the polypeptide. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAPTM Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening an antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Biotechnology (NY)* 9:1369-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et*

al. (1993) *EMBO J.* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrard *et al.* (1991) *Biotechnology (NY)* 9:1373-1377; Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al.* (1990) *Nature* 348:552-554.

Since it is well-known in the art that antibody heavy and light chain CDR3 domains play a particularly important role in the binding specificity/affinity of an antibody for an antigen, the recombinant monoclonal antibodies of the present invention prepared as set forth above preferably comprise the heavy and light chain CDR3s of variable regions of the antibodies described herein and well-known in the art. Similarly, the antibodies can further comprise the CDR2s of variable regions of said antibodies. The antibodies can further comprise the CDR1s of variable regions of said antibodies. In other embodiments, the antibodies can comprise any combinations of the CDRs.

The CDR1, 2, and/or 3 regions of the engineered antibodies described above can comprise the exact amino acid sequence(s) as those of variable regions of the present invention disclosed herein. However, the ordinarily skilled artisan will appreciate that some deviation from the exact CDR sequences may be possible while still retaining the ability of the antibody to bind a desired target, such as CX3CR1 and/or a binding partner thereof, either alone or in combination with an immune checkpoint target and/or a binding partner thereof, such as CX3CR1, CX3CL1, PD-L1, PD-L2, and/or PD-1 effectively (*e.g.*, conservative sequence modifications). Accordingly, in another embodiment, the engineered antibody may be composed of one or more CDRs that are, for example, 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% identical to one or more CDRs of the present invention described herein or otherwise publicly available.

For example, the structural features of non-human or human antibodies (*e.g.*, a rat anti-mouse/anti-human antibody) can be used to create structurally related human antibodies that retain at least one functional property of the antibodies of the present invention, such as binding to CX3CR1, CX3CL1, PD-L1, PD-L2, and/or PD-1. Another functional property includes inhibiting binding of the original known, non-human or human antibodies in a competition ELISA assay.

In some embodiments, monoclonal antibodies capable of binding and inhibiting/blocking CX3CR1, CX3CL1, PD-L1, PD-L2, and/or PD-1 are provided,

comprising a heavy chain wherein the variable domain comprises at least a CDR having a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% identical from the group of heavy chain variable domain CDRs presented herein or otherwise publicly available.

5 Similarly, monoclonal antibodies binding and inhibiting/blocking CX3CR1, CX3CL1, PD-L1, PD-L2, and/or PD-1, comprising a light chain wherein the variable domain comprises at least a CDR having a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% identical from the group of light chain variable domain CDRs presented herein or otherwise publicly available, are also
10 provided.

Monoclonal antibodies capable of binding and inhibiting/blocking CX3CR1, CX3CL1, PD-L1, PD-L2, and/or PD-1, comprising a heavy chain wherein the variable domain comprises at least a CDR having a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% identical from the group of
15 heavy chain variable domain CDRs presented herein or otherwise publicly available; and comprising a light chain wherein the variable domain comprises at least a CDR having a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% identical from the group of light chain variable domain CDRs presented herein or otherwise publicly available, are also provided.

20 A skilled artisan will note that such percentage homology is equivalent to and can be achieved by introducing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more conservative amino acid substitutions within a given CDR.

The monoclonal antibodies of the present invention can comprise a heavy chain, wherein the variable domain comprises at least a CDR having a sequence selected from the
25 group consisting of the heavy chain variable domain CDRs presented herein or otherwise publicly available and a light chain, wherein the variable domain comprises at least a CDR having a sequence selected from the group consisting of the light chain variable domain CDRs presented herein or otherwise publicly available.

Such monoclonal antibodies can comprise a light chain, wherein the variable
30 domain comprises at least a CDR having a sequence selected from the group consisting of CDR-L1, CDR-L2, and CDR-L3, as described herein; and/or a heavy chain, wherein the variable domain comprises at least a CDR having a sequence selected from the group consisting of CDR-H1, CDR-H2, and CDR-H3, as described herein. In some

embodiments, the monoclonal antibodies capable of binding human CX3CR1, CX3CL1, PD-L1, PD-L2, and/or PD-1 comprises or consists of CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2, and CDR-H3, as described herein.

5 The heavy chain variable domain of the monoclonal antibodies of the present invention can comprise or consist of the vH amino acid sequence set forth herein or otherwise publicly available and/or the light chain variable domain of the monoclonal antibodies of the present invention can comprise or consist of the vk amino acid sequence set forth herein or otherwise publicly available.

10 The present invention further provides fragments of said monoclonal antibodies which include, but are not limited to, Fv, Fab, F(ab')₂, Fab', dsFv, scFv, sc(Fv)₂ and diabodies; and multispecific antibodies formed from antibody fragments.

Other fragments of the monoclonal antibodies of the present invention are also contemplated. For example, individual immunoglobulin heavy and/or light chains are provided, wherein the variable domains thereof comprise at least a CDR presented herein or
15 otherwise publicly available. In one embodiment, the immunoglobulin heavy chain comprises at least a CDR having a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% identical from the group of heavy chain or light chain variable domain CDRs presented herein or otherwise publicly available. In another embodiment, an immunoglobulin light chain comprises at least a CDR having a
20 sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% identical from the group of light chain or heavy chain variable domain CDRs presented herein or otherwise publicly available, are also provided.

In some embodiments, the immunoglobulin heavy and/or light chain comprises a variable domain comprising at least one of CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2, or CDR-H3 described herein. Such immunoglobulin heavy chains can comprise or
25 consist of at least one of CDR-H1, CDR-H2, and CDR-H3. Such immunoglobulin light chains can comprise or consist of at least one of CDR-L1, CDR-L2, and CDR-L3.

In other embodiments, an immunoglobulin heavy and/or light chain according to the present invention comprises or consists of a vH or vk variable domain sequence,
30 respectively, provided herein or otherwise publicly available.

The present invention further provides polypeptides which have a sequence selected from the group consisting of vH variable domain, vk variable domain, CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2, and CDR-H3 sequences described herein.

Antibodies, immunoglobulins, and polypeptides of the invention can be used in an isolated (*e.g.*, purified) form or contained in a vector, such as a membrane or lipid vesicle (*e.g.* a liposome).

Amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. It is known that when a humanized antibody is produced by simply grafting only CDRs in VH and VL of an antibody derived from a non-human animal in FRs of the VH and VL of a human antibody, the antigen binding activity is reduced in comparison with that of the original antibody derived from a non-human animal. It is considered that several amino acid residues of the VH and VL of the non-human antibody, not only in CDRs but also in FRs, are directly or indirectly associated with the antigen binding activity. Hence, substitution of these amino acid residues with different amino acid residues derived from FRs of the VH and VL of the human antibody would reduce binding activity and can be corrected by replacing the amino acids with amino acid residues of the original antibody derived from a non-human animal.

Modifications and changes may be made in the structure of the antibodies described herein, and in the DNA sequences encoding them, and still obtain a functional molecule that encodes an antibody and polypeptide with desirable characteristics. For example, certain amino acids may be substituted by other amino acids in a protein structure without appreciable loss of activity. Since the interactive capacity and nature of a protein define the protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and, of course, in its DNA encoding sequence, while nevertheless obtaining a protein with like properties. It is thus contemplated that various changes may be made in the antibodies sequences of the invention, or corresponding DNA sequences which encode said polypeptides, without appreciable loss of their biological activity.

In making the changes in the amino sequences of polypeptide, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art. It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics these are: isoleucine (+4.5);

valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophane (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (<RTI 3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

5 It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e. still obtain a biological functionally equivalent protein.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their
10 hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well-known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

Another type of amino acid modification of the antibody of the invention may be
15 useful for altering the original glycosylation pattern of the antibody to, for example, increase stability. By “altering” is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody. Glycosylation of antibodies is typically N-linked. “N-linked” refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The
20 tripeptide sequences asparagine-X-serine and asparagines-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid
25 sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). Another type of covalent modification involves chemically or enzymatically coupling glycosides to the antibody. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the
30 sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine,

tyrosine, or tryptophan, or (f) the amide group of glutamine. For example, such methods are described in WO87/05330.

Similarly, removal of any carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact. Chemical deglycosylation is described by *Sojahn et al.* (1987) and by *Edge et al.* (1981). Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo- and exo-glycosidases as described by *Thotakura et al.* (1987).

Other modifications can involve the formation of immunoconjugates. For example, in one type of covalent modification, antibodies or proteins are covalently linked to one of a variety of non proteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. No. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Conjugation of antibodies or other proteins of the present invention with heterologous agents can be made using a variety of bifunctional protein coupling agents including but not limited to N-succinimidyl (2-pyridyldithio) propionate (SPDP), succinimidyl (N-maleimidomethyl)cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, carbon labeled 1-isothiocyanatobenzyl methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody (WO 94/11026).

In another aspect, the present invention features antibodies conjugated to a therapeutic moiety, such as a cytotoxin, a drug, and/or a radioisotope. When conjugated to a cytotoxin, these antibody conjugates are referred to as "immunotoxins." A cytotoxin or cytotoxic agent includes any agent that is detrimental to (*e.g.*, kills) cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy

anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine),
 5 alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin
 10 (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine). An antibody of the present invention can be conjugated to a radioisotope, *e.g.*, radioactive iodine, to generate cytotoxic radiopharmaceuticals for treating a related disorder, such as a cancer.

Conjugated antibodies, in addition to therapeutic utility, can be useful for diagnostically or prognostically to monitor polypeptide levels in tissue as part of a clinical
 15 testing procedure, *e.g.*, to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline
 20 phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate (FITC), rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin (PE); an example of a luminescent material includes luminol; examples of bioluminescent materials include
 25 luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , or ^3H . [0134] As used herein, the term “labeled”, with regard to the antibody, is intended to encompass direct labeling of the antibody by coupling (*i.e.*, physically linking) a detectable substance, such as a radioactive agent or a fluorophore (*e.g.* fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the
 30 antibody, as well as indirect labeling of the antibody by reactivity with a detectable substance.

The antibody conjugates of the present invention can be used to modify a given biological response. The therapeutic moiety is not to be construed as limited to classical

chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active toxin, or active fragment thereof, such as abrin, ricin A, *Pseudomonas* exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor or interferon- γ ; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other cytokines or growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well-known, see, *e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld *et al.* (eds.), pp. 243 56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson *et al.* (eds.), pp. 623 53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera *et al.* (eds.), pp. 475 506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin *et al.* (eds.), pp. 303 16 (Academic Press 1985), and Thorpe *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.*, 62:119 58 (1982).

In some embodiments, conjugations can be made using a "cleavable linker" facilitating release of the cytotoxic agent or growth inhibitory agent in a cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (See *e.g.* U.S. Pat. No. 5,208,020) may be used. Alternatively, a fusion protein comprising the antibody and cytotoxic agent or growth inhibitory agent may be made, by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

Additionally, recombinant polypeptide antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant

DNA techniques known in the art, for example using methods described in Robinson *et al.* International Patent Publication PCT/US86/02269; Akira *et al.* European Patent Application 184,187; Taniguchi, M. European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci.* 84:214-218; Nishimura *et al.* (1987) *Cancer Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *Biotechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeven *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

In addition, humanized antibodies can be made according to standard protocols such as those disclosed in U.S. Patent 5,565,332. In another embodiment, antibody chains or specific binding pair members can be produced by recombination between vectors comprising nucleic acid molecules encoding a fusion of a polypeptide chain of a specific binding pair member and a component of a replicable generic display package and vectors containing nucleic acid molecules encoding a second polypeptide chain of a single binding pair member using techniques known in the art, *e.g.*, as described in U.S. Patents 5,565,332, 5,871,907, or 5,733,743. The use of intracellular antibodies to inhibit protein function in a cell is also known in the art (see *e.g.*, Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T. M. *et al.* (1990) *FEBS Lett.* 274:193-198; Carlson, J. R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W. A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Biotechnology (NY)* 12:396-399; Chen, S-Y. *et al.* (1994) *Hum. Gene Ther.* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-5936; Beerli, R. R. *et al.* (1994) *J. Biol. Chem.* 269:23931-23936; Beerli, R. R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A. M. *et al.* (1995) *EMBO J.* 14:1542-1551; Richardson, J. H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

Additionally, fully human antibodies could be made against biomarkers of the invention, including the biomarkers listed in Table 1, or fragments thereof. Fully human

antibodies can be made in mice that are transgenic for human immunoglobulin genes, *e.g.* according to Hogan, *et al.*, “Manipulating the Mouse Embryo: A Laboratory Manual,” Cold Spring Harbor Laboratory. Briefly, transgenic mice are immunized with purified immunogen. Spleen cells are harvested and fused to myeloma cells to produce hybridomas. Hybridomas are selected based on their ability to produce antibodies which bind to the immunogen. Fully human antibodies would reduce the immunogenicity of such antibodies in a human.

In one embodiment, an antibody for use in the instant invention is a bispecific or multispecific antibody. A bispecific antibody has binding sites for two different antigens within a single antibody polypeptide. Antigen binding may be simultaneous or sequential. Triomas and hybrid hybridomas are two examples of cell lines that can secrete bispecific antibodies. Examples of bispecific antibodies produced by a hybrid hybridoma or a trioma are disclosed in U.S. Patent 4,474,893. Bispecific antibodies have been constructed by chemical means (Staerz *et al.* (1985) *Nature* 314:628, and Perez *et al.* (1985) *Nature* 316:354) and hybridoma technology (Staerz and Bevan (1986) *Proc. Natl. Acad. Sci. USA*, 83:1453, and Staerz and Bevan (1986) *Immunol. Today* 7:241). Bispecific antibodies are also described in U.S. Patent 5,959,084. Fragments of bispecific antibodies are described in U.S. Patent 5,798,229.

Bispecific agents can also be generated by making heterohybridomas by fusing hybridomas or other cells making different antibodies, followed by identification of clones producing and co-assembling both antibodies. They can also be generated by chemical or genetic conjugation of complete immunoglobulin chains or portions thereof such as Fab and Fv sequences. The antibody component can bind to a polypeptide or a fragment thereof of one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or a fragment thereof. In one embodiment, the bispecific antibody could specifically bind to both a polypeptide or a fragment thereof and its natural binding partner(s) or a fragment(s) thereof.

In another aspect of this invention, peptides or peptide mimetics can be used to antagonize the activity of one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or a fragment(s) thereof. In one embodiment, variants of one or more biomarkers listed in Table 1 which function as a modulating agent for the respective full length protein, can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, for antagonist activity. In one embodiment, a variegated

library of variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of variants can be produced, for instance, by enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential polypeptide sequences is expressible as individual polypeptides containing the set of polypeptide sequences therein. There are a variety of methods which can be used to produce libraries of polypeptide variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential polypeptide sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang, S. A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of a polypeptide coding sequence can be used to generate a variegated population of polypeptide fragments for screening and subsequent selection of variants of a given polypeptide. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a polypeptide coding sequence with a nuclease under conditions wherein nicking occurs only about once per polypeptide, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the polypeptide.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of polypeptides. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was

detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants of interest (Arkin and Youvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delagrave *et al.* (1993) *Protein Eng.* 6(3):327-331). In one embodiment, cell based assays can be exploited to analyze a variegated polypeptide library. For example, a library of expression vectors can be transfected into a cell line which ordinarily synthesizes one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or a fragment thereof. The transfected cells are then cultured such that the full length polypeptide and a particular mutant polypeptide are produced and the effect of expression of the mutant on the full length polypeptide activity in cell supernatants can be detected, *e.g.*, by any of a number of functional assays. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of full length polypeptide activity, and the individual clones further characterized.

Systematic substitution of one or more amino acids of a polypeptide amino acid sequence with a D-amino acid of the same type (*e.g.*, D-lysine in place of L-lysine) can be used to generate more stable peptides. In addition, constrained peptides comprising a polypeptide amino acid sequence of interest or a substantially identical sequence variation can be generated by methods known in the art (Rizo and Gierasch (1992) *Annu. Rev. Biochem.* 61:387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

The amino acid sequences disclosed herein will enable those of skill in the art to produce polypeptides corresponding peptide sequences and sequence variants thereof.

Such polypeptides can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding the peptide sequence, frequently as part of a larger polypeptide. Alternatively, such peptides can be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well-known in the art and are described further in Maniatis *et al. Molecular Cloning: A Laboratory Manual* (1989), 2nd Ed., Cold Spring Harbor, N.Y.; Berger and Kimmel, *Methods in Enzymology*, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, Calif.; Merrifield, J. (1969) *J. Am. Chem. Soc.* 91:501; Chaiken I. M. (1981) *CRC Crit. Rev. Biochem.* 11:

255; Kaiser *et al.* (1989) *Science* 243:187; Merrifield, B. (1986) *Science* 232:342; Kent, S. B. H. (1988) *Annu. Rev. Biochem.* 57:957; and Offord, R. E. (1980) *Semisynthetic Proteins*, Wiley Publishing, which are incorporated herein by reference).

Peptides can be produced, typically by direct chemical synthesis. Peptides can be produced as modified peptides, with nonpeptide moieties attached by covalent linkage to the N-terminus and/or C-terminus. In certain preferred embodiments, either the carboxy-terminus or the amino-terminus, or both, are chemically modified. The most common modifications of the terminal amino and carboxyl groups are acetylation and amidation, respectively. Amino-terminal modifications such as acylation (*e.g.*, acetylation) or alkylation (*e.g.*, methylation) and carboxy-terminal-modifications such as amidation, as well as other terminal modifications, including cyclization, can be incorporated into various embodiments of the invention. Certain amino-terminal and/or carboxy-terminal modifications and/or peptide extensions to the core sequence can provide advantageous physical, chemical, biochemical, and pharmacological properties, such as: enhanced stability, increased potency and/or efficacy, resistance to serum proteases, desirable pharmacokinetic properties, and others. Peptides disclosed herein can be used therapeutically to treat disease, *e.g.*, by altering costimulation in a patient.

Peptidomimetics (Fauchere (1986) *Adv. Drug Res.* 15:29; Veber and Freidinger (1985) *TINS* p.392; and Evans *et al.* (1987) *J. Med. Chem.* 30:1229, which are incorporated herein by reference) are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides can be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biological or pharmacological activity), but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH₂NH-, -CH₂S-, -CH₂-CH₂-, -CH=CH- (cis and trans), -COCH₂-, -CH(OH)CH₂-, and -CH₂SO-, by methods known in the art and further described in the following references: Spatola, A. F. in “*Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*” Weinstein, B., ed., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., *Vega Data* (March 1983), Vol. 1, Issue 3, “Peptide Backbone Modifications” (general review); Morley, J. S. (1980) *Trends Pharm. Sci.* pp. 463-468 (general review); Hudson, D. *et al.* (1979) *Int. J. Pept. Prot. Res.* 14:177-185 (-CH₂NH-, CH₂CH₂-); Spatola, A. F. *et al.* (1986) *Life Sci.* 38:1243-1249 (-CH₂-S); Hann, M. M. (1982) *J. Chem. Soc. Perkin Trans.*

I. 307-314 (-CH-CH-, cis and trans); Almquist, R. G. *et al.* (190) *J. Med. Chem.* 23:1392-1398 (-COCH₂-); Jennings-White, C. *et al.* (1982) *Tetrahedron Lett.* 23:2533 (-COCH₂-); Szelke, M. *et al.* European Appln. EP 45665 (1982) CA: 97:39405 (1982)(-CH(OH)CH₂-); Holladay, M. W. *et al.* (1983) *Tetrahedron Lett.* (1983) 24:4401-4404 (-C(OH)CH₂-);
5 and Hruby, V. J. (1982) *Life Sci.* (1982) 31:189-199 (-CH₂-S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH₂NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy,
10 etc.), altered specificity (*e.g.*, a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions
15 generally are positions that do not form direct contacts with the macropolypeptides(s) to which the peptidomimetic binds to produce the therapeutic effect. Derivatization (*e.g.*, labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

Also encompassed by the present invention are small molecules which can
20 modulate (either enhance or inhibit) interactions, *e.g.*, between biomarkers described herein or listed in Table 1 and their natural binding partners. The small molecules of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-
25 compound' library method; and synthetic library methods using affinity chromatography selection. (Lam, K. S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994) *J. Med. Chem.* 37:2678;
30 Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds can be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*). Compounds can be screened in cell based or non-cell based assays. Compounds can be screened in pools (*e.g.* multiple compounds in each testing sample) or as individual compounds.

The invention also relates to chimeric or fusion proteins of the biomarkers of the invention, including the biomarkers listed in Table 1, or fragments thereof. As used herein, a "chimeric protein" or "fusion protein" comprises one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or a fragment thereof, operatively linked to another polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the respective biomarker. In a preferred embodiment, the fusion protein comprises at least one biologically active portion of one or more biomarkers of the invention, including one or more biomarkers listed in Table 1, or fragments thereof. Within the fusion protein, the term "operatively linked" is intended to indicate that the biomarker sequences and the non-biomarker sequences are fused in-frame to each other in such a way as to preserve functions exhibited when expressed independently of the fusion. The "another" sequences can be fused to the N-terminus or C-terminus of the biomarker sequences, respectively.

Such a fusion protein can be produced by recombinant expression of a nucleotide sequence encoding the first peptide and a nucleotide sequence encoding the second peptide. The second peptide may optionally correspond to a moiety that alters the solubility, affinity, stability or valency of the first peptide, for example, an immunoglobulin constant region. In another preferred embodiment, the first peptide consists of a portion of a biologically active molecule (*e.g.* the extracellular portion of the polypeptide or the ligand binding portion). The second peptide can include an immunoglobulin constant region, for example, a human C γ 1 domain or C γ 4 domain (*e.g.*, the hinge, CH2 and CH3 regions of human IgC γ 1, or human IgC γ 4, see *e.g.*, Capon *et al.* U.S. Patents 5,116,964; 5,580,756; 5,844,095 and the like, incorporated herein by reference). Such constant regions may retain regions which mediate effector function (*e.g.* Fc receptor binding) or may be altered to reduce effector

function. A resulting fusion protein may have altered solubility, binding affinity, stability and/or valency (*i.e.*, the number of binding sites available per polypeptide) as compared to the independently expressed first peptide, and may increase the efficiency of protein purification. Fusion proteins and peptides produced by recombinant techniques can be
5 secreted and isolated from a mixture of cells and medium containing the protein or peptide. Alternatively, the protein or peptide can be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture typically includes host cells, media and other byproducts. Suitable media for cell culture are well-known in the art. Protein and peptides can be isolated from cell culture media, host cells, or both using techniques known in the art
10 for purifying proteins and peptides. Techniques for transfecting host cells and purifying proteins and peptides are known in the art.

Preferably, a fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for
15 example employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene
20 fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992).

Particularly preferred Ig fusion proteins include the extracellular domain portion or
25 variable region-like domain of CX3CR1, PD-1, or other biomarker listed in Table 1, coupled to an immunoglobulin constant region (*e.g.*, the Fc region). The immunoglobulin constant region may contain genetic modifications which reduce or eliminate effector activity inherent in the immunoglobulin structure. For example, DNA encoding the extracellular portion of a polypeptide of interest can be joined to DNA encoding the hinge,
30 CH2 and CH3 regions of human IgG γ 1 and/or IgG γ 4 modified by site directed mutagenesis, *e.g.*, as taught in WO 97/28267.

In another embodiment, the fusion protein contains a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of a polypeptide can be increased through use of a heterologous signal sequence.

The fusion proteins of the invention can be used as immunogens to produce
 5 antibodies in a subject. Such antibodies may be used to purify the respective natural polypeptides from which the fusion proteins were generated, or in screening assays to identify polypeptides which inhibit the interactions between one or more biomarkers polypeptide or a fragment thereof and its natural binding partner(s) or a fragment(s) thereof.

Also provided herein are compositions comprising one or more nucleic acids
 10 comprising or capable of expressing at least 1, 2, 3, 4, 5, 10, 20 or more small nucleic acids or antisense oligonucleotides or derivatives thereof, wherein said small nucleic acids or antisense oligonucleotides or derivatives thereof in a cell specifically hybridize (*e.g.*, bind) under cellular conditions, with cellular nucleic acids (*e.g.*, small non-coding RNAs such as miRNAs, pre-miRNAs, pri-miRNAs, miRNA*, anti-miRNA, a miRNA binding site, a
 15 variant and/or functional variant thereof, cellular mRNAs or a fragments thereof). In one embodiment, expression of the small nucleic acids or antisense oligonucleotides or derivatives thereof in a cell can inhibit expression or biological activity of cellular nucleic acids and/or proteins, *e.g.*, by inhibiting transcription, translation and/or small nucleic acid processing of, for example, one or more biomarkers of the invention, including one or more
 20 biomarkers listed in Table 1, or fragment(s) thereof. In one embodiment, the small nucleic acids or antisense oligonucleotides or derivatives thereof are small RNAs (*e.g.*, microRNAs) or complements of small RNAs. In another embodiment, the small nucleic acids or antisense oligonucleotides or derivatives thereof can be single or double stranded and are at least six nucleotides in length and are less than about 1000, 900, 800, 700, 600,
 25 500, 400, 300, 200, 100, 50, 40, 30, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, or 10 nucleotides in length. In another embodiment, a composition may comprise a library of nucleic acids comprising or capable of expressing small nucleic acids or antisense oligonucleotides or derivatives thereof, or pools of said small nucleic acids or antisense oligonucleotides or derivatives thereof. A pool of nucleic acids may comprise about 2-5, 5-
 30 10, 10-20, 10-30 or more nucleic acids comprising or capable of expressing small nucleic acids or antisense oligonucleotides or derivatives thereof.

In one embodiment, binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the

major groove of the double helix. In general, “antisense” refers to the range of techniques generally employed in the art, and includes any process that relies on specific binding to oligonucleotide sequences.

It is well-known in the art that modifications can be made to the sequence of a miRNA or a pre-miRNA without disrupting miRNA activity. As used herein, the term “functional variant” of a miRNA sequence refers to an oligonucleotide sequence that varies from the natural miRNA sequence, but retains one or more functional characteristics of the miRNA (*e.g.* cancer cell proliferation inhibition, induction of cancer cell apoptosis, enhancement of cancer cell susceptibility to chemotherapeutic agents, specific miRNA target inhibition). In some embodiments, a functional variant of a miRNA sequence retains all of the functional characteristics of the miRNA. In certain embodiments, a functional variant of a miRNA has a nucleobase sequence that is at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the miRNA or precursor thereof over a region of about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleobases, or that the functional variant hybridizes to the complement of the miRNA or precursor thereof under stringent hybridization conditions. Accordingly, in certain embodiments the nucleobase sequence of a functional variant is capable of hybridizing to one or more target sequences of the miRNA.

miRNAs and their corresponding stem-loop sequences described herein may be found in miRBase, an online searchable database of miRNA sequences and annotation, found on the world wide web at microrna.sanger.ac.uk. Entries in the miRBase Sequence database represent a predicted hairpin portion of a miRNA transcript (the stem-loop), with information on the location and sequence of the mature miRNA sequence. The miRNA stem-loop sequences in the database are not strictly precursor miRNAs (pre-miRNAs), and may in some instances include the pre-miRNA and some flanking sequence from the presumed primary transcript. The miRNA nucleobase sequences described herein encompass any version of the miRNA, including the sequences described in Release 10.0 of the miRBase sequence database and sequences described in any earlier Release of the miRBase sequence database. A sequence database release may result in the re-naming of certain miRNAs. A sequence database release may result in a variation of a mature miRNA sequence.

In some embodiments, miRNA sequences of the invention may be associated with a second RNA sequence that may be located on the same RNA molecule or on a separate RNA molecule as the miRNA sequence. In such cases, the miRNA sequence may be referred to as the active strand, while the second RNA sequence, which is at least partially complementary to the miRNA sequence, may be referred to as the complementary strand. The active and complementary strands are hybridized to create a double-stranded RNA that is similar to a naturally occurring miRNA precursor. The activity of a miRNA may be optimized by maximizing uptake of the active strand and minimizing uptake of the complementary strand by the miRNA protein complex that regulates gene translation. This can be done through modification and/or design of the complementary strand.

In some embodiments, the complementary strand is modified so that a chemical group other than a phosphate or hydroxyl at its 5' terminus. The presence of the 5' modification apparently eliminates uptake of the complementary strand and subsequently favors uptake of the active strand by the miRNA protein complex. The 5' modification can be any of a variety of molecules known in the art, including NH₂, NHCOCH₃, and biotin.

In another embodiment, the uptake of the complementary strand by the miRNA pathway is reduced by incorporating nucleotides with sugar modifications in the first 2-6 nucleotides of the complementary strand. It should be noted that such sugar modifications can be combined with the 5' terminal modifications described above to further enhance miRNA activities.

In some embodiments, the complementary strand is designed so that nucleotides in the 3' end of the complementary strand are not complementary to the active strand. This results in double-strand hybrid RNAs that are stable at the 3' end of the active strand but relatively unstable at the 5' end of the active strand. This difference in stability enhances the uptake of the active strand by the miRNA pathway, while reducing uptake of the complementary strand, thereby enhancing miRNA activity.

Small nucleic acid and/or antisense constructs of the methods and compositions presented herein can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of cellular nucleic acids (*e.g.*, small RNAs, mRNA, and/or genomic DNA). Alternatively, the small nucleic acid molecules can produce RNA which encodes mRNA, miRNA, pre-miRNA, pri-miRNA, miRNA*, anti-miRNA, or a miRNA binding site, or a variant thereof. For example, selection of plasmids suitable for expressing the miRNAs, methods for

inserting nucleic acid sequences into the plasmid, and methods of delivering the recombinant plasmid to the cells of interest are within the skill in the art. See, for example, Zeng *et al.* (2002) *Mol. Cell* 9:1327-1333; Tuschl (2002), *Nat. Biotechnol.* 20:446-448; Brummelkamp *et al.* (2002) *Science* 296:550-553; Miyagishi *et al.* (2002) *Nat. Biotechnol.* 20:497-500; Paddison *et al.* (2002) *Genes Dev.* 16:948-958; Lee *et al.* (2002) *Nat. Biotechnol.* 20:500-505; and Paul *et al.* (2002) *Nat. Biotechnol.* 20:505-508, the entire disclosures of which are herein incorporated by reference.

Alternatively, small nucleic acids and/or antisense constructs are oligonucleotide probes that are generated *ex vivo* and which, when introduced into the cell, results in hybridization with cellular nucleic acids. Such oligonucleotide probes are preferably modified oligonucleotides that are resistant to endogenous nucleases, *e.g.*, exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as small nucleic acids and/or antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol *et al.* (1988) *BioTechniques* 6:958-976; and Stein *et al.* (1988) *Cancer Res* 48:2659-2668.

Antisense approaches may involve the design of oligonucleotides (either DNA or RNA) that are complementary to cellular nucleic acids (*e.g.*, complementary to biomarkers listed in Table 1). Absolute complementarity is not required. In the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a nucleic acid (*e.g.*, RNA) it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the mRNA, *e.g.*, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently been shown to be effective at inhibiting translation of mRNAs as well (Wagner (1994) *Nature* 372:333). Therefore, oligonucleotides complementary to either the 5' or 3' untranslated, non-coding regions of

genes could be used in an antisense approach to inhibit translation of endogenous mRNAs. Oligonucleotides complementary to the 5' untranslated region of the mRNA may include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could also be used in accordance with the methods and compositions presented herein. Whether designed to hybridize to the 5', 3' or coding region of cellular mRNAs, small nucleic acids and/or antisense nucleic acids should be at least six nucleotides in length, and can be less than about 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50, 40, 30, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, or 10 nucleotides in length.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. In one embodiment these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. In another embodiment these studies compare levels of the target nucleic acid or protein with that of an internal control nucleic acid or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

Small nucleic acids and/or antisense oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. Small nucleic acids and/or antisense oligonucleotides can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc., and may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556; Lemaitre *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134), hybridization-triggered cleavage agents. (See, *e.g.*, Krol *et al.* (1988) *BioTech.* 6:958-976) or intercalating agents. (See, *e.g.*, Zon (1988) *Pharm. Res.* 5:539-549). To this end, small nucleic acids and/or antisense oligonucleotides may be conjugated to another molecule,

e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

Small nucleic acids and/or antisense oligonucleotides may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxyethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Small nucleic acids and/or antisense oligonucleotides may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In certain embodiments, a compound comprises an oligonucleotide (*e.g.*, a miRNA or miRNA encoding oligonucleotide) conjugated to one or more moieties which enhance the activity, cellular distribution or cellular uptake of the resulting oligonucleotide. In certain such embodiments, the moiety is a cholesterol moiety (*e.g.*, antagomirs) or a lipid moiety or liposome conjugate. Additional moieties for conjugation include carbohydrates, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. In certain embodiments, a conjugate group is attached directly to the oligonucleotide. In certain embodiments, a conjugate group is attached to the oligonucleotide by a linking moiety selected from amino, hydroxyl, carboxylic acid, thiol, unsaturations (*e.g.*, double or triple bonds), 8-amino-3,6-dioxaoctanoic acid (ADO), succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), 6-aminohexanoic acid (AHEx or AHA), substituted C1-C10 alkyl, substituted or unsubstituted C2-C10 alkenyl, and substituted or unsubstituted C2-C10 alkynyl. In certain such embodiments, a substituent group is selected from hydroxyl,

amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl.

In certain such embodiments, the compound comprises the oligonucleotide having one or more stabilizing groups that are attached to one or both termini of the oligonucleotide to enhance properties such as, for example, nuclease stability. Included in stabilizing groups are cap structures. These terminal modifications protect the oligonucleotide from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap), or at the 3'-terminus (3'-cap), or can be present on both termini. Cap structures include, for example, inverted deoxy abasic caps.

Suitable cap structures include a 4',5'-methylene nucleotide, a 1-(beta-D-erythrofuransyl) nucleotide, a 4'-thio nucleotide, a carbocyclic nucleotide, a 1,5-anhydrohexitol nucleotide, an L-nucleotide, an alpha-nucleotide, a modified base nucleotide, a phosphorodithioate linkage, a threo-pentofuransyl nucleotide, an acyclic 3',4'-seco nucleotide, an acyclic 3,4-dihydroxybutyl nucleotide, an acyclic 3,5-dihydroxypentyl nucleotide, a 3'-3'-inverted nucleotide moiety, a 3'-3'-inverted abasic moiety, a 3'-2'-inverted nucleotide moiety, a 3'-2'-inverted abasic moiety, a 1,4-butanediol phosphate, a 3'-phosphoramidate, a hexylphosphate, an aminohexyl phosphate, a 3'-phosphate, a 3'-phosphorothioate, a phosphorodithioate, a bridging methylphosphonate moiety, and a non-bridging methylphosphonate moiety 5'-amino-alkyl phosphate, a 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, a 6-aminohexyl phosphate, a 1,2-aminododecyl phosphate, a hydroxypropyl phosphate, a 5'-5'-inverted nucleotide moiety, a 5'-5'-inverted abasic moiety, a 5'-phosphoramidate, a 5'-phosphorothioate, a 5'-amino, a bridging and/or non-bridging 5'-phosphoramidate, a phosphorothioate, and a 5'-mercapto moiety.

Small nucleic acids and/or antisense oligonucleotides can also contain a neutral peptide-like backbone. Such molecules are termed peptide nucleic acid (PNA)-oligomers and are described, *e.g.*, in Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93:14670 and in Eglom et al. (1993) Nature 365:566. One advantage of PNA oligomers is their capability to bind to complementary DNA essentially independently from the ionic strength of the medium due to the neutral backbone of the DNA. In yet another embodiment, small nucleic acids and/or antisense oligonucleotides comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a

phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In a further embodiment, small nucleic acids and/or antisense oligonucleotides are α -anomeric oligonucleotides. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al. (1987) Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al. (1987) Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al. (1987) FEBS Lett. 215:327-330).

Small nucleic acids and/or antisense oligonucleotides of the methods and compositions presented herein may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988) Nucl. Acids Res. 16:3209, methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc. For example, an isolated miRNA can be chemically synthesized or recombinantly produced using methods known in the art. In some instances, miRNA are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. Commercial suppliers of synthetic RNA molecules or synthesis reagents include, *e.g.*, Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, Colo., USA), Pierce Chemical (part of Perbio Science, Rockford, Ill., USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA), Cruachem (Glasgow, UK), and Exiqon (Vedbaek, Denmark).

Small nucleic acids and/or antisense oligonucleotides can be delivered to cells *in vivo*. A number of methods have been developed for delivering small nucleic acids and/or antisense oligonucleotides DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systematically.

In one embodiment, small nucleic acids and/or antisense oligonucleotides may comprise or be generated from double stranded small interfering RNAs (siRNAs), in which sequences fully complementary to cellular nucleic acids (*e.g.* mRNAs) sequences mediate

degradation or in which sequences incompletely complementary to cellular nucleic acids (e.g., mRNAs) mediate translational repression when expressed within cells, or piwiRNAs. In another embodiment, double stranded siRNAs can be processed into single stranded antisense RNAs that bind single stranded cellular RNAs (e.g., microRNAs) and inhibit their expression. RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. *in vivo*, long dsRNA is cleaved by ribonuclease III to generate 21- and 22-nucleotide siRNAs. It has been shown that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney (293) and HeLa cells (Elbashir et al. (2001) *Nature* 411:494-498). Accordingly, translation of a gene in a cell can be inhibited by contacting the cell with short double stranded RNAs having a length of about 15 to 30 nucleotides or of about 18 to 21 nucleotides or of about 19 to 21 nucleotides. Alternatively, a vector encoding for such siRNAs or short hairpin RNAs (shRNAs) that are metabolized into siRNAs can be introduced into a target cell (see, e.g., McManus et al. (2002) *RNA* 8:842; Xia et al. (2002) *Nature Biotechnology* 20:1006; and Brummelkamp et al. (2002) *Science* 296:550). Vectors that can be used are commercially available, e.g., from OligoEngine under the name pSuper RNAi SystemTM.

Ribozyme molecules designed to catalytically cleave cellular mRNA transcripts can also be used to prevent translation of cellular mRNAs and expression of cellular polypeptides, or both (See, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al. (1990) *Science* 247:1222-1225 and U.S. Patent No. 5,093,246). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy cellular mRNAs, the use of hammerhead ribozymes is preferred.

Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well-known in the art and is described more fully in Haseloff and Gerlach (1988) *Nature* 334:585-591. The ribozyme may be engineered so that the cleavage recognition site is located near the 5' end of cellular mRNAs; *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the methods presented herein also include RNA endoribonucleases (hereinafter “Cech-type ribozymes”) such as the one which occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug *et al.* (1984) *Science* 224:574-578; Zaug *et al.* (1986) *Science* 231:470-475; Zaug *et al.* (1986) *Nature* 324:429-433; WO 88/04300; and Been *et al.* (1986) *Cell* 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The methods and compositions presented herein encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in cellular genes.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.). A preferred method of delivery involves using a DNA construct “encoding” the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous cellular messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription of cellular genes are preferably single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides should promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in CGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called “switchback” nucleic acid molecule. Switchback molecules are synthesized in an alternating 5’-3’, 3’-5’ manner, such that they base pair

with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

Small nucleic acids (*e.g.*, miRNAs, pre-miRNAs, pri-miRNAs, miRNA*, anti-miRNA, or a miRNA binding site, or a variant thereof), antisense oligonucleotides, ribozymes, and triple helix molecules of the methods and compositions presented herein may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well-known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Moreover, various well-known modifications to nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone. One of skill in the art will readily understand that polypeptides, small nucleic acids, and antisense oligonucleotides can be further linked to another peptide or polypeptide (*e.g.*, a heterologous peptide), *e.g.*, that serves as a means of protein detection. Non-limiting examples of label peptide or polypeptide moieties useful for detection in the invention include, without limitation, suitable enzymes such as horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; epitope tags, such as FLAG, MYC, HA, or HIS tags; fluorophores such as green fluorescent protein; dyes; radioisotopes; digoxigenin; biotin; antibodies; polymers; as well as others known in the art, for example, in Principles of Fluorescence Spectroscopy, Joseph R. Lakowicz (Editor), Plenum Pub Corp, 2nd edition (July 1999).

The modulatory agents described herein (*e.g.*, antibodies, small molecules, peptides, fusion proteins, or small nucleic acids) can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. The compositions may contain a single

such molecule or agent or any combination of agents described herein. “Single active agents” described herein can be combined with other pharmacologically active compounds (“second active agents”) known in the art according to the methods and compositions provided herein.

5 The production and use of biomarker nucleic acid and/or biomarker polypeptide molecules described herein can be facilitated by using standard recombinant techniques. In some embodiments, such techniques use vectors, preferably expression vectors, containing a nucleic acid encoding a biomarker polypeptide or a portion of such a polypeptide. As used herein, the term “vector” refers to a nucleic acid molecule capable of transporting
10 another nucleic acid to which it has been linked. One type of vector is a “plasmid”, which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of
15 replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors, namely expression vectors, are capable of directing the expression of genes to which they are operably linked. In general, expression vectors of utility in recombinant DNA
20 techniques are often in the form of plasmids (vectors). However, the present invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

 The recombinant expression vectors of the present invention comprise a nucleic acid
25 of the present invention in a form suitable for expression of the nucleic acid in a host cell. This means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operably linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, “operably linked” is intended to mean that the nucleotide sequence of
30 interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term “regulatory sequence” is intended to include promoters, enhancers and other expression control elements (*e.g.*,

polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel, *Methods in Enzymology: Gene Expression Technology* vol.185, Academic Press, San Diego, CA (1991). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct
5 expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the present invention can be introduced into host cells to thereby produce proteins or
10 peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein.

The recombinant expression vectors for use in the present invention can be designed for expression of a polypeptide corresponding to a marker of the present invention in prokaryotic (*e.g.*, *E. coli*) or eukaryotic cells (*e.g.*, insect cells {using baculovirus
15 expression vectors}, yeast cells or mammalian cells). Suitable host cells are discussed further in Goeddel, *supra*. Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with
20 vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the
25 recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical
30 fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson, 1988, *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, 1988, *Gene* 69:301-315) and pET 11d (Studier *et al.*, p. 60-89, In *Gene Expression Technology: Methods in Enzymology* vol.185, Academic Press, San Diego, CA, 1991). Target biomarker nucleic acid expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target biomarker nucleic acid expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a co-expressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21 (DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacterium with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, p. 119-128, In *Gene Expression Technology: Methods in Enzymology* vol. 185, Academic Press, San Diego, CA, 1990. Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, 1992, *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the present invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the expression vector is a yeast expression vector.

Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari *et al.*, 1987, *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, 1982, *Cell* 30:933-943), pJRY88 (Schultz *et al.*, 1987, *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and pPicZ (Invitrogen Corp, San Diego, CA).

Alternatively, the expression vector is a baculovirus expression vector. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.*, 1983, *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers, 1989, *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the present invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, 1987, *Nature* 329:840) and pMT2PC (Kaufman *et al.*, 1987, *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian

Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook *et al.*, *supra*.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.*, 1987, *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton, 1988, *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989, *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.*, 1983, *Cell* 33:729-740; Queen and Baltimore, 1983, *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle, 1989, *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.*, 1985, *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss, 1990, *Science* 249:374-379) and the α -fetoprotein promoter (Camper and Tilghman, 1989, *Genes Dev.* 3:537-546).

The present invention further provides a recombinant expression vector comprising a DNA molecule cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operably linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to the mRNA encoding a polypeptide of the present invention. Regulatory sequences operably linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue-specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid, or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes (see Weintraub *et al.*, 1986, *Trends in Genetics*, Vol. 1(1)).

Another aspect of the present invention pertains to host cells into which a recombinant expression vector of the present invention has been introduced. The terms “host cell” and “recombinant host cell” are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential
5 progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic (*e.g.*, *E. coli*) or eukaryotic cell (*e.g.*, insect cells, yeast or mammalian cells).

10 Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms “transformation” and “transfection” are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation.

15 Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*supra*), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these
20 integrants, a gene that encodes a selectable marker (*e.g.*, for resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive,
25 while the other cells die).

V. Analyzing Biomarker Nucleic Acids and Polypeptides

Biomarker nucleic acids and/or biomarker polypeptides can be analyzed according to the methods described herein and techniques known to the skilled artisan to identify such
30 genetic or expression alterations useful for the present invention including, but not limited to, 1) an alteration in the level of a biomarker transcript or polypeptide, 2) a deletion or addition of one or more nucleotides from a biomarker gene, 4) a substitution of one or more

nucleotides of a biomarker gene, 5) aberrant modification of a biomarker gene, such as an expression regulatory region, and the like.

a. Methods for Detection of Copy Number

Methods of evaluating the copy number of a biomarker nucleic acid are well-known
5 to those of skill in the art. The presence or absence of chromosomal gain or loss can be evaluated simply by a determination of copy number of the regions or markers identified herein.

In one embodiment, a biological sample is tested for the presence of copy number changes in genomic loci containing the genomic marker. A copy number of at least 3, 4, 5,
10 6, 7, 8, 9, or 10 is predictive of poorer outcome of CX3CR1 and immune checkpoint combination inhibitor treatment.

Methods of evaluating the copy number of a biomarker locus include, but are not limited to, hybridization-based assays. Hybridization-based assays include, but are not limited to, traditional “direct probe” methods, such as Southern blots, *in situ* hybridization
15 (*e.g.*, FISH and FISH plus SKY) methods, and “comparative probe” methods, such as comparative genomic hybridization (CGH), *e.g.*, cDNA-based or oligonucleotide-based CGH. The methods can be used in a wide variety of formats including, but not limited to, substrate (*e.g.* membrane or glass) bound methods or array-based approaches.

In one embodiment, evaluating the biomarker gene copy number in a sample
20 involves a Southern Blot. In a Southern Blot, the genomic DNA (typically fragmented and separated on an electrophoretic gel) is hybridized to a probe specific for the target region. Comparison of the intensity of the hybridization signal from the probe for the target region with control probe signal from analysis of normal genomic DNA (*e.g.*, a non-amplified portion of the same or related cell, tissue, organ, *etc.*) provides an estimate of the relative
25 copy number of the target nucleic acid. Alternatively, a Northern blot may be utilized for evaluating the copy number of encoding nucleic acid in a sample. In a Northern blot, mRNA is hybridized to a probe specific for the target region. Comparison of the intensity of the hybridization signal from the probe for the target region with control probe signal from analysis of normal RNA (*e.g.*, a non-amplified portion of the same or related cell,
30 tissue, organ, *etc.*) provides an estimate of the relative copy number of the target nucleic acid. Alternatively, other methods well-known in the art to detect RNA can be used, such that higher or lower expression relative to an appropriate control (*e.g.*, a non-amplified

portion of the same or related cell tissue, organ, etc.) provides an estimate of the relative copy number of the target nucleic acid.

An alternative means for determining genomic copy number is *in situ* hybridization (*e.g.*, Angerer (1987) *Meth. Enzymol* 152: 649). Generally, *in situ* hybridization comprises the following steps: (1) fixation of tissue or biological structure to be analyzed; (2) prehybridization treatment of the biological structure to increase accessibility of target DNA, and to reduce nonspecific binding; (3) hybridization of the mixture of nucleic acids to the nucleic acid in the biological structure or tissue; (4) post-hybridization washes to remove nucleic acid fragments not bound in the hybridization and (5) detection of the hybridized nucleic acid fragments. The reagent used in each of these steps and the conditions for use vary depending on the particular application. In a typical *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If a nucleic acid is to be probed, the cells are typically denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled probes specific to the nucleic acid sequence encoding the protein. The targets (*e.g.*, cells) are then typically washed at a predetermined stringency or at an increasing stringency until an appropriate signal to noise ratio is obtained. The probes are typically labeled, *e.g.*, with radioisotopes or fluorescent reporters. In one embodiment, probes are sufficiently long so as to specifically hybridize with the target nucleic acid(s) under stringent conditions. Probes generally range in length from about 200 bases to about 1000 bases. In some applications it is necessary to block the hybridization capacity of repetitive sequences. Thus, in some embodiments, tRNA, human genomic DNA, or Cot-I DNA is used to block non-specific hybridization.

An alternative means for determining genomic copy number is comparative genomic hybridization. In general, genomic DNA is isolated from normal reference cells, as well as from test cells (*e.g.*, tumor cells) and amplified, if necessary. The two nucleic acids are differentially labeled and then hybridized *in situ* to metaphase chromosomes of a reference cell. The repetitive sequences in both the reference and test DNAs are either removed or their hybridization capacity is reduced by some means, for example by prehybridization with appropriate blocking nucleic acids and/or including such blocking nucleic acid sequences for said repetitive sequences during said hybridization. The bound, labeled DNA sequences are then rendered in a visualizable form, if necessary. Chromosomal regions in the test cells which are at increased or decreased copy number can

be identified by detecting regions where the ratio of signal from the two DNAs is altered. For example, those regions that have decreased in copy number in the test cells will show relatively lower signal from the test DNA than the reference compared to other regions of the genome. Regions that have been increased in copy number in the test cells will show relatively higher signal from the test DNA. Where there are chromosomal deletions or multiplications, differences in the ratio of the signals from the two labels will be detected and the ratio will provide a measure of the copy number. In another embodiment of CGH, array CGH (aCGH), the immobilized chromosome element is replaced with a collection of solid support bound target nucleic acids on an array, allowing for a large or complete percentage of the genome to be represented in the collection of solid support bound targets. Target nucleic acids may comprise cDNAs, genomic DNAs, oligonucleotides (*e.g.*, to detect single nucleotide polymorphisms) and the like. Array-based CGH may also be performed with single-color labeling (as opposed to labeling the control and the possible tumor sample with two different dyes and mixing them prior to hybridization, which will yield a ratio due to competitive hybridization of probes on the arrays). In single color CGH, the control is labeled and hybridized to one array and absolute signals are read, and the possible tumor sample is labeled and hybridized to a second array (with identical content) and absolute signals are read. Copy number difference is calculated based on absolute signals from the two arrays. Methods of preparing immobilized chromosomes or arrays and performing comparative genomic hybridization are well-known in the art (see, *e.g.*, U.S. Pat. Nos: 6,335,167; 6,197,501; 5,830,645; and 5,665,549 and Albertson (1984) *EMBO J.* 3: 1227-1234; Pinkel (1988) *Proc. Natl. Acad. Sci. USA* 85: 9138-9142; EPO Pub. No. 430,402; *Methods in Molecular Biology*, Vol. 33: *In situ* Hybridization Protocols, Choo, ed., Humana Press, Totowa, N.J. (1994), *etc.*) In another embodiment, the hybridization protocol of Pinkel, *et al.* (1998) *Nature Genetics* 20: 207-211, or of Kallioniemi (1992) *Proc. Natl Acad Sci USA* 89:5321-5325 (1992) is used.

In still another embodiment, amplification-based assays can be used to measure copy number. In such amplification-based assays, the nucleic acid sequences act as a template in an amplification reaction (*e.g.*, Polymerase Chain Reaction (PCR)). In a quantitative amplification, the amount of amplification product will be proportional to the amount of template in the original sample. Comparison to appropriate controls, *e.g.* healthy tissue, provides a measure of the copy number.

Methods of “quantitative” amplification are well-known to those of skill in the art.

For example, quantitative PCR involves simultaneously co-amplifying a known quantity of a control sequence using the same primers. This provides an internal standard that may be used to calibrate the PCR reaction. Detailed protocols for quantitative PCR are provided in Innis, *et al.* (1990) *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y.). Measurement of DNA copy number at microsatellite loci using quantitative PCR analysis is described in Ginzonger, *et al.* (2000) *Cancer Research* 60:5405-5409. The known nucleic acid sequence for the genes is sufficient to enable one of skill in the art to routinely select primers to amplify any portion of the gene. Fluorogenic quantitative PCR may also be used in the methods of the present invention. In fluorogenic quantitative PCR, quantitation is based on amount of fluorescence signals, *e.g.*, TaqMan and SYBR green.

Other suitable amplification methods include, but are not limited to, ligase chain reaction (LCR) (see Wu and Wallace (1989) *Genomics* 4: 560, Landegren, *et al.* (1988) *Science* 241:1077, and Barringer *et al.* (1990) *Gene* 89: 117), transcription amplification (Kwoh, *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 1173), self-sustained sequence replication (Guatelli, *et al.* (1990) *Proc. Nat. Acad. Sci. USA* 87: 1874), dot PCR, and linker adapter PCR, *etc.*

Loss of heterozygosity (LOH) and major copy proportion (MCP) mapping (Wang, Z.C., *et al.* (2004) *Cancer Res* 64(1):64-71; Seymour, A. B., *et al.* (1994) *Cancer Res* 54, 2761-4; Hahn, S. A., *et al.* (1995) *Cancer Res* 55, 4670-5; Kimura, M., *et al.* (1996) *Genes Chromosomes Cancer* 17, 88-93; Li *et al.*, (2008) *MBC Bioinform.* 9, 204-219) may also be used to identify regions of amplification or deletion.

b. Methods for Detection of Biomarker Nucleic Acid Expression

Biomarker expression may be assessed by any of a wide variety of well-known methods for detecting expression of a transcribed molecule or protein. Non-limiting examples of such methods include immunological methods for detection of secreted, cell-surface, cytoplasmic, or nuclear proteins, protein purification methods, protein function or activity assays, nucleic acid hybridization methods, nucleic acid reverse transcription methods, and nucleic acid amplification methods.

In preferred embodiments, activity of a particular gene is characterized by a measure of gene transcript (*e.g.* mRNA), by a measure of the quantity of translated protein, or by a measure of gene product activity. Marker expression can be monitored in a variety of ways, including by detecting mRNA levels, protein levels, or protein activity, any of which can be measured using standard techniques. Detection can involve quantification of

the level of gene expression (*e.g.*, genomic DNA, cDNA, mRNA, protein, or enzyme activity), or, alternatively, can be a qualitative assessment of the level of gene expression, in particular in comparison with a control level. The type of level being detected will be clear from the context.

5 In another embodiment, detecting or determining expression levels of a biomarker and functionally similar homologs thereof, including a fragment or genetic alteration thereof (*e.g.*, in regulatory or promoter regions thereof) comprises detecting or determining RNA levels for the marker of interest. In one embodiment, one or more cells from the subject to be tested are obtained and RNA is isolated from the cells. In a preferred
10 embodiment, a sample of breast tissue cells is obtained from the subject.

 In one embodiment, RNA is obtained from a single cell. For example, a cell can be isolated from a tissue sample by laser capture microdissection (LCM). Using this technique, a cell can be isolated from a tissue section, including a stained tissue section, thereby assuring that the desired cell is isolated (*see, e.g.*, Bonner *et al.* (1997) Science 278:
15 1481; Emmert-Buck *et al.* (1996) Science 274:998; Fend *et al.* (1999) Am. J. Path. 154: 61 and Murakami *et al.* (2000) Kidney Int. 58:1346). For example, Murakami *et al.*, *supra*, describe isolation of a cell from a previously immunostained tissue section.

 It is also possible to obtain cells from a subject and culture the cells *in vitro*, such as to obtain a larger population of cells from which RNA can be extracted. Methods for
20 establishing cultures of non-transformed cells, *i.e.*, primary cell cultures, are known in the art.

 When isolating RNA from tissue samples or cells from individuals, it may be important to prevent any further changes in gene expression after the tissue or cells has been removed from the subject. Changes in expression levels are known to change rapidly
25 following perturbations, *e.g.*, heat shock or activation with lipopolysaccharide (LPS) or other reagents. In addition, the RNA in the tissue and cells may quickly become degraded. Accordingly, in a preferred embodiment, the tissue or cells obtained from a subject is snap frozen as soon as possible.

 RNA can be extracted from the tissue sample by a variety of methods, *e.g.*, the
30 guanidium thiocyanate lysis followed by CsCl centrifugation (Chirgwin *et al.*, 1979, Biochemistry 18:5294-5299). RNA from single cells can be obtained as described in methods for preparing cDNA libraries from single cells, such as those described in Dulac,

C. (1998) Curr. Top. Dev. Biol. 36, 245 and Jena *et al.* (1996) J. Immunol. Methods 190:199. Care to avoid RNA degradation must be taken, *e.g.*, by inclusion of RNAsin.

The RNA sample can then be enriched in particular species. In one embodiment, poly(A)+ RNA is isolated from the RNA sample. In general, such purification takes
5 advantage of the poly-A tails on mRNA. In particular and as noted above, poly-T oligonucleotides may be immobilized within on a solid support to serve as affinity ligands for mRNA. Kits for this purpose are commercially available, *e.g.*, the MessageMaker kit (Life Technologies, Grand Island, NY).

In a preferred embodiment, the RNA population is enriched in marker sequences.
10 Enrichment can be undertaken, *e.g.*, by primer-specific cDNA synthesis, or multiple rounds of linear amplification based on cDNA synthesis and template-directed *in vitro* transcription (*see, e.g.*, Wang *et al.* (1989) PNAS 86, 9717; Dulac *et al.*, *supra*, and Jena *et al.*, *supra*).

The population of RNA, enriched or not in particular species or sequences, can
15 further be amplified. As defined herein, an “amplification process” is designed to strengthen, increase, or augment a molecule within the RNA. For example, where RNA is mRNA, an amplification process such as RT-PCR can be utilized to amplify the mRNA, such that a signal is detectable or detection is enhanced. Such an amplification process is beneficial particularly when the biological, tissue, or tumor sample is of a small size or
20 volume.

Various amplification and detection methods can be used. For example, it is within the scope of the present invention to reverse transcribe mRNA into cDNA followed by polymerase chain reaction (RT-PCR); or, to use a single enzyme for both steps as described in U.S. Pat. No. 5,322,770, or reverse transcribe mRNA into cDNA followed by symmetric
25 gap ligase chain reaction (RT-AGLCR) as described by R. L. Marshall, *et al.*, PCR Methods and Applications 4: 80-84 (1994). Real time PCR may also be used.

Other known amplification methods which can be utilized herein include but are not limited to the so-called “NASBA” or “3SR” technique described in PNAS USA 87: 1874-1878 (1990) and also described in Nature 350 (No. 6313): 91-92 (1991); Q-beta
30 amplification as described in published European Patent Application (EPA) No. 4544610; strand displacement amplification (as described in G. T. Walker *et al.*, Clin. Chem. 42: 9-13 (1996) and European Patent Application No. 684315; target mediated amplification, as described by PCT Publication WO9322461; PCR; ligase chain reaction (LCR) (*see, e.g.*,

Wu and Wallace, *Genomics* 4, 560 (1989), Landegren *et al.*, *Science* 241, 1077 (1988)); self-sustained sequence replication (SSR) (*see, e.g.*, Guatelli *et al.*, *Proc. Nat. Acad. Sci. USA*, 87, 1874 (1990)); and transcription amplification (*see, e.g.*, Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA* 86, 1173 (1989)).

5 Many techniques are known in the state of the art for determining absolute and relative levels of gene expression, commonly used techniques suitable for use in the present invention include Northern analysis, RNase protection assays (RPA), microarrays and PCR-based techniques, such as quantitative PCR and differential display PCR. For example, Northern blotting involves running a preparation of RNA on a denaturing agarose gel, and
10 transferring it to a suitable support, such as activated cellulose, nitrocellulose or glass or nylon membranes. Radiolabeled cDNA or RNA is then hybridized to the preparation, washed and analyzed by autoradiography.

In situ hybridization visualization may also be employed, wherein a radioactively labeled antisense RNA probe is hybridized with a thin section of a biopsy sample, washed,
15 cleaved with RNase and exposed to a sensitive emulsion for autoradiography. The samples may be stained with hematoxylin to demonstrate the histological composition of the sample, and dark field imaging with a suitable light filter shows the developed emulsion. Non-radioactive labels such as digoxigenin may also be used.

Alternatively, mRNA expression can be detected on a DNA array, chip or a
20 microarray. Labeled nucleic acids of a test sample obtained from a subject may be hybridized to a solid surface comprising biomarker DNA. Positive hybridization signal is obtained with the sample containing biomarker transcripts. Methods of preparing DNA arrays and their use are well-known in the art (*see, e.g.*, U.S. Pat. Nos: 6,618,6796; 6,379,897; 6,664,377; 6,451,536; 548,257; U.S. 20030157485 and Schena *et al.* (1995)
25 *Science* 20, 467-470; Gerhold *et al.* (1999) *Trends In Biochem. Sci.* 24, 168-173; and Lennon *et al.* (2000) *Drug Discovery Today* 5, 59-65, which are herein incorporated by reference in their entirety). Serial Analysis of Gene Expression (SAGE) can also be performed (See for example U.S. Patent Application 20030215858).

To monitor mRNA levels, for example, mRNA is extracted from the biological
30 sample to be tested, reverse transcribed, and fluorescently-labeled cDNA probes are generated. The microarrays capable of hybridizing to marker cDNA are then probed with the labeled cDNA probes, the slides scanned and fluorescence intensity measured. This intensity correlates with the hybridization intensity and expression levels.

Types of probes that can be used in the methods described herein include cDNA, riboprobes, synthetic oligonucleotides and genomic probes. The type of probe used will generally be dictated by the particular situation, such as riboprobes for *in situ* hybridization, and cDNA for Northern blotting, for example. In one embodiment, the probe is directed to nucleotide regions unique to the RNA. The probes may be as short as is required to differentially recognize marker mRNA transcripts, and may be as short as, for example, 15 bases; however, probes of at least 17, 18, 19 or 20 or more bases can be used. In one embodiment, the primers and probes hybridize specifically under stringent conditions to a DNA fragment having the nucleotide sequence corresponding to the marker. As herein used, the term “stringent conditions” means hybridization will occur only if there is at least 95% identity in nucleotide sequences. In another embodiment, hybridization under “stringent conditions” occurs when there is at least 97% identity between the sequences.

The form of labeling of the probes may be any that is appropriate, such as the use of radioisotopes, for example, ^{32}P and ^{35}S . Labeling with radioisotopes may be achieved, whether the probe is synthesized chemically or biologically, by the use of suitably labeled bases.

In one embodiment, the biological sample contains polypeptide molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting marker polypeptide, mRNA, genomic DNA, or fragments thereof, such that the presence of the marker polypeptide, mRNA, genomic DNA, or fragments thereof, is detected in the biological sample, and comparing the presence of the marker polypeptide, mRNA, genomic DNA, or fragments thereof, in the control sample with the presence of the marker polypeptide, mRNA, genomic DNA, or fragments thereof in the test sample.

c. Methods for Detection of Biomarker Protein Expression

The activity or level of a biomarker protein can be detected and/or quantified by detecting or quantifying the expressed polypeptide. The polypeptide can be detected and quantified by any of a number of means well-known to those of skill in the art. Aberrant levels of polypeptide expression of the polypeptides encoded by a biomarker nucleic acid and functionally similar homologs thereof, including a fragment or genetic alteration thereof (*e.g.*, in regulatory or promoter regions thereof) are associated with the likelihood of

response of a cancer to CX3CR1/immune checkpoint combination inhibitor therapy. Any method known in the art for detecting polypeptides can be used. Such methods include, but are not limited to, immunodiffusion, immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, Western blotting, binder-ligand assays, immunohistochemical techniques, agglutination, complement assays, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like (*e.g.*, Basic and Clinical Immunology, Sites and Terr, eds., Appleton and Lange, Norwalk, Conn. pp 217-262, 1991 which is incorporated by reference). Preferred are binder-ligand immunoassay methods including reacting antibodies with an epitope or epitopes and competitively displacing a labeled polypeptide or derivative thereof.

For example, ELISA and RIA procedures may be conducted such that a desired biomarker protein standard is labeled (with a radioisotope such as ^{125}I or ^{35}S , or an assayable enzyme, such as horseradish peroxidase or alkaline phosphatase), and, together with the unlabeled sample, brought into contact with the corresponding antibody, whereon a second antibody is used to bind the first, and radioactivity or the immobilized enzyme assayed (competitive assay). Alternatively, the biomarker protein in the sample is allowed to react with the corresponding immobilized antibody, radioisotope- or enzyme-labeled anti-biomarker protein antibody is allowed to react with the system, and radioactivity or the enzyme assayed (ELISA-sandwich assay). Other conventional methods may also be employed as suitable.

The above techniques may be conducted essentially as a “one-step” or “two-step” assay. A “one-step” assay involves contacting antigen with immobilized antibody and, without washing, contacting the mixture with labeled antibody. A “two-step” assay involves washing before contacting, the mixture with labeled antibody. Other conventional methods may also be employed as suitable.

In one embodiment, a method for measuring biomarker protein levels comprises the steps of: contacting a biological specimen with an antibody or variant (*e.g.*, fragment) thereof which selectively binds the biomarker protein, and detecting whether said antibody or variant thereof is bound to said sample and thereby measuring the levels of the biomarker protein.

Enzymatic and radiolabeling of biomarker protein and/or the antibodies may be effected by conventional means. Such means will generally include covalent linking of the

enzyme to the antigen or the antibody in question, such as by glutaraldehyde, specifically so as not to adversely affect the activity of the enzyme, by which is meant that the enzyme must still be capable of interacting with its substrate, although it is not necessary for all of the enzyme to be active, provided that enough remains active to permit the assay to be effected. Indeed, some techniques for binding enzyme are non-specific (such as using formaldehyde), and will only yield a proportion of active enzyme.

It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed without laborious and time-consuming labor. It is possible for a second phase to be immobilized away from the first, but one phase is usually sufficient.

It is possible to immobilize the enzyme itself on a support, but if solid-phase enzyme is required, then this is generally best achieved by binding to antibody and affixing the antibody to a support, models and systems for which are well-known in the art. Simple polyethylene may provide a suitable support.

Enzymes employable for labeling are not particularly limited, but may be selected from the members of the oxidase group, for example. These catalyze production of hydrogen peroxide by reaction with their substrates, and glucose oxidase is often used for its good stability, ease of availability and cheapness, as well as the ready availability of its substrate (glucose). Activity of the oxidase may be assayed by measuring the concentration of hydrogen peroxide formed after reaction of the enzyme-labeled antibody with the substrate under controlled conditions well-known in the art.

Other techniques may be used to detect biomarker protein according to a practitioner's preference based upon the present disclosure. One such technique is Western blotting (Towbin et al., Proc. Nat. Acad. Sci. 76:4350 (1979)), wherein a suitably treated sample is run on an SDS-PAGE gel before being transferred to a solid support, such as a nitrocellulose filter. Anti-biomarker protein antibodies (unlabeled) are then brought into contact with the support and assayed by a secondary immunological reagent, such as labeled protein A or anti-immunoglobulin (suitable labels including ^{125}I , horseradish peroxidase and alkaline phosphatase). Chromatographic detection may also be used.

Immunohistochemistry may be used to detect expression of biomarker protein, *e.g.*, in a biopsy sample. A suitable antibody is brought into contact with, for example, a thin layer of cells, washed, and then contacted with a second, labeled antibody. Labeling may

be by fluorescent markers, enzymes, such as peroxidase, avidin, or radiolabeling. The assay is scored visually, using microscopy.

Anti-biomarker protein antibodies, such as intrabodies, may also be used for imaging purposes, for example, to detect the presence of biomarker protein in cells and tissues of a subject. Suitable labels include radioisotopes, iodine (^{125}I , ^{121}I), carbon (^{14}C), sulphur (^{35}S), tritium (^3H), indium (^{112}In), and technetium ($^{99\text{m}}\text{Tc}$), fluorescent labels, such as fluorescein and rhodamine, and biotin.

For *in vivo* imaging purposes, antibodies are not detectable, as such, from outside the body, and so must be labeled, or otherwise modified, to permit detection. Markers for this purpose may be any that do not substantially interfere with the antibody binding, but which allow external detection. Suitable markers may include those that may be detected by X-radiography, NMR or MRI. For X-radiographic techniques, suitable markers include any radioisotope that emits detectable radiation but that is not overtly harmful to the subject, such as barium or cesium, for example. Suitable markers for NMR and MRI generally include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by suitable labeling of nutrients for the relevant hybridoma, for example.

The size of the subject, and the imaging system used, will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of technetium-99. The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain biomarker protein. The labeled antibody or antibody fragment can then be detected using known techniques.

Antibodies that may be used to detect biomarker protein include any antibody, whether natural or synthetic, full length or a fragment thereof, monoclonal or polyclonal, that binds sufficiently strongly and specifically to the biomarker protein to be detected. An antibody may have a K_d of at most about 10^{-6}M , 10^{-7}M , 10^{-8}M , 10^{-9}M , 10^{-10}M , 10^{-11}M , 10^{-12}M . The phrase “specifically binds” refers to binding of, for example, an antibody to an epitope or antigen or antigenic determinant in such a manner that binding can be displaced or competed with a second preparation of identical or similar epitope, antigen or antigenic determinant. An antibody may bind preferentially to the biomarker protein relative to other proteins, such as related proteins.

Antibodies are commercially available or may be prepared according to methods known in the art.

Antibodies and derivatives thereof that may be used encompass polyclonal or monoclonal antibodies, chimeric, human, humanized, primatized (CDR-grafted), veneered or single-chain antibodies as well as functional fragments, *i.e.*, biomarker protein binding fragments, of antibodies. For example, antibody fragments capable of binding to a biomarker protein or portions thereof, including, but not limited to, Fv, Fab, Fab' and F(ab')₂ fragments can be used. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For example, papain or pepsin cleavage can generate Fab or F(ab')₂ fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or F(ab')₂ fragments. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a chimeric gene encoding a F(ab')₂ heavy chain portion can be designed to include DNA sequences encoding the CH₁ domain and hinge region of the heavy chain.

Synthetic and engineered antibodies are described in, *e.g.*, Cabilly *et al.*, U.S. Pat. No. 4,816,567 Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S. Pat. No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M. S. *et al.*, WO 86/01533; Neuberger, M. S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Pat. No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, European Patent No. 0451216 B1; and Padlan, E. A. *et al.*, EP 0519596 A1. See also, Newman, R. *et al.*, BioTechnology, 10: 1455-1460 (1992), regarding primatized antibody, and Ladner *et al.*, U.S. Pat. No. 4,946,778 and Bird, R. E. *et al.*, Science, 242: 423-426 (1988)) regarding single-chain antibodies. Antibodies produced from a library, *e.g.*, phage display library, may also be used.

In some embodiments, agents that specifically bind to a biomarker protein other than antibodies are used, such as peptides. Peptides that specifically bind to a biomarker protein can be identified by any means known in the art. For example, specific peptide binders of a biomarker protein can be screened for using peptide phage display libraries.

d. Methods for Detection of Biomarker Structural Alterations

The following illustrative methods can be used to identify the presence of a structural alteration in a biomarker nucleic acid and/or biomarker polypeptide molecule in

order to, for example, identify CX3CR1/immune checkpoint pathway proteins that are overexpressed, overfunctional, and the like.

In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:360-364), the latter of which can be particularly useful for detecting point mutations in a biomarker nucleic acid such as a biomarker gene (see Abravaya *et al.* (1995) *Nucleic Acids Res.* 23:675-682). This method can include the steps of collecting a sample of cells from a subject, isolating nucleic acid (*e.g.*, genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a biomarker gene under conditions such that hybridization and amplification of the biomarker gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self-sustained sequence replication (Guatelli, J. C. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh, D. Y. *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi, P. M. *et al.* (1988) *Bio-Technology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well-known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a biomarker nucleic acid from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Pat. No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in biomarker nucleic acid can be identified by hybridizing a sample and control nucleic acids, *e.g.*, DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotide probes (Cronin, M. T. *et al.* (1996) *Hum. Mutat.* 7:244-255; Kozal, M. J. *et al.* (1996) *Nat. Med.* 2:753-759). For
5 example, biomarker genetic mutations can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin *et al.* (1996) *supra*. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential, overlapping probes. This step allows the identification of point mutations.
10 This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene. Such biomarker genetic mutations can be identified in a variety of contexts, including, for
15 example, germline and somatic mutations.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence a biomarker gene and detect mutations by comparing the sequence of the sample biomarker with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam
20 and Gilbert (1977) *Proc. Natl. Acad. Sci. USA* 74:560 or Sanger (1977) *Proc. Natl. Acad. Sci. USA* 74:5463. It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays (Naeve (1995) *Biotechniques* 19:448-53), including sequencing by mass spectrometry (see, *e.g.*, PCT International Publication No. WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-
25 162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in a biomarker gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) *Science* 230:1242). In general, the art technique of “mismatch cleavage” starts by providing heteroduplexes formed by
30 hybridizing (labeled) RNA or DNA containing the wild-type biomarker sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to base pair mismatches between the control and sample strands.

For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with SI nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4397 and Saleeba *et al.* (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called “DNA mismatch repair” enzymes) in defined systems for detecting and mapping point mutations in biomarker cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 15:1657-1662). According to an exemplary embodiment, a probe based on a biomarker sequence, *e.g.*, a wild-type biomarker treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like (*e.g.*, U.S. Pat. No. 5,459,039.)

In other embodiments, alterations in electrophoretic mobility can be used to identify mutations in biomarker genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci USA* 86:2766; see also Cotton (1993) *Mutat. Res.* 285:125-144 and Hayashi (1992) *Genet. Anal. Tech. Appl.* 9:73-79). Single-stranded DNA fragments of sample and control biomarker nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet.* 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA will be modified to ensure that it does not
5 completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys. Chem.* 265:12753).

Examples of other techniques for detecting point mutations include, but are not
10 limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature* 324:163; Saiki *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6230). Such allele specific
15 oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides
20 used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel
25 restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a
30 specific site by looking for the presence or absence of amplification.

3. Anti-Cancer Therapies

The efficacy of CX3CR1 and immune checkpoint combination inhibitor therapy is

predicted according to biomarker amount and/or activity associated with a cancer in a subject according to the methods described herein. In one embodiment, such CX3CR1/immune checkpoint combination inhibitor therapy or combinations of therapies (*e.g.*, one or more CX3CR1/immune checkpoint combination inhibitors in combination with one or more additional anti-cancer therapies, such as another immune checkpoint inhibitor) can be administered, particularly if a subject has first been indicated as being a likely responder to CX3CR1/immune checkpoint combination inhibitor therapy. In another embodiment, such CX3CR1/immune checkpoint combination inhibitor therapy can be avoided once a subject is indicated as not being a likely responder to CX3CR1/immune checkpoint combination inhibitor therapy and an alternative treatment regimen, such as targeted and/or untargeted anti-cancer therapies can be administered. Combination therapies are also contemplated and can comprise, for example, one or more chemotherapeutic agents and radiation, one or more chemotherapeutic agents and immunotherapy, or one or more chemotherapeutic agents, radiation and chemotherapy, each combination of which can be with anti-immune checkpoint therapy. In addition, any representative embodiment of an agent to modulate a particular target can be adapted to any other target described herein by the ordinarily skilled artisan (*e.g.*, direct and indirect PD-1 inhibitors described herein can be applied to other immune checkpoint inhibitors and/or CX3CR1, such as monospecific antibodies, bispecific antibodies, non-activating forms, small molecules, peptides, interfering nucleic acids, and the like).

The term “targeted therapy” refers to administration of agents that selectively interact with a chosen biomolecule to thereby treat cancer. One example includes immune checkpoint inhibitors, which are well-known in the art. For example, anti-PD-1 pathway agents, such as therapeutic monoclonal blocking antibodies, which are well-known in the art and described above, can be used to target tumor microenvironments and cells expressing unwanted components of the PD-1 pathway, such as PD-1, PD-L1, and/or PD-L2.

For example, the term “PD-1 pathway” refers to the PD-1 receptor and its ligands, PD-L1 and PD-L2. “PD-1 pathway inhibitors” block or otherwise reduce the interaction between PD-1 and one or both of its ligands such that the immunoinhibitory signaling otherwise generated by the interaction is blocked or otherwise reduced. Anti-immune checkpoint inhibitors can be direct or indirect. Direct anti-immune checkpoint inhibitors block or otherwise reduce the interaction between an immune checkpoint and at least one of

its ligands. For example, PD-1 inhibitors can block PD-1 binding with one or both of its ligands. Direct PD-1 combination inhibitors are well-known in the art, especially since the natural binding partners of PD-1 (*e.g.*, PD-L1 and PD-L2), PD-L1 (*e.g.*, PD-1 and B7-1), and PD-L2 (*e.g.*, PD-1 and RGMb) are known.

5 For example, agents which directly block the interaction between PD-1 and PD-L1, PD-1 and PD-L2, PD-1 and both PD-L1 and PD-L2, such as a bispecific antibody, can prevent inhibitory signaling and upregulate an immune response (*i.e.*, as a PD-1 pathway inhibitor). Alternatively, agents that indirectly block the interaction between PD-1 and one or both of its ligands can prevent inhibitory signaling and upregulate an immune response.

10 For example, B7-1 or a soluble form thereof, by binding to a PD-L1 polypeptide indirectly reduces the effective concentration of PD-L1 polypeptide available to bind to PD-1. Exemplary agents include monospecific or bispecific blocking antibodies against PD-1, PD-L1, and/or PD-L2 that block the interaction between the receptor and ligand(s); a non-activating form of PD-1, PD-L1, and/or PD-L2 (*e.g.*, a dominant negative or soluble polypeptide), small molecules or peptides that block the interaction between PD-1, PD-L1, and/or PD-L2; fusion proteins (*e.g.* the extracellular portion of PD-1, PD-L1, and/or PD-L2, fused to the Fc portion of an antibody or immunoglobulin) that bind to PD-1, PD-L1, and/or PD-L2 and inhibit the interaction between the receptor and ligand(s); a non-activating form of a natural PD-1, PD-L2, and/or PD-L2 ligand, and a soluble form of a natural PD-1, PD-
15 L2, and/or PD-L2 ligand.

Indirect anti-immune checkpoint inhibitors block or otherwise reduce the immunoinhibitory signaling generated by the interaction between the immune checkpoint and at least one of its ligands. For example, an inhibitor can block the interaction between PD-1 and one or both of its ligands without necessarily directly blocking the interaction
25 between PD-1 and one or both of its ligands. For example, indirect inhibitors include intrabodies that bind the intracellular portion of PD-1 and/or PD-L1 required to signal to block or otherwise reduce the immunoinhibitory signaling. Similarly, nucleic acids that reduce the expression of PD-1, PD-L1, and/or PD-L2 can indirectly inhibit the interaction between PD-1 and one or both of its ligands by removing the availability of components for
30 interaction. Such nucleic acid molecules can block PD-L1, PD-L2, and/or PD-L2 transcription or translation.

Similarly, agents which directly block the interaction between CX3CL1 and CX3CR1, such as an anti-CX3CR1 antibody, an anti-CX3CL1 antibody, an anti-

CX3CR1/anti-immune checkpoint bispecific antibody, and the like, can prevent the CX3CL1/CX3CR1 signaling and its downstream immune responses. Alternatively, agents that indirectly block the interaction between CX3CL1 and CX3CR1 can prevent the CX3CL1/CX3CR1 signaling and its downstream immune responses. For example, a
5 soluble form of CX3CR1, such as an extracellular domain of CX3CR1, by binding to a CX3CL1 polypeptide indirectly reduces the effective concentration of CX3CL1 polypeptide available to bind to CX3CR1 on cell surface. Exemplary agents include monospecific or bispecific blocking antibodies against CX3CR1 and/or CX3CL1 that block the interaction between the receptor and ligand(s); a non-activating form of CX3CR1 and/or
10 CX3CL1 (*e.g.*, a dominant negative or soluble polypeptide), small molecules or peptides that block the interaction between CX3CR1 and CX3CL1; fusion proteins (*e.g.* the extracellular portion of CX3CR1 or CX3CL1, fused to the Fc portion of an antibody or immunoglobulin) that bind to CX3CL1 or CX3CR1 and inhibit the interaction between the receptor and ligand(s); a non-activating form of a natural CX3CR1 and/or CX3CL1, and a
15 soluble form of a natural CX3CR1 and/or CX3CL1.

Immunotherapies that are designed to elicit or amplify an immune response are referred to as “activation immunotherapies.” Immunotherapies that are designed to reduce or suppress an immune response are referred to as “suppression immunotherapies.” Any agent believed to have an immune system effect on the genetically modified transplanted
20 cancer cells can be assayed to determine whether the agent is an immunotherapy and the effect that a given genetic modification has on the modulation of immune response. In some embodiments, the immunotherapy is cancer cell-specific. In some embodiments, immunotherapy can be “untargeted,” which refers to administration of agents that do not selectively interact with immune system cells, yet modulates immune system function.
25 Representative examples of untargeted therapies include, without limitation, chemotherapy, gene therapy, and radiation therapy.

Immunotherapy can involve passive immunity for short-term protection of a host, achieved by the administration of pre-formed antibody directed against a cancer antigen or disease antigen (*e.g.*, administration of a monoclonal antibody, optionally linked to a
30 chemotherapeutic agent or toxin, to a tumor antigen). Immunotherapy can also focus on using the cytotoxic lymphocyte-recognized epitopes of cancer cell lines. Alternatively, antisense polynucleotides, ribozymes, RNA interference molecules, triple helix

polynucleotides and the like, can be used to selectively modulate biomolecules that are linked to the initiation, progression, and/or pathology of a tumor or cancer.

In one embodiment, immunotherapy comprises adoptive cell-based immunotherapies. Well-known adoptive cell-based immunotherapeutic modalities, including, without limitation, Irradiated autologous or allogeneic tumor cells, tumor lysates or apoptotic tumor cells, antigen-presenting cell-based immunotherapy, dendritic cell-based immunotherapy, adoptive T cell transfer, adoptive CAR T cell therapy, autologous immune enhancement therapy (AIET), cancer vaccines, and/or antigen presenting cells. Such cell-based immunotherapies can be further modified to express one or more gene products to further modulate immune responses, such as expressing cytokines like GM-CSF, and/or to express tumor-associated antigen (TAA) antigens, such as Mage-1, gp-100, patient-specific neoantigen vaccines, and the like.

In another embodiment, immunotherapy comprises non-cell-based immunotherapies. In one embodiment, compositions comprising antigens with or without vaccine-enhancing adjuvants are used. Such compositions exist in many well-known forms, such as peptide compositions, oncolytic viruses, recombinant antigen comprising fusion proteins, and the like. In still another embodiment, immunomodulatory interleukins, such as IL-2, IL-6, IL-7, IL-12, IL-17, IL-23, and the like, as well as modulators thereof (*e.g.*, blocking antibodies or more potent or longer lasting forms) are used. In yet another embodiment, immunomodulatory cytokines, such as interferons, G-CSF, imiquimod, TNFalpha, and the like, as well as modulators thereof (*e.g.*, blocking antibodies or more potent or longer lasting forms) are used. In another embodiment, immunomodulatory chemokines, such as CCL3, CCL26, and CXCL7, and the like, as well as modulators thereof (*e.g.*, blocking antibodies or more potent or longer lasting forms) are used. In another embodiment, immunomodulatory molecules targeting immunosuppression, such as STAT3 signaling modulators, NFkappaB signaling modulators, and immune checkpoint modulators, are used. The terms “immune checkpoint” and “anti-immune checkpoint therapy” are described above.

In still another embodiment, immunomodulatory drugs, such as immunocytostatic drugs, glucocorticoids, cytostatics, immunophilins and modulators thereof (*e.g.*, rapamycin, a calcineurin inhibitor, tacrolimus, ciclosporin (cyclosporin), pimecrolimus, abetimus, gusperimus, ridaforolimus, everolimus, temsirolimus, zotarolimus, etc.), hydrocortisone (cortisol), cortisone acetate, prednisone, prednisolone, methylprednisolone, dexamethasone,

betamethasone, triamcinolone, beclometasone, fludrocortisone acetate, deoxycorticosterone acetate (doca) aldosterone, a non-glucocorticoid steroid, a pyrimidine synthesis inhibitor, leflunomide, teriflunomide, a folic acid analog, methotrexate, anti-thymocyte globulin, anti-lymphocyte globulin, thalidomide, lenalidomide, pentoxifylline, bupropion, curcumin, catechin, an opioid, an IMPDH inhibitor, mycophenolic acid, myriocin, fingolimod, an NF- κ B inhibitor, raloxifene, drotrecogin alfa, denosumab, an NF- κ B signaling cascade inhibitor, disulfiram, olmesartan, dithiocarbamate, a proteasome inhibitor, bortezomib, MG132, Prol, NPI-0052, curcumin, genistein, resveratrol, parthenolide, thalidomide, lenalidomide, flavopiridol, non-steroidal anti-inflammatory drugs (NSAIDs), arsenic trioxide, dehydroxymethylepoxyquinomycin (DHMEQ), I3C(indole-3-carbinol)/DIM(di-indolmethane) (I3C/DIM), Bay 11-7082, luteolin, cell permeable peptide SN-50, I κ B α -super repressor overexpression, NF κ B decoy oligodeoxynucleotide (ODN), or a derivative or analog of any thereof, are used. In yet another embodiment, immunomodulatory antibodies or protein are used. For example, antibodies that bind to CD40, Toll-like receptor (TLR), OX40, GITR, CD27, or to 4-1BB, T-cell bispecific antibodies, an anti-IL-2 receptor antibody, an anti-CD3 antibody, OKT3 (muromonab), orelizumab, teplizumab, visilizumab, an anti-CD4 antibody, clenoliximab, keliximab, zanolimumab, an anti-CD11a antibody, efalizumab, an anti-CD18 antibody, erlizumab, rovelizumab, an anti-CD20 antibody, afutuzumab, ocrelizumab, ofatumumab, pascolizumab, rituximab, an anti-CD23 antibody, lumiliximab, an anti-CD40 antibody, teneliximab, toralizumab, an anti-CD40L antibody, ruplizumab, an anti-CD62L antibody, aselizumab, an anti-CD80 antibody, galiximab, an anti-CD147 antibody, gavilimomab, a B-Lymphocyte stimulator (BLyS) inhibiting antibody, belimumab, an CTLA4-Ig fusion protein, abatacept, belatacept, an anti-CTLA4 antibody, ipilimumab, tremelimumab, an anti-eotaxin 1 antibody, bertilimumab, an anti- α 4-integrin antibody, natalizumab, an anti-IL-6R antibody, tocilizumab, an anti-LFA-1 antibody, odulimomab, an anti-CD25 antibody, basiliximab, daclizumab, inolimomab, an anti-CD5 antibody, zolimomab, an anti-CD2 antibody, sipilizumab, nerelimomab, faralimomab, atlizumab, atorolimumab, cedelizumab, dorlimomab aritox, dorlixizumab, fontolizumab, gantenerumab, gomiliximab, lebrilizumab, maslimomab, morolimumab, pexelizumab, reslizumab, rovelizumab, talizumab, telimomab aritox, vapaliximab, vepalimumab, aflibercept, alefacept, rilonacept, an IL-1 receptor antagonist, anakinra, an anti-IL-5 antibody, mepolizumab, an IgE inhibitor, omalizumab, talizumab, an IL12 inhibitor, an IL23 inhibitor, ustekinumab, and the like.

Nutritional supplements that enhance immune responses, such as vitamin A, vitamin E, vitamin C, and the like, are well-known in the art (see, for example, U.S. Pat. Nos. 4,981,844 and 5,230,902 and PCT Publ. No. WO 2004/004483) can be used in the methods described herein.

5 Similarly, agents and therapies other than immunotherapy or in combination thereof can be used with in combination with anti-CX3CR1/immune checkpoint agents to stimulate an immune response to thereby treat a condition that would benefit therefrom. For example, chemotherapy, radiation, epigenetic modifiers (*e.g.*, histone deacetylase (HDAC) modifiers, methylation modifiers, phosphorylation modifiers, and the like), targeted
10 therapy, and the like are well-known in the art.

 The term “untargeted therapy” refers to administration of agents that do not selectively interact with a chosen biomolecule yet treat cancer. Representative examples of untargeted therapies include, without limitation, chemotherapy, gene therapy, and radiation therapy.

15 In one embodiment, chemotherapy is used. Chemotherapy includes the administration of a chemotherapeutic agent. Such a chemotherapeutic agent may be, but is not limited to, those selected from among the following groups of compounds: platinum compounds, cytotoxic antibiotics, antimetabolites, anti-mitotic agents, alkylating agents, arsenic compounds, DNA topoisomerase inhibitors, taxanes, nucleoside analogues, plant
20 alkaloids, and toxins; and synthetic derivatives thereof. Exemplary compounds include, but are not limited to, alkylating agents: cisplatin, treosulfan, and trofosfamide; plant alkaloids: vinblastine, paclitaxel, docetaxol; DNA topoisomerase inhibitors: teniposide, crisnatol, and mitomycin; anti-folates: methotrexate, mycophenolic acid, and hydroxyurea; pyrimidine analogs: 5-fluorouracil, doxifluridine, and cytosine arabinoside; purine analogs:
25 mercaptapurine and thioguanine; DNA antimetabolites: 2'-deoxy-5-fluorouridine, aphidicolin glycinate, and pyrazoloimidazole; and antimitotic agents: halichondrin, colchicine, and rhizoxin. Compositions comprising one or more chemotherapeutic agents (*e.g.*, FLAG, CHOP) may also be used. FLAG comprises fludarabine, cytosine arabinoside (Ara-C) and G-CSF. CHOP comprises cyclophosphamide, vincristine, doxorubicin, and
30 prednisone. In another embodiments, PARP (*e.g.*, PARP-1 and/or PARP-2) inhibitors are used and such inhibitors are well-known in the art (*e.g.*, Olaparib, ABT-888, BSI-201, BGP-15 (N-Gene Research Laboratories, Inc.); INO-1001 (Inotek Pharmaceuticals Inc.); PJ34 (Soriano *et al.*, 2001; Pacher *et al.*, 2002b); 3-aminobenzamide (Trevigen); 4-amino-

1,8-naphthalimide; (Trevigen); 6(5H)-phenanthridinone (Trevigen); benzamide (U.S. Pat. Re. 36,397); and NU1025 (Bowman *et al.*). The mechanism of action is generally related to the ability of PARP inhibitors to bind PARP and decrease its activity. PARP catalyzes the conversion of .beta.-nicotinamide adenine dinucleotide (NAD⁺) into nicotinamide and poly-ADP-ribose (PAR). Both poly (ADP-ribose) and PARP have been linked to regulation of transcription, cell proliferation, genomic stability, and carcinogenesis (Bouchard V. J. *et al.* Experimental Hematology, Volume 31, Number 6, June 2003, pp. 446-454(9); Herceg Z.; Wang Z.-Q. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, Volume 477, Number 1, 2 Jun. 2001, pp. 97-110(14)).

10 Poly(ADP-ribose) polymerase 1 (PARP1) is a key molecule in the repair of DNA single-strand breaks (SSBs) (de Murcia J. *et al.* 1997. Proc Natl Acad Sci USA 94:7303-7307; Schreiber V, Dantzer F, Ame J C, de Murcia G (2006) Nat Rev Mol Cell Biol 7:517-528; Wang Z Q, *et al.* (1997) Genes Dev 11:2347-2358). Knockout of SSB repair by inhibition of PARP1 function induces DNA double-strand breaks (DSBs) that can trigger synthetic

15 lethality in cancer cells with defective homology-directed DSB repair (Bryant H E, *et al.* (2005) Nature 434:913-917; Farmer H, *et al.* (2005) Nature 434:917-921). The foregoing examples of chemotherapeutic agents are illustrative, and are not intended to be limiting.

In another embodiment, radiation therapy is used. The radiation used in radiation therapy can be ionizing radiation. Radiation therapy can also be gamma rays, X-rays, or proton beams. Examples of radiation therapy include, but are not limited to, external-beam radiation therapy, interstitial implantation of radioisotopes (I-125, palladium, iridium), radioisotopes such as strontium-89, thoracic radiation therapy, intraperitoneal P-32 radiation therapy, and/or total abdominal and pelvic radiation therapy. For a general overview of radiation therapy, see Hellman, Chapter 16: Principles of Cancer Management:

25 Radiation Therapy, 6th edition, 2001, DeVita *et al.*, eds., J. B. Lippencott Company, Philadelphia. The radiation therapy can be administered as external beam radiation or teletherapy wherein the radiation is directed from a remote source. The radiation treatment can also be administered as internal therapy or brachytherapy wherein a radioactive source is placed inside the body close to cancer cells or a tumor mass. Also encompassed is the use

30 of photodynamic therapy comprising the administration of photosensitizers, such as hematoporphyrin and its derivatives, Vertoporphin (BPD-MA), phthalocyanine, photosensitizer Pc4, demethoxy-hypocrellin A; and 2BA-2-DMHA.

In another embodiment, surgical intervention can occur to physically remove cancerous cells and/or tissues.

In still another embodiment, hormone therapy is used. Hormonal therapeutic treatments can comprise, for example, hormonal agonists, hormonal antagonists (*e.g.*,
5 flutamide, bicalutamide, tamoxifen, raloxifene, leuprolide acetate (LUPRON), LH-RH antagonists), inhibitors of hormone biosynthesis and processing, and steroids (*e.g.*, dexamethasone, retinoids, deltoids, betamethasone, cortisol, cortisone, prednisone, dehydrotestosterone, glucocorticoids, mineralocorticoids, estrogen, testosterone, progestins), vitamin A derivatives (*e.g.*, all-trans retinoic acid (ATRA)); vitamin D3
10 analogs; antigestagens (*e.g.*, mifepristone, onapristone), or antiandrogens (*e.g.*, cyproterone acetate).

In yet another embodiment, hyperthermia, a procedure in which body tissue is exposed to high temperatures (up to 106°F.) is used. Heat may help shrink tumors by damaging cells or depriving them of substances they need to live. Hyperthermia therapy
15 can be local, regional, and whole-body hyperthermia, using external and internal heating devices. Hyperthermia is almost always used with other forms of therapy (*e.g.*, radiation therapy, chemotherapy, and biological therapy) to try to increase their effectiveness. Local hyperthermia refers to heat that is applied to a very small area, such as a tumor. The area may be heated externally with high-frequency waves aimed at a tumor from a device
20 outside the body. To achieve internal heating, one of several types of sterile probes may be used, including thin, heated wires or hollow tubes filled with warm water; implanted microwave antennae; and radiofrequency electrodes. In regional hyperthermia, an organ or a limb is heated. Magnets and devices that produce high energy are placed over the region to be heated. In another approach, called perfusion, some of the patient's blood is removed,
25 heated, and then pumped (perfused) into the region that is to be heated internally. Whole-body heating is used to treat metastatic cancer that has spread throughout the body. It can be accomplished using warm-water blankets, hot wax, inductive coils (like those in electric blankets), or thermal chambers (similar to large incubators). Hyperthermia does not cause any marked increase in radiation side effects or complications. Heat applied directly to the
30 skin, however, can cause discomfort or even significant local pain in about half the patients treated. It can also cause blisters, which generally heal rapidly.

In still another embodiment, photodynamic therapy (also called PDT, photoradiation therapy, phototherapy, or photochemotherapy) is used for the treatment of some types of

cancer. It is based on the discovery that certain chemicals known as photosensitizing agents can kill one-celled organisms when the organisms are exposed to a particular type of light. PDT destroys cancer cells through the use of a fixed-frequency laser light in combination with a photosensitizing agent. In PDT, the photosensitizing agent is injected into the

5 bloodstream and absorbed by cells all over the body. The agent remains in cancer cells for a longer time than it does in normal cells. When the treated cancer cells are exposed to laser light, the photosensitizing agent absorbs the light and produces an active form of oxygen that destroys the treated cancer cells. Light exposure must be timed carefully so that it occurs when most of the photosensitizing agent has left healthy cells but is still

10 present in the cancer cells. The laser light used in PDT can be directed through a fiber-optic (a very thin glass strand). The fiber-optic is placed close to the cancer to deliver the proper amount of light. The fiber-optic can be directed through a bronchoscope into the lungs for the treatment of lung cancer or through an endoscope into the esophagus for the treatment of esophageal cancer. An advantage of PDT is that it causes minimal damage to

15 healthy tissue. However, because the laser light currently in use cannot pass through more than about 3 centimeters of tissue (a little more than one and an eighth inch), PDT is mainly used to treat tumors on or just under the skin or on the lining of internal organs.

Photodynamic therapy makes the skin and eyes sensitive to light for 6 weeks or more after treatment. Patients are advised to avoid direct sunlight and bright indoor light for at least 6

20 weeks. If patients must go outdoors, they need to wear protective clothing, including sunglasses. Other temporary side effects of PDT are related to the treatment of specific areas and can include coughing, trouble swallowing, abdominal pain, and painful breathing or shortness of breath. In December 1995, the U.S. Food and Drug Administration (FDA) approved a photosensitizing agent called porfimer sodium, or Photofrin®, to relieve

25 symptoms of esophageal cancer that is causing an obstruction and for esophageal cancer that cannot be satisfactorily treated with lasers alone. In January 1998, the FDA approved porfimer sodium for the treatment of early non-small cell lung cancer in patients for whom the usual treatments for lung cancer are not appropriate. The National Cancer Institute and other institutions are supporting clinical trials (research studies) to evaluate the use of

30 photodynamic therapy for several types of cancer, including cancers of the bladder, brain, larynx, and oral cavity.

In yet another embodiment, laser therapy is used to harness high-intensity light to destroy cancer cells. This technique is often used to relieve symptoms of cancer such as

bleeding or obstruction, especially when the cancer cannot be cured by other treatments. It may also be used to treat cancer by shrinking or destroying tumors. The term “laser” stands for light amplification by stimulated emission of radiation. Ordinary light, such as that from a light bulb, has many wavelengths and spreads in all directions. Laser light, on the other hand, has a specific wavelength and is focused in a narrow beam. This type of high-intensity light contains a lot of energy. Lasers are very powerful and may be used to cut through steel or to shape diamonds. Lasers also can be used for very precise surgical work, such as repairing a damaged retina in the eye or cutting through tissue (in place of a scalpel). Although there are several different kinds of lasers, only three kinds have gained wide use in medicine: Carbon dioxide (CO₂) laser--This type of laser can remove thin layers from the skin's surface without penetrating the deeper layers. This technique is particularly useful in treating tumors that have not spread deep into the skin and certain precancerous conditions. As an alternative to traditional scalpel surgery, the CO₂ laser is also able to cut the skin. The laser is used in this way to remove skin cancers.

Neodymium:yttrium-aluminum-garnet (Nd:YAG) laser-- Light from this laser can penetrate deeper into tissue than light from the other types of lasers, and it can cause blood to clot quickly. It can be carried through optical fibers to less accessible parts of the body. This type of laser is sometimes used to treat throat cancers. Argon laser--This laser can pass through only superficial layers of tissue and is therefore useful in dermatology and in eye surgery. It also is used with light-sensitive dyes to treat tumors in a procedure known as photodynamic therapy (PDT). Lasers have several advantages over standard surgical tools, including: Lasers are more precise than scalpels. Tissue near an incision is protected, since there is little contact with surrounding skin or other tissue. The heat produced by lasers sterilizes the surgery site, thus reducing the risk of infection. Less operating time may be needed because the precision of the laser allows for a smaller incision. Healing time is often shortened; since laser heat seals blood vessels, there is less bleeding, swelling, or scarring. Laser surgery may be less complicated. For example, with fiber optics, laser light can be directed to parts of the body without making a large incision. More procedures may be done on an outpatient basis. Lasers can be used in two ways to treat cancer: by shrinking or destroying a tumor with heat, or by activating a chemical--known as a photosensitizing agent--that destroys cancer cells. In PDT, a photosensitizing agent is retained in cancer cells and can be stimulated by light to cause a reaction that kills cancer cells. CO₂ and Nd:YAG lasers are used to shrink or destroy tumors. They may be used

with endoscopes, tubes that allow physicians to see into certain areas of the body, such as the bladder. The light from some lasers can be transmitted through a flexible endoscope fitted with fiber optics. This allows physicians to see and work in parts of the body that could not otherwise be reached except by surgery and therefore allows very precise aiming of the laser beam. Lasers also may be used with low-power microscopes, giving the doctor a clear view of the site being treated. Used with other instruments, laser systems can produce a cutting area as small as 200 microns in diameter--less than the width of a very fine thread. Lasers are used to treat many types of cancer. Laser surgery is a standard treatment for certain stages of glottis (vocal cord), cervical, skin, lung, vaginal, vulvar, and penile cancers. In addition to its use to destroy the cancer, laser surgery is also used to help relieve symptoms caused by cancer (palliative care). For example, lasers may be used to shrink or destroy a tumor that is blocking a patient's trachea (windpipe), making it easier to breathe. It is also sometimes used for palliation in colorectal and anal cancer. Laser-induced interstitial thermotherapy (LITT) is one of the most recent developments in laser therapy. LITT uses the same idea as a cancer treatment called hyperthermia; that heat may help shrink tumors by damaging cells or depriving them of substances they need to live. In this treatment, lasers are directed to interstitial areas (areas between organs) in the body. The laser light then raises the temperature of the tumor, which damages or destroys cancer cells.

The duration and/or dose of treatment with therapies may vary according to the particular therapeutic agent or combination thereof. An appropriate treatment time for a particular cancer therapeutic agent will be appreciated by the skilled artisan. The present invention contemplates the continued assessment of optimal treatment schedules for each cancer therapeutic agent, where the phenotype of the cancer of the subject as determined by the methods of the present invention is a factor in determining optimal treatment doses and schedules.

Any means for the introduction of a polynucleotide into mammals, human or non-human, or cells thereof may be adapted to the practice of this invention for the delivery of the various constructs of the present invention into the intended recipient. In one embodiment of the present invention, the DNA constructs are delivered to cells by transfection, *i.e.*, by delivery of "naked" DNA or in a complex with a colloidal dispersion system. A colloidal system includes macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles,

mixed micelles, and liposomes. The preferred colloidal system of this invention is a lipid-complexed or liposome-formulated DNA. In the former approach, prior to formulation of DNA, *e.g.*, with lipid, a plasmid containing a transgene bearing the desired DNA constructs may first be experimentally optimized for expression (*e.g.*, inclusion of an intron in the 5' untranslated region and elimination of unnecessary sequences (Felgner, *et al.*, Ann NY Acad Sci 126-139, 1995). Formulation of DNA, *e.g.* with various lipid or liposome materials, may then be effected using known methods and materials and delivered to the recipient mammal. See, *e.g.*, Canonico et al, Am J Respir Cell Mol Biol 10:24-29, 1994; Tsan et al, Am J Physiol 268; Alton *et al.*, Nat Genet. 5:135-142, 1993 and U.S. patent No. 5,679,647 by Carson *et al.*

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs, which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand. Naked DNA or DNA associated with a delivery vehicle, *e.g.*, liposomes, can be administered to several sites in a subject (see below).

Nucleic acids can be delivered in any desired vector. These include viral or non-viral vectors, including adenovirus vectors, adeno-associated virus vectors, retrovirus vectors, lentivirus vectors, and plasmid vectors. Exemplary types of viruses include HSV (herpes simplex virus), AAV (adeno associated virus), HIV (human immunodeficiency virus), BIV (bovine immunodeficiency virus), and MLV (murine leukemia virus). Nucleic acids can be administered in any desired format that provides sufficiently efficient delivery

levels, including in virus particles, in liposomes, in nanoparticles, and complexed to polymers.

The nucleic acids encoding a protein or nucleic acid of interest may be in a plasmid or viral vector, or other vector as is known in the art. Such vectors are well-known and any
5 can be selected for a particular application. In one embodiment of the present invention, the gene delivery vehicle comprises a promoter and a demethylase coding sequence. Preferred promoters are tissue-specific promoters and promoters which are activated by cellular proliferation, such as the thymidine kinase and thymidylate synthase promoters. Other preferred promoters include promoters which are activatable by infection with a
10 virus, such as the α - and β -interferon promoters, and promoters which are activatable by a hormone, such as estrogen. Other promoters which can be used include the Moloney virus LTR, the CMV promoter, and the mouse albumin promoter. A promoter may be constitutive or inducible.

In another embodiment, naked polynucleotide molecules are used as gene delivery
15 vehicles, as described in WO 90/11092 and U.S. Patent 5,580,859. Such gene delivery vehicles can be either growth factor DNA or RNA and, in certain embodiments, are linked to killed adenovirus. Curiel *et al.*, Hum. Gene. Ther. 3:147-154, 1992. Other vehicles which can optionally be used include DNA-ligand (Wu *et al.*, J. Biol. Chem. 264:16985-16987, 1989), lipid-DNA combinations (Felgner *et al.*, Proc. Natl. Acad. Sci. USA 84:7413-7417, 1989), liposomes (Wang *et al.*, Proc. Natl. Acad. Sci. 84:7851-7855, 1987) and microprojectiles (Williams *et al.*, Proc. Natl. Acad. Sci. 88:2726-2730, 1991).
20

A gene delivery vehicle can optionally comprise viral sequences such as a viral origin of replication or packaging signal. These viral sequences can be selected from viruses such as astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, retrovirus, togavirus or adenovirus. In a preferred
25 embodiment, the growth factor gene delivery vehicle is a recombinant retroviral vector. Recombinant retroviruses and various uses thereof have been described in numerous references including, for example, Mann *et al.*, Cell 33:153, 1983, Cane and Mulligan, Proc. Nat'l. Acad. Sci. USA 81:6349, 1984, Miller *et al.*, Human Gene Therapy 1:5-14, 1990, U.S. Patent Nos. 4,405,712, 4,861,719, and 4,980,289, and PCT Application Nos. WO 89/02,468, WO 89/05,349, and WO 90/02,806. Numerous retroviral gene delivery vehicles can be utilized in the present invention, including for example those described in
30 EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent

No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart, Cancer Res. 53:3860-3864, 1993; Vile and Hart, Cancer Res. 53:962-967, 1993; Ram *et al.*, Cancer Res. 53:83-88, 1993; Takamiya *et al.*, J. Neurosci. Res. 33:493-503, 1992; Baba *et al.*, J. Neurosurg. 79:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and
5 WO91/02805).

Other viral vector systems that can be used to deliver a polynucleotide of the present invention have been derived from herpes virus, *e.g.*, Herpes Simplex Virus (U.S. Patent No. 5,631,236 by Woo *et al.*, issued May 20, 1997 and WO 00/08191 by Neurovex), vaccinia virus (Ridgeway (1988) Ridgeway, "Mammalian expression vectors," In: Rodriguez R L,
10 Denhardt D T, ed. Vectors: A survey of molecular cloning vectors and their uses. Stoneham: Butterworth,; Baichwal and Sugden (1986) "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," In: Kucherlapati R, ed. Gene transfer. New York: Plenum Press; Coupar *et al.* (1988) Gene, 68:1-10), and several RNA viruses. Preferred viruses include an alphavirus, a poxvirus, an
15 arena virus, a vaccinia virus, a polio virus, and the like. They offer several attractive features for various mammalian cells (Friedmann (1989) Science, 244:1275-1281; Ridgeway, 1988, *supra*; Baichwal and Sugden, 1986, *supra*; Coupar *et al.*, 1988; Horwich *et al.* (1990) J. Virol., 64:642-650).

In other embodiments, target DNA in the genome can be manipulated using well-
20 known methods in the art. For example, the target DNA in the genome can be manipulated by deletion, insertion, and/or mutation are retroviral insertion, artificial chromosome techniques, gene insertion, random insertion with tissue specific promoters, gene targeting, transposable elements and/or any other method for introducing foreign DNA or producing modified DNA/modified nuclear DNA. Other modification techniques include deleting
25 DNA sequences from a genome and/or altering nuclear DNA sequences. Nuclear DNA sequences, for example, may be altered by site-directed mutagenesis.

In other embodiments, recombinant biomarker polypeptides, and fragments thereof, can be administered to subjects. In some embodiments, fusion proteins can be constructed and administered which have enhanced biological properties. In addition, the biomarker
30 polypeptides, and fragment thereof, can be modified according to well-known pharmacological methods in the art (*e.g.*, pegylation, glycosylation, oligomerization, *etc.*) in order to further enhance desirable biological activities, such as increased bioavailability and decreased proteolytic degradation.

4. Clinical Efficacy

Clinical efficacy can be measured by any method known in the art. For example, the response to a therapy, such as CX3CR1/immune checkpoint combination inhibitor therapies, relates to any response of the cancer, *e.g.*, a tumor, to the therapy, preferably to a change in tumor mass and/or volume after initiation of neoadjuvant or adjuvant chemotherapy. Tumor response may be assessed in a neoadjuvant or adjuvant situation where the size of a tumor after systemic intervention can be compared to the initial size and dimensions as measured by CT, PET, mammogram, ultrasound or palpation and the cellularity of a tumor can be estimated histologically and compared to the cellularity of a tumor biopsy taken before initiation of treatment. Response may also be assessed by caliper measurement or pathological examination of the tumor after biopsy or surgical resection. Response may be recorded in a quantitative fashion like percentage change in tumor volume or cellularity or using a semi-quantitative scoring system such as residual cancer burden (Symmans *et al.*, *J. Clin. Oncol.* (2007) 25:4414-4422) or Miller-Payne score (Ogston *et al.*, (2003) *Breast* (Edinburgh, Scotland) 12:320-327) in a qualitative fashion like “pathological complete response” (pCR), “clinical complete remission” (cCR), “clinical partial remission” (cPR), “clinical stable disease” (cSD), “clinical progressive disease” (cPD) or other qualitative criteria. Assessment of tumor response may be performed early after the onset of neoadjuvant or adjuvant therapy, *e.g.*, after a few hours, days, weeks or preferably after a few months. A typical endpoint for response assessment is upon termination of neoadjuvant chemotherapy or upon surgical removal of residual tumor cells and/or the tumor bed.

In some embodiments, clinical efficacy of the therapeutic treatments described herein may be determined by measuring the clinical benefit rate (CBR). The clinical benefit rate is measured by determining the sum of the percentage of patients who are in complete remission (CR), the number of patients who are in partial remission (PR) and the number of patients having stable disease (SD) at a time point at least 6 months out from the end of therapy. The shorthand for this formula is $CBR = CR + PR + SD$ over 6 months. In some embodiments, the CBR for a particular anti-immune checkpoint therapeutic regimen is at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or more.

Additional criteria for evaluating the response to anti-immune checkpoint therapies are related to “survival,” which includes all of the following: survival until mortality, also known as overall survival (wherein said mortality may be either irrespective of cause or tumor related); “recurrence-free survival” (wherein the term recurrence shall include both
5 localized and distant recurrence); metastasis free survival; disease free survival (wherein the term disease shall include cancer and diseases associated therewith). The length of said survival may be calculated by reference to a defined start point (*e.g.*, time of diagnosis or start of treatment) and end point (*e.g.*, death, recurrence or metastasis). In addition, criteria for efficacy of treatment can be expanded to include response to chemotherapy, probability
10 of survival, probability of metastasis within a given time period, and probability of tumor recurrence.

For example, in order to determine appropriate threshold values, a particular anti-cancer therapeutic regimen can be administered to a population of subjects and the outcome can be correlated to biomarker measurements that were determined prior to administration
15 of any anti-immune checkpoint therapy. The outcome measurement may be pathologic response to therapy given in the neoadjuvant setting. Alternatively, outcome measures, such as overall survival and disease-free survival can be monitored over a period of time for subjects following anti-immune checkpoint therapy for whom biomarker measurement values are known. In certain embodiments, the same doses of anti-immune checkpoint
20 agents are administered to each subject. In related embodiments, the doses administered are standard doses known in the art for anti-immune checkpoint agents. The period of time for which subjects are monitored can vary. For example, subjects may be monitored for at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, or 60 months. Biomarker measurement threshold values that correlate to outcome of an anti-immune checkpoint
25 therapy can be determined using methods such as those described in the Examples section.

5. Further Uses and Methods of the Present Invention

The compositions described herein can be used in a variety of diagnostic, prognostic, and therapeutic applications. In any method described herein, such as a
30 diagnostic method, prognostic method, therapeutic method, or combination thereof, all steps of the method can be performed by a single actor or, alternatively, by more than one actor. For example, diagnosis can be performed directly by the actor providing therapeutic treatment. Alternatively, a person providing a therapeutic agent can request that a

diagnostic assay be performed. The diagnostician and/or the therapeutic interventionist can interpret the diagnostic assay results to determine a therapeutic strategy. Similarly, such alternative processes can apply to other assays, such as prognostic assays.

a. Screening Methods

5 One aspect of the present invention relates to screening assays, including non-cell based assays and xenograft animal model assays. In one embodiment, the assays provide a method for identifying whether a cancer is likely to respond to CX3CR1/immune checkpoint combination inhibitor therapy, such as in a human by using a xenograft animal model assay, and/or whether an agent can inhibit the growth of or kill a cancer cell that is
10 unlikely to respond to CX3CR1/immune checkpoint combination inhibitor therapy.

In one embodiment, the present invention relates to assays for screening test agents which bind to, or modulate the biological activity of, at least one biomarker described herein (*e.g.*, in the tables, figures, examples, or otherwise in the specification). In one embodiment, a method for identifying such an agent entails determining the ability of the
15 agent to modulate, *e.g.* inhibit, the at least one biomarker described herein.

In one embodiment, an assay is a cell-free or cell-based assay, comprising contacting at least one biomarker described herein, with a test agent, and determining the ability of the test agent to modulate (*e.g.*, inhibit) the enzymatic activity of the biomarker, such as by measuring direct binding of substrates or by measuring indirect parameters as
20 described below.

For example, in a direct binding assay, biomarker protein (or their respective target polypeptides or molecules) can be coupled with a radioisotope or enzymatic label such that binding can be determined by detecting the labeled protein or molecule in a complex. For example, the targets can be labeled with ^{125}I , ^{35}S , ^{14}C , or ^3H , either directly or indirectly,
25 and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, the targets can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product. Determining the interaction between biomarker and substrate can also be accomplished
30 using standard binding or enzymatic analysis assays. In one or more embodiments of the above described assay methods, it may be desirable to immobilize polypeptides or molecules to facilitate separation of complexed from uncomplexed forms of one or both of the proteins or molecules, as well as to accommodate automation of the assay.

Binding of a test agent to a target can be accomplished in any vessel suitable for containing the reactants. Non-limiting examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. Immobilized forms of the antibodies described herein can also include antibodies bound to a solid phase like a porous, microporous (with an average pore diameter less than about one micron) or macroporous (with an average pore diameter of more than about 10 microns) material, such as a membrane, cellulose, nitrocellulose, or glass fibers; a bead, such as that made of agarose or polyacrylamide or latex; or a surface of a dish, plate, or well, such as one made of polystyrene.

In an alternative embodiment, determining the ability of the agent to modulate the interaction between the biomarker and a substrate or a biomarker and its natural binding partner can be accomplished by determining the ability of the test agent to modulate the activity of a polypeptide or other product that functions downstream or upstream of its position within the signaling pathway (*e.g.*, feedback loops). Such feedback loops are well-known in the art (see, for example, Chen and Guillemin (2009) *Int. J. Tryptophan Res.* 2:1-19).

The present invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein, such as in an appropriate animal model. For example, an agent identified as described herein can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an antibody identified as described herein can be used in an animal model to determine the mechanism of action of such an agent.

b. Predictive Medicine

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining the amount and/or activity level of a biomarker described herein in the context of a biological sample (*e.g.*, blood, serum, cells, or tissue) to thereby determine whether an individual afflicted with a cancer is likely to respond to CX3CR1/immune checkpoint combination inhibitor therapy, such as in a cancer. Such assays can be used for prognostic or predictive purpose alone, or can be coupled with a therapeutic intervention to thereby prophylactically treat an individual prior to the onset or after recurrence of a disorder characterized by or associated

with biomarker polypeptide, nucleic acid expression or activity. The skilled artisan will appreciate that any method can use one or more (*e.g.*, combinations) of biomarkers described herein, such as those in the tables, figures, examples, and otherwise described in the specification.

5 Another aspect of the present invention pertains to monitoring the influence of agents (*e.g.*, drugs, compounds, and small nucleic acid-based molecules) on the expression or activity of a biomarker described herein. These and other agents are described in further detail in the following sections.

10 The skilled artisan will also appreciate that, in certain embodiments, the methods of the present invention implement a computer program and computer system. For example, a computer program can be used to perform the algorithms described herein. A computer system can also store and manipulate data generated by the methods of the present invention which comprises a plurality of biomarker signal changes/profiles which can be used by a computer system in implementing the methods of this invention. In
15 certain embodiments, a computer system receives biomarker expression data; (ii) stores the data; and (iii) compares the data in any number of ways described herein (*e.g.*, analysis relative to appropriate controls) to determine the state of informative biomarkers from cancerous or pre-cancerous tissue. In other embodiments, a computer system (i) compares the determined expression biomarker level to a threshold value; and (ii) outputs an
20 indication of whether said biomarker level is significantly modulated (*e.g.*, above or below) the threshold value, or a phenotype based on said indication.

 In certain embodiments, such computer systems are also considered part of the present invention. Numerous types of computer systems can be used to implement the analytic methods of this invention according to knowledge possessed by a skilled artisan in
25 the bioinformatics and/or computer arts. Several software components can be loaded into memory during operation of such a computer system. The software components can comprise both software components that are standard in the art and components that are special to the present invention (*e.g.*, dCHIP software described in Lin *et al.* (2004) *Bioinformatics* 20, 1233-1240; radial basis machine learning algorithms (RBM) known in
30 the art).

 The methods of the present invention can also be programmed or modeled in mathematical software packages that allow symbolic entry of equations and high-level specification of processing, including specific algorithms to be used, thereby freeing a user

of the need to procedurally program individual equations and algorithms. Such packages include, *e.g.*, Matlab from Mathworks (Natick, Mass.), Mathematica from Wolfram Research (Champaign, Ill.) or S-Plus from MathSoft (Seattle, Wash.).

In certain embodiments, the computer comprises a database for storage of biomarker data. Such stored profiles can be accessed and used to perform comparisons of interest at a later point in time. For example, biomarker expression profiles of a sample derived from the non-cancerous tissue of a subject and/or profiles generated from population-based distributions of informative loci of interest in relevant populations of the same species can be stored and later compared to that of a sample derived from the cancerous tissue of the subject or tissue suspected of being cancerous of the subject.

In addition to the exemplary program structures and computer systems described herein, other, alternative program structures and computer systems will be readily apparent to the skilled artisan. Such alternative systems, which do not depart from the above described computer system and programs structures either in spirit or in scope, are therefore intended to be comprehended within the accompanying claims.

c. Diagnostic Assays

The present invention provides, in part, methods, systems, and code for accurately classifying whether a biological sample is associated with a cancer that is likely to respond to CX3CR1/immune checkpoint combination inhibitor therapy. In some embodiments, the present invention is useful for classifying a sample (*e.g.*, from a subject) as associated with or at risk for responding to or not responding to CX3CR1/immune checkpoint combination inhibitor therapy using a statistical algorithm and/or empirical data (*e.g.*, the amount or activity of a biomarker described herein, such as in the tables, figures, examples, and otherwise described in the specification).

An exemplary method for detecting the amount or activity of a biomarker described herein, and thus useful for classifying whether a sample is likely or unlikely to respond to CX3CR1/immune checkpoint combination inhibitor therapy involves obtaining a biological sample from a test subject and contacting the biological sample with an agent, such as a protein-binding agent like an antibody or antigen-binding fragment thereof, or a nucleic acid-binding agent like an oligonucleotide, capable of detecting the amount or activity of the biomarker in the biological sample. In some embodiments, at least one antibody or antigen-binding fragment thereof is used, wherein two, three, four, five, six, seven, eight, nine, ten, or more such antibodies or antibody fragments can be used in

combination (*e.g.*, in sandwich ELISAs) or in serial. In certain instances, the statistical algorithm is a single learning statistical classifier system. For example, a single learning statistical classifier system can be used to classify a sample as a based upon a prediction or probability value and the presence or level of the biomarker. The use of a single learning statistical classifier system typically classifies the sample as, for example, a likely anti-immune checkpoint therapy responder or progressor sample with a sensitivity, specificity, positive predictive value, negative predictive value, and/or overall accuracy of at least about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

Other suitable statistical algorithms are well-known to those of skill in the art. For example, learning statistical classifier systems include a machine learning algorithmic technique capable of adapting to complex data sets (*e.g.*, panel of markers of interest) and making decisions based upon such data sets. In some embodiments, a single learning statistical classifier system such as a classification tree (*e.g.*, random forest) is used. In other embodiments, a combination of 2, 3, 4, 5, 6, 7, 8, 9, 10, or more learning statistical classifier systems are used, preferably in tandem. Examples of learning statistical classifier systems include, but are not limited to, those using inductive learning (*e.g.*, decision/classification trees such as random forests, classification and regression trees (C&RT), boosted trees, etc.), Probably Approximately Correct (PAC) learning, connectionist learning (*e.g.*, neural networks (NN), artificial neural networks (ANN), neuro fuzzy networks (NFN), network structures, perceptrons such as multi-layer perceptrons, multi-layer feed-forward networks, applications of neural networks, Bayesian learning in belief networks, etc.), reinforcement learning (*e.g.*, passive learning in a known environment such as naive learning, adaptive dynamic learning, and temporal difference learning, passive learning in an unknown environment, active learning in an unknown environment, learning action-value functions, applications of reinforcement learning, etc.), and genetic algorithms and evolutionary programming. Other learning statistical classifier systems include support vector machines (*e.g.*, Kernel methods), multivariate adaptive regression splines (MARS), Levenberg-Marquardt algorithms, Gauss-Newton algorithms, mixtures of Gaussians, gradient descent algorithms, and learning vector quantization (LVQ). In certain embodiments, the method of the present invention further comprises sending the sample classification results to a clinician, *e.g.*, an oncologist.

In another embodiment, the diagnosis of a subject is followed by administering to the individual a therapeutically effective amount of a defined treatment based upon the diagnosis.

5 In one embodiment, the methods further involve obtaining a control biological sample (*e.g.*, biological sample from a subject who does not have a cancer or whose cancer is susceptible to CX3CR1/immune checkpoint combination inhibitor therapy), a biological sample from the subject during remission, or a biological sample from the subject during treatment for developing a cancer progressing despite CX3CR1/immune checkpoint combination inhibitor therapy.

10 d. Prognostic Assays

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a cancer that is likely or unlikely to be responsive to CX3CR1/immune checkpoint combination inhibitor therapy. The assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to
15 identify a subject having or at risk of developing a disorder associated with a misregulation of the amount or activity of at least one biomarker described herein, such as in cancer. Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing a disorder associated with a misregulation of the at least one biomarker described herein, such as in cancer. Furthermore, the prognostic assays described herein
20 can be used to determine whether a subject can be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, polypeptide, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with the aberrant biomarker expression or activity.

e. Treatment Methods

25 The therapeutic compositions described herein, such as the combination of CX3CR1 inhibitors and immune checkpoint inhibitors, can be used in a variety of *in vitro* and *in vivo* therapeutic applications using the formulations and/or combinations described herein. In one embodiment, the therapeutic agents can be used to treat cancers determined to be responsive thereto. For example, single or multiple agents that inhibit or block both
30 CX3CR1 and an immune checkpoint can be used to treat cancers in subjects identified as likely responders thereto.

Modulatory methods of the present invention involve contacting a cell, such as an immune cell with an agent that inhibits or blocks the expression and/or activity of CX3CR1

and an immune checkpoint, such as PD-1. Exemplary agents useful in such methods are described above. Such agents can be administered *in vitro* or *ex vivo* (e.g., by contacting the cell with the agent) or, alternatively, *in vivo* (e.g., by administering the agent to a subject). As such, the present invention provides methods useful for treating an individual
5 afflicted with a condition that would benefit from an increased immune response, such as an infection or a cancer like colorectal cancer.

Agents that upregulate immune responses can be in the form of enhancing an existing immune response or eliciting an initial immune response. Thus, enhancing an immune response using the subject compositions and methods is useful for treating cancer,
10 but can also be useful for treating an infectious disease (e.g., bacteria, viruses, or parasites), a parasitic infection, an inflammatory condition, and an immunosuppressive disease.

Exemplary infectious disorders include viral skin diseases, such as Herpes or shingles, in which case such an agent can be delivered topically to the skin. In addition, systemic viral diseases, such as influenza, the common cold, and encephalitis might be
15 alleviated by systemic administration of such agents. In one preferred embodiment, agents that upregulate the immune response described herein are useful for modulating the arginase/iNOS balance during *Trypanosoma cruzi* infection in order to facilitate a protective immune response against the parasite.

Immune responses can also be enhanced in an infected patient through an *ex vivo*
20 approach, for instance, by removing immune cells from the patient, contacting immune cells *in vitro* with an agent described herein and reintroducing the *in vitro* stimulated immune cells into the patient.

In certain instances, it may be desirable to further administer other agents that upregulate immune responses, for example, forms of other B7 family members that
25 transduce signals via costimulatory receptors, in order to further augment the immune response. Such additional agents and therapies are described further below.

Agents that upregulate an immune response can be used prophylactically in vaccines against various polypeptides (e.g., polypeptides derived from pathogens). Immunity against a pathogen (e.g., a virus) can be induced by vaccinating with a viral
30 protein along with an agent that upregulates an immune response, in an appropriate adjuvant.

In another embodiment, upregulation or enhancement of an immune response function, as described herein, is useful in the induction of tumor immunity.

In another embodiment, the immune response can be stimulated by the methods described herein, such that preexisting tolerance, clonal deletion, and/or exhaustion (*e.g.*, T cell exhaustion) is overcome. For example, immune responses against antigens to which a subject cannot mount a significant immune response, *e.g.*, to an autologous antigen, such as a tumor specific antigens can be induced by administering appropriate agents described herein that upregulate the immune response. In one embodiment, an autologous antigen, such as a tumor-specific antigen, can be coadministered. In another embodiment, the subject agents can be used as adjuvants to boost responses to foreign antigens in the process of active immunization.

In one embodiment, immune cells are obtained from a subject and cultured *ex vivo* in the presence of an agent as described herein, to expand the population of immune cells and/or to enhance immune cell activation. In a further embodiment the immune cells are then administered to a subject. Immune cells can be stimulated *in vitro* by, for example, providing to the immune cells a primary activation signal and a costimulatory signal, as is known in the art. Various agents can also be used to costimulate proliferation of immune cells. In one embodiment immune cells are cultured *ex vivo* according to the method described in PCT Application No. WO 94/29436. The costimulatory polypeptide can be soluble, attached to a cell membrane, or attached to a solid surface, such as a bead.

6. Administration of Agents

The immune modulating agents of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo*, to enhance immune cell mediated immune responses. By “biologically compatible form suitable for administration *in vivo*” is meant a form to be administered in which any toxic effects are outweighed by the therapeutic effects. The term “subject” is intended to include living organisms in which an immune response can be elicited, *e.g.*, mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of an agent as described herein can be in any pharmacological form including a therapeutically active amount of an agent alone or in combination with a pharmaceutically acceptable carrier.

Administration of a therapeutically active amount of the therapeutic composition of the present invention is defined as an amount effective, at dosages and for periods of time necessary, to achieve the desired result. For example, a therapeutically active amount of an

agent may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of peptide to elicit a desired response in the individual. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses can be administered daily or the dose can be proportionally reduced
5 as indicated by the exigencies of the therapeutic situation.

Inhibiting or blocking both CX3CR1 and an immune checkpoint expression and/or activity can be accomplished by combination therapy with the modulatory agents described herein. Combination therapy describes a therapy in which both CX3CR1 and an immune checkpoint are inhibited or blocked simultaneously. Simultaneous inhibition or blockade
10 may be achieved by administration of the modulatory agents described herein simultaneously (*e.g.*, in a combination dosage form or by simultaneous administration of single agents) or by administration of single agents according to a schedule that results in effective amounts of each modulatory agent present in the patient at the same time.

The therapeutic agents described herein can be administered in a convenient manner
15 such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound can be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. For example, for administration of agents, by other than parenteral administration, it may be
20 desirable to coat the agent with, or co-administer the agent with, a material to prevent its inactivation.

An agent can be administered to an individual in an appropriate carrier, diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer
25 solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional
30 liposomes (Sterna *et al.* (1984) *J. Neuroimmunol.* 7:27).

As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or

non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; (4) intravaginally or
5 intrarectally, for example, as a pessary, cream or foam; or (5) aerosol, for example, as an aqueous aerosol, liposomal preparation or solid particles containing the compound.

The phrase “therapeutically-effective amount” as used herein means that amount of an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, or expression and/or activity of the complex, or composition comprising an agent that modulates (*e.g.*,
10 inhibits) biomarker expression and/or activity, or expression and/or activity of the complex, which is effective for producing some desired therapeutic effect, *e.g.*, cancer treatment, at a reasonable benefit/risk ratio.

The phrase “pharmaceutically acceptable” is employed herein to refer to those agents, materials, compositions, and/or dosage forms which are, within the scope of sound
15 medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase “pharmaceutically-acceptable carrier” as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid
20 filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1)
25 sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols,
30 such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20)

phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

The term “pharmaceutically-acceptable salts” refers to the relatively non-toxic, inorganic and organic acid addition salts of the agents that modulates (*e.g.*, inhibits)

5 biomarker expression and/or activity, or expression and/or activity of the complex encompassed by the present invention. These salts can be prepared *in situ* during the final isolation and purification of the therapeutic agents, or by separately reacting a purified therapeutic agent in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed. Representative salts include the hydrobromide,
10 hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like (See, for example, Berge *et al.* (1977) “Pharmaceutical Salts”, *J. Pharm. Sci.* 66:1-19).

15 In other cases, the agents useful in the methods of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable bases. The term “pharmaceutically-acceptable salts” in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of agents that modulates (*e.g.*, inhibits) biomarker expression and/or
20 activity, or expression and/or activity of the complex. These salts can likewise be prepared *in situ* during the final isolation and purification of the therapeutic agents, or by separately reacting the purified therapeutic agent in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary or tertiary
25 amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like (see, for example, Berge *et al.*, *supra*).

30 Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations useful in the methods of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal, aerosol and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well-known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient, which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a therapeutic agent with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a therapeutic agent as an active ingredient. A compound may also be administered as a bolus, electuary or paste.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, acetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered peptide or peptidomimetic moistened with an inert liquid diluent.

Tablets, and other solid dosage forms, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well-known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions, which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions

may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions, which can be used include polymeric substances and waxes. The active
5 ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the
10 art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

15 Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active agent may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters,
20 microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more therapeutic agents with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene
25 glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active agent.

Formulations which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are
30 known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active

component may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to a therapeutic agent, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch,
5 tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these
10 substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

The agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, can be alternatively administered by aerosol. This is accomplished by preparing an aqueous
15 aerosol, liposomal preparation or solid particles containing the compound. A nonaqueous (*e.g.*, fluorocarbon propellant) suspension could be used. Sonic nebulizers are preferred because they minimize exposing the agent to shear, which can result in degradation of the compound.

Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or
20 suspension of the agent together with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers vary with the requirements of the particular compound, but typically include nonionic surfactants (Tweens, Pluronics, or polyethylene glycol), innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. Aerosols generally are
25 prepared from isotonic solutions.

Transdermal patches have the added advantage of providing controlled delivery of a therapeutic agent to the body. Such dosage forms can be made by dissolving or dispersing the agent in the proper medium. Absorption enhancers can also be used to increase the flux of the peptidomimetic across the skin. The rate of such flux can be controlled by either
30 providing a rate controlling membrane or dispersing the peptidomimetic in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more therapeutic agents in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the present invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution, which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of an agent that modulates (*e.g.*, inhibits) biomarker expression and/or activity, in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be

controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions, which are compatible with body tissue.

When the therapeutic agents of the present invention are administered as
5 pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be determined by the methods of the present invention so as to obtain an
10 amount of the active ingredient, which is effective to achieve the desired therapeutic response for a particular subject, composition, and mode of administration, without being toxic to the subject.

The nucleic acid molecules of the present invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for
15 example, intravenous injection, local administration (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054 3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector
20 can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

In one embodiment, an agent of the invention is an antibody. As defined herein, a therapeutically effective amount of antibody (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more
25 preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present.
30 Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8

weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result from the results of diagnostic assays.

5

7. Kits

The present invention also encompasses kits for detecting and/or modulating biomarkers described herein. A kit of the present invention may also include instructional materials disclosing or describing the use of the kit or an antibody of the disclosed
10 invention in a method of the disclosed invention as provided herein. A kit may also include additional components to facilitate the particular application for which the kit is designed. For example, a kit may additionally contain means of detecting the label (*e.g.*, enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a sheep anti-mouse-HRP, *etc.*) and reagents necessary for controls (*e.g.*,
15 control biological samples or standards). A kit may additionally include buffers and other reagents recognized for use in a method of the disclosed invention. Non-limiting examples include agents to reduce non-specific binding, such as a carrier protein or a detergent.

EXAMPLES

20 Example 1: Materials and methods for Examples 2-5

a. Cells and cell culture

CT26 colon mouse tumor cell line was purchased from ATCC (CT26.WT ATCC CRL-2638). CT26 was maintained in RPMI-1640 with 10% heat-inactivated FCS, 1% GlutaMax®, 1% pen-strep, and 15 µg/ml gentamicin, at 5% CO₂. Jurkat cells transfected
25 with mCX3CR1 were made and maintained in RPMI-1640 media with 5 µg/ml puromycin in 5% CO₂. Similarly, 300 cells transfected with mCX3CR1 and 300 cells transfected with mPD-L1 were made and maintained in RPMI-1640 media with 5 µg/ml puromycin and 50 µM 2-mercaptoethanol in 5% CO₂.

b. Antibody

30 The antibodies for mCX3CR1, clones 455.2E8 and 455.1C11, were generated by immunizing CX3CR1 knockout mice (kindly provided by Uli von Andrian, Harvard Medical School) with 300-mCX3CR1 cells. Jurkat-CX3CR1 cells were used to screen for reactivity with CX3CR1 and untransfected Jurkat cells were used to screen for lack of

reactivity with untransfected Jurkat cells. The monoclonal antibodies are of IgG2c isotype, an isotype found in the knockout mouse of C57BL/6 origin. Goat anti-mouse IgG PE was purchased from Southern Biotech (Birmingham, AL). MOPC21, IgG1, and C1.18, mIgG2a were purchased from BioXcell. CX3CL1-human IgG1 Fc fusion protein was a kind gift of Uli von Andrian at Harvard Medical School. For flow cytometry, fluorochrome-conjugated antibodies were made by Biolegend (San Diego, CA) and their isotype controls were purchased from BioLegend (San Diego, CA).

c. Antibody titration experiments

The cell line, Jurkat mCX3CR1, was incubated with the 455.1C11 and 455.2E8 antibody. The antibody binding was tested at 10 µg/ml and a series of two-fold dilutions. A flow cytometric analysis using BD FACS Canto™ was done. The detection reagent used was goat anti-mouse IgG PE at 10 µg/ml.

d. Antibody blocking of ligand binding experiments

The cell line, Jurkat mCX3CR1, was incubated for 30 min. with hybridoma supernatant of 1C11 and 2E8 antibody in a two fold-dilution series. This was followed by the subsequent addition of CX3CL1-hIgG at 2 µg/ml. The binding of the CX3CL1 fusion protein was detected by goat anti-hIgG PE at 10 µg/ml and flow cytometry.

e. CX3CR1 expression

The tumor cell line, CT26, was assayed for CX3CR1 expression using 1C11 antibody and PD-L1 expression using 298B.8E2 antibody (Chaudhri *et al.* (2018) *Cancer Immunol. Res.* 6:21-929). A flow cytometric analysis using BD FACSCanto™ II was performed and data were analysed using FlowJo software.

f. Tumor infiltrating lymphocyte isolation

For immune infiltrate studies, mice were sacrificed at tumor size of 1 cm. Tumors were disaggregated using collagenase and red blood cells were lysed using RBC lysis buffer. Cells were Fc-blocked and stained with fluorochrome-conjugated antibodies for 30 min, and permeabilized for intracellular staining. Acquisition was performed on a Becton Dickinson LSRFortessa™ SORP HTS flow cytometer. Data analysis was performed using FlowJo X software.

g. Antibody treatment

CT26 tumor cells were injected at 0.2-0.25 million cells per mouse in Balb/c mice. At day 7, the antibody treatments were started for a series of 5 treatments once every 3 days. 200 µg of antibodies were injected per treatment. Mice were monitored for tumor

size and survival. The treatment groups were 1C11 anti mCX3CR1, 1A12 anti mPD-1, 2A3 rat IgG2a isotype control. For study of tumor immune infiltrate, mice were sacrificed when the tumors were about 1 cm in size.

h. Western blot

5 Cell lysates were resolved on NuPAGE® Novex® bis tris 4-12% gel followed by transfer to nitrocellulose membrane by dry transfer method (Bio-Rad). The membrane was blocked in 5% milk, 1% goat serum in TBST. The primary antibody, anti-mouse CX3CR1 455.1C11 used at 1 µg/ml, was incubated at 4°C overnight. The secondary antibody was goat anti-mouse IgG HRP used at 1 to 5,000 dilution. The blot was developed with
10 SuperSignal™ West Pico stable peroxide solution/SuperSignal™ West Pico luminal enhancer.

Example 2: Anti-mCX3CR1 monoclonal antibodies bind with high affinity to mCX3CR1

15 CX3CR1 monoclonal antibodies were generated by immunizing CX3CR1 knockout mice. In order to determine the binding affinity of generated anti-mCX3CR1 antibodies, two clones, named 1C11 and 2E8, were tested for their respective binding affinities to cell surface CX3CR1 expressed by transfection on Jurkat cells. As expected, the two clones had a very high binding affinity with CX3CR1, while no binding was observed with the
20 mIgG1 control (MOPC21). The antibody binding followed a concentration-dependent dose curve. Antibody 1C11 had the highest affinity with an EC₅₀ of 0.102 µg/ml (FIG. 1). This antibody was selected for further studies. A blocking assay was performed to see if 1C11 blocked CX3CL1 from binding to CX3CR1. Both of the 1C11 and 2E8 antibodies blocked the binding of a CX3CL1 fusion protein to Jurkat cells transfected to express mCX3CR1
25 (FIG. 2).

Example 3: Expression of CX3CR1 and PD-L1 on tumor cell lines

CT26 colon carcinoma cells were used to test whether tumor cell line expressed CX3CR1 and PD-L1. By flow cytometry with the 1C11 anti-mCX3CR1 antibody and the
30 8E2 anti-mPD-L1 antibody, at 10 µg/ml, it was determined that CT26 cells express CX3CR1 and PD-L1.(FIGS. 3A-3B). The IgG controls did not show any binding.

Western blot analyses further confirmed total CX3CR1 protein expression in CT26 (FIG. 7). Cell surface CX3CR1 expression was tested on additional tumor mouse cell lines,

4T1 (metastatic breast) and GL261 (glioblastoma), by flow cytometry. These additional tumor cell lines also expressed CX3CR1 (FIG. 8). In addition, total CX3CR1 protein expression was tested and confirmed in 4T1, GL261 and ID8 (ovarian) mouse tumor lines (FIG. 9).

5

Example 4: Expression of CX3CR1 in the tumor immune infiltrate

Tumor immune infiltrate generated as described in FIG. 4A was analyzed in order to determine whether CX3CR1 was expressed. It was determined that both the tumor immune infiltrate and tumor cells expressed CX3CR1 (FIGS. 4A, 4B, 5A, and 5B). It was observed that a variety of immune cell types including B cells, NK cells, CD4 T cells, CD8 T cells, regulatory T cells (Tregs), M1 macrophages (M1), M2 macrophages (M2), and dendritic cells (DC), express CX3CR1 (FIGS. 5A and 5B).

10

Example 5: Tumor immunotherapy with CX3CR1 monoclonal antibody

A mouse CT26 tumor immunotherapy experiment was carried out with antibody blockade therapy starting at day 7 with 200 µg mAb per mouse. It was determined that combination blockade of CX3CR1 and PD-1 synergistically enhanced survival of tumor bearing mice as compared to anti-mPD-1 alone (FIG. 6). As expected, the IgG-treated control mice died at early time points (FIG. 6).

15

CX3CR1 is a chemokine receptor that is important for homing of leukocytes, NK cells, B cells, effector T cells, and CD14⁺ monocytes. Previous studies have indicated that the CX3CR1-CX3CL1 axis might be a candidate for tumor immunotherapy but good modulating agents were not available. Though this pathway is a chemoattractant for leukocyte migration, its function for the adhesion and migration of tumor cells has also been shown (Celesti *et al.* (2013) *Br. J. Cancer* 109: 2424-2433). CX3CR1 was reported to be highly expressed on a wide variety of tumor cell types (Ferretti *et al.* (2014) *Mediators Inflamm.* 480941). The expression of CX3CR1 on cancer cell lines, as well as ex vivo mouse tumors, is demonstrated herein by flow cytometry. There is greater expression of CX3CR1 on the tumor cells than that of the infiltrating immune cells.

20

25

The data described herein are consistent with the previous results of CX3CR1 being highly expressed on M2 macrophages within the tumor immune infiltrate (Zheng *et al.* (2013) *Mol. Cancer* 12:141). It has been shown that mice lacking CX3CR1 have a decrease in liver metastasis of colon carcinoma, with a decrease in the number of

30

macrophages within the tumor microenvironment (Zheng *et al.* (2013) *Mol. Cancer* 12:141). The same study also shows that CX3CR1 induces angiogenesis and enhances macrophage survival by interfering with the pro-apoptotic pathway in macrophages. The depletion of M2 macrophages can be an effective anti-tumor strategy

5 New anti-mouse CX3CR1 antibodies were developed for use in tumor models. CX3CR1 antibodies were shown to block the interaction of CX3CR1 with its ligand CX3CL1. The mechanisms of action of the CX3CR1 antibody can be by targeting the immune suppressive cell types such as M2 macrophages and the tumor itself. The anti-CX3CR1 antibodies may be of a killer characteristic since they have IgG2c as the Fc
10 domain.

 Studies have shown the involvement of CX3CR1 across different tumors, for instance, it has been shown that malignant breast cancer cells overexpress CX3CR1 and this aids in the migration of cancer cells to bone and brain which release soluble CX3CL1 (Jamieson-Gladney *et al.* (2011) *Breast Cancer Res.* 13:R91). The blockade approach
15 demonstrated herein can have therapeutic effect across different tumor types.

 More effective PD1 combination therapies are needed to increase the number of responders to PD1 therapy in cancer (Freeman and Sharpe. (2012) *Nat. Immunol.* 13:113-115). The data described herein supports the use of anti-CX3CR1 antibody in combination with anti-PD-1 antibody to enhance anti-tumor immunity in a mouse colon carcinoma
20 model. The study described herein indicated that the therapeutic effect of this combination therapy can be clinically significant.

Example 6: Characterization of CX3CR1 monoclonal antibodies

 Additional characterization of CX3CR1 antibodies can be performed. For example,
25 in one embodiment, the combination therapy demonstrated herein can be applied to additional tumor types. In certain embodiments, the combination therapy described herein can be tested in mouse tumor models that have an effective response to PD1 therapy, or in tumors where tumor-associated macrophages play a dominant immunosuppressive role.

 In another embodiment, a study of the tumor infiltrates upon CX3CR1 antibody
30 treatment is carried out to understand the mechanism of action of the CX3CR1 antibody. For example, a comparison between the group treated with single agent and the group treated with the combination blockade indicates the immune subsets and the cell types depleted by the CX3CR1 antibody blockade. In still another embodiment, CX3CR1

antibodies can be injected into the tumor microenvironment rather than systematically administered in order to prevent the depletion of various immune cells.

Additional assays can be used to evaluate CX3CR1 antibodies. For example, ADCC (antibody dependent cellular cytotoxicity) and CDCC (complement dependent cellular cytotoxicity) *in vitro* assays are performed to further characterize the depletion mechanism of the antibodies.

Incorporation by Reference

All publications, patents, and patent applications mentioned herein are hereby incorporated by reference in their entirety as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Also incorporated by reference in their entirety are any polynucleotide and polypeptide sequences which reference an accession number correlating to an entry in a public database, such as those maintained by The Institute for Genomic Research (TIGR) on the world wide web at tigr.org and/or the National Center for Biotechnology Information (NCBI) on the World Wide Web at ncbi.nlm.nih.gov.

Equivalents

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

What is claimed is:

1. A method of treating a subject afflicted with a cancer comprising administering to the subject a therapeutically effective amount of at least one agent that inhibits or blocks both chemokine (C-X3-C motif) receptor 1 (CX3CR1) and an immune checkpoint.
2. The method of claim 1, wherein the at least one agent is a single agent that inhibits or blocks both CX3CR1 and the immune checkpoint.
3. The method of claim 1, wherein the at least one agent comprises a first agent that selectively inhibits or blocks CX3CR1, chemokine (C-X3-C motif) ligand 1 (CX3CL1), and/or the interaction between CX3CR1 and CX3CL1, and a second agent that selectively inhibits or blocks the immune checkpoint.
4. The method of claim 3, wherein said first agent and said second agent comprise a small molecule that inhibits or blocks CX3CR1, CX3CL1, the interaction between CX3CR1 and CX3CL1, and/or the immune checkpoint.
5. The method of claim 1 or 3, wherein the at least one agent comprises an RNA interfering agent which inhibits expression of CX3CR1, CX3CL1, and/or the immune checkpoint.
6. The method of claim 5, wherein the RNA interfering agent is a small interfering RNA (siRNA), small hairpin RNA (shRNA), or a microRNA (miRNA).
7. The method of claim 1 or 3, wherein the at least one agent comprises an antisense oligonucleotide complementary to CX3CR1, CX3CL1, and/or the immune checkpoint.
8. The method of claim 1 or 3, wherein the at least one agent comprises a peptide or peptidomimetic that inhibits or blocks CX3CR1, CX3CL1, the interaction between CX3CR1 and CX3CL1, and/or the immune checkpoint.
9. The method of claim 1 or 3, wherein the at least one agent comprises an aptamer that inhibits or blocks CX3CR1, CX3CL1, the interaction between CX3CR1 and CX3CL1, and/or the immune checkpoint.

10. The method of claim 1 or 3, wherein the at least one agent is an antibody and/or an intrabody, or an antigen binding fragment thereof, which specifically binds to CX3CR1, CX3CL1, and/or the immune checkpoint.
11. The method of claim 10, wherein the antibody, or antigen binding fragment thereof, selectively or specifically binds the extracellular domain of CX3CR1 and/or the immune checkpoint.
12. The method of claim 10 or 11, wherein the antibody and/or intrabody, or antigen binding fragment thereof, is an IgG2c, IgG1, IgG2, or IgG4 antibody.
13. The method of any one of claims 10-12, wherein the antibody and/or intrabody, or antigen binding fragment thereof, is murine, chimeric, humanized, composite, or human.
14. The method of any one of claims 10-13, wherein the antibody and/or intrabody, or antigen binding fragment thereof, is detectably labeled, comprises an effector domain, comprises an Fc domain, and/or is selected from the group consisting of Fv, Fav, F(ab')₂, Fab', dsFv, scFv, sc(Fv)₂, and diabodies fragments.
15. The method of any one of claims 10-14, wherein the antibody and/or intrabody, or antigen binding fragment thereof, is conjugated to a cytotoxic agent.
16. The method of claim 15, wherein the cytotoxic agent is selected from the group consisting of a chemotherapeutic agent, a biologic agent, a toxin, and a radioactive isotope.
17. The method of any one of claims 1-16, wherein the at least one agent inhibits the expression and/or the function of the extracellular domain of CX3CR1.
18. The method of any one of claims 1-17, wherein the immune checkpoint is selected from the group consisting of CTLA-4, PD-1, VISTA, B7-H2, B7-H3, PD-L1, B7-H4, B7-H6, ICOS, HVEM, PD-L2, CD160, gp49B, PIR-B, KIR family receptors, TIM-1, TIM-3, TIM-4, LAG-3, GITR, 4-1BB, OX-40, BTLA, SIRPalpha (CD47), CD48, 2B4 (CD244), B7.1, B7.2, ILT-2, ILT-4, TIGIT, HHLA2, butyrophilins, and A2aR.
19. The method of claim 18, wherein the immune checkpoint is selected from the group consisting of CTLA-4, PD-1, PD-L1, PD-L2, TIM-3, and LAG-3.

20. The method of claim 19, wherein the immune checkpoint is PD-1.
21. The method of any one of claims 1-20, wherein the at least one agent reduces the number of proliferating cells in the cancer and/or reduces the volume or size of a tumor of the cancer.
22. The method of any one of claims 1-21, wherein the at least one agent is administered in a pharmaceutically acceptable formulation.
23. The method of any one of claims 1-22, further comprising administering to the subject a therapeutic agent or regimen for treating the cancer.
24. The method of any one of claims 1-23, wherein the cancer cells in the subject express CX3CR1 and/or PD-L1.
25. The method of any one of claims 1-24, wherein tumor immune infiltrating cells in the subject express CX3CR1.
26. The method of claim 25, wherein the tumor immune infiltrating cells are CD45+ cells, optionally the CD45+ cells are selected from the group consisting of B cells, NK cells, CD4 T cells, CD8 T cells, regulatory T cells (Tregs), M1 macrophages (M1), M2 macrophages (M2), and dendritic cells (DC).
27. The method of any one of claims 1-26, wherein the cancer is selected from the group consisting of colorectal cancer, gliomas, neuroblastoma, prostate cancer, breast cancer, pancreatic ductal carcinoma, ovarian cancer, B-CLL, leukemia, B cell lymphoma, renal cell carcinoma, lung cancer, osteosarcoma, hepatoblastoma, hepatocellular carcinoma, head and neck cancer, adenocarcinoma, gastric carcinoma, myeloma, squamous cell carcinoma, melanoma, and testicular germ cell tumor.
28. The method of claim 27, wherein the cancer is colorectal cancer.
29. The method of any one of claims 1-28, wherein the subject is an animal model of the cancer.
30. The method of claim 29, wherein the animal model is a mouse model.

31. The method of any one of claims 1-30, wherein the subject is a mammal.
32. The method of claim 31, wherein the mammal is a mouse or a human.
33. The method of claim 32, wherein the mammal is a human.
34. The method of any one of claims 1-33, wherein the antibody and/or intrabody, or antigen binding fragment thereof, is generated in a CX3CR1 knockout, CX3CL1 knockout, and/or immune checkpoint knockout host cell or animal.
35. The method of any one of claims 1-34, wherein the at least one agent is an antibody and/or an intrabody, or an antigen binding fragment thereof, which specifically binds to CX3CR1.
36. A monoclonal antibody, or antigen-binding fragment thereof, wherein the monoclonal antibody comprises:
- a) a heavy chain sequence with at least about 95% identity to a heavy chain sequence selected from the group consisting of the sequences listed in Table 2 or
 - b) a light chain sequence with at least about 95% identity to a light chain sequence selected from the group consisting of the sequences listed in Table 2.
37. The monoclonal antibody, or antigen-binding fragment thereof, of claim 36, wherein the monoclonal antibody comprises:
- a) a heavy chain CDR sequence with at least about 95% identity to a heavy chain CDR sequence selected from the group consisting of the sequences listed in Table 2 or
 - b) a light chain CDR sequence with at least about 95% identity to a light chain CDR sequence selected from the group consisting of the sequences listed in Table 2.
38. The monoclonal antibody, or antigen-binding fragment thereof, of claim 36, wherein the monoclonal antibody comprises:
- a) a heavy chain sequence selected from the group consisting of the sequences listed in Table 2; or
 - b) a light chain sequence selected from the group consisting of the sequences listed in Table 2.

39. The monoclonal antibody, or antigen-binding fragment thereof, of claim 36, wherein the monoclonal antibody comprises:
- a) a heavy chain CDR sequence selected from the group consisting of the sequences listed in Table 2; or
 - b) a light chain CDR sequence selected from the group consisting the sequences listed in Table 2.
40. The monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-39, wherein the monoclonal antibody or antigen-binding fragment thereof is chimeric, humanized, composite, murine, or human.
41. The monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-40, wherein the monoclonal antibody, or antigen-binding fragment thereof, is detectably labeled, comprises an effector domain, comprises an Fc domain, and/or is selected from the group consisting of Fv, Fav, F(ab')₂, Fab', dsFv, scFv, sc(Fv)₂, and diabodies fragments.
42. The monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-41, wherein the monoclonal antibody or antigen-binding fragment thereof inhibits the binding of commercial antibody to CX3CR1.
43. The monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-42, wherein said antibody is obtainable from hybridoma _____ deposited under deposit accession number _____.
44. An immunoglobulin heavy and/or light chain of any one of claims 36-43.
45. The method of claim 35, wherein the at least one agent is the monoclonal antibody of any one of claims 36-44.
46. An isolated nucleic acid molecule that hybridizes, under stringent conditions, with the complement of a nucleic acid encoding a polypeptide selected from the group consisting of the sequences listed in Table 2, or a sequence with at least about 95% homology to a nucleic acid encoding a polypeptide selected from the group consisting of the sequences listed in Table 2.
47. A vector comprising the isolated nucleic acid of claim 46.

48. A host cell which comprises the isolated nucleic acid of claim 46, comprises the vector of claim 47, expresses the antibody or antigen-binding fragment thereof of any one of claims 36-44, or is accessible under deposit accession number _____.

49. A device or kit comprising at least one monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-44, said device or kit optionally comprising a label to detect the at least one monoclonal antibody or antigen-binding fragment thereof, or a complex comprising the monoclonal antibody or antigen-binding fragment thereof.

50. A method of producing at least one monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-44, which method comprises the steps of: (i) culturing a transformed host cell which has been transformed by a nucleic acid comprising a sequence encoding a monoclonal antibody of any one of claims 36-44 under conditions suitable to allow expression of said antibody, or antigen-binding fragment thereof; and (ii) recovering the expressed antibody, or antigen-binding fragment thereof.

51. A method of detecting the presence or level of a CX3CR1 polypeptide said method comprising obtaining a sample and detecting said polypeptide in a sample by use of at least one monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-44.

52. The method of claim 51, wherein the at least one monoclonal antibody, or antigen-binding fragment thereof, forms a complex with a CX3CR1 polypeptide and the complex is detected in the form of an enzyme linked immunosorbent assay (ELISA), radioimmune assay (RIA), immunochemically, or using an intracellular flow assay.

53. A method for monitoring the progression of a disorder associated with aberrant CX3CR1 expression in a subject, the method comprising:

- a) detecting in a subject sample at a first point in time the level of expression of CX3CR1 using at least one monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-44;
- b) repeating step a) at a subsequent point in time; and
- c) comparing the level of expression of said CX3CR1 detected in steps a) and b) to monitor the progression of the disorder in the subject.

54. The method of claim 53, wherein between the first point in time and the subsequent point in time, the subject has undergone treatment to ameliorate the disorder.

55. A method for predicting the clinical outcome of a subject afflicted with a disorder associated with aberrant CX3CR1, the method comprising:

a) determining the level of expression of CX3CR1 in a patient sample using at least one monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-44;

b) determining the level of expression of CX3CR1 in a sample from a control subject having a good clinical outcome using at least one monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-44; and

c) comparing the level of expression of CX3CR1 in the patient sample and in the sample from the control subject;

wherein a significantly higher level of expression in the patient sample as compared to the expression level in the sample from the control subject is an indication that the patient has a poor clinical outcome.

56. A method of assessing the efficacy of a therapy for a disorder associated with aberrant CX3CR1 in a subject, the method comprising comparing:

a) the level of expression of CX3CR1 using at least one monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-44, in a first sample obtained from the subject prior to providing at least a portion of the therapy to the subject, and

b) the level of expression of CX3CR1 in a second sample obtained from the subject following provision of the portion of the therapy,

wherein a significantly lower level of expression of CX3CR1 in the second sample, relative to the first sample, is an indication that the therapy is efficacious for inhibiting the disorder in the subject.

57. A method of assessing the efficacy of a test compound for inhibiting a disorder associated with aberrant CX3CR1 in a subject, the method comprising comparing:

a) the level of expression of CX3CR1 using at least one monoclonal antibody, or antigen-binding fragment thereof, of any one of claims 36-44, in a first sample obtained from the subject and exposed to the test compound; and

b) the level of expression of CX3CR1 in a second sample obtained from the subject,

wherein the second sample is not exposed to the test compound, and a significantly lower level of expression of CX3CR1, relative to the second sample, is an indication that the test compound is efficacious for inhibiting the disorder in the subject.

58. The method of claim 57, wherein the first and second samples are portions of a single sample obtained from the subject or portions of pooled samples obtained from the subject.

59. The method of any one of claims 53-58, wherein the disorder is a cancer, optionally wherein the cancer is selected from the group consisting of colorectal cancer, gliomas, neuroblastoma, prostate cancer, breast cancer, pancreatic ductal carcinoma, ovarian cancer, B-CLL, leukemia, B cell lymphoma, renal cell carcinoma, lung cancer, osteosarcoma, hepatoblastoma, hepatocellular carcinoma, head and neck cancer, adenocarcinoma, gastric carcinoma, myeloma, squamous cell carcinoma, melanoma, and testicular germ cell tumor.

60. The method of any one of claims 53-59, wherein the sample comprises cells, serum, peritumoral tissue, and/or intratumoral tissue obtained from the subject.

61. The method of any one of claims 53-60, wherein said significant increase comprises an at least twenty percent increase between the level of expression of CX3CR1 in the subject sample relative to the normal level of expression of CX3CR1 in the sample from the control subject.

62. The method of any one of claims 53-61, wherein the subject is an animal model of a cancer.

63. The method of claim 62, wherein the animal model is a mouse model.

64. The method of any one of claims 53-63, wherein the subject is a mammal.

65. The method of claim 64, wherein the mammal is a mouse or a human.

FIG. 1

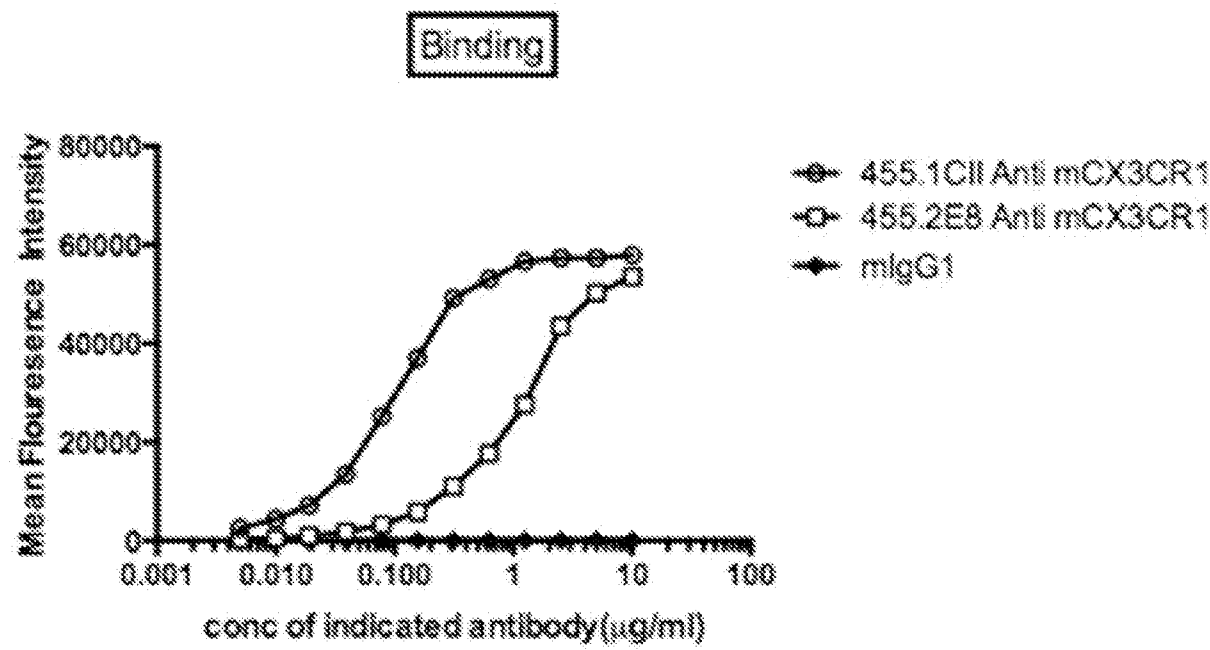


FIG. 2

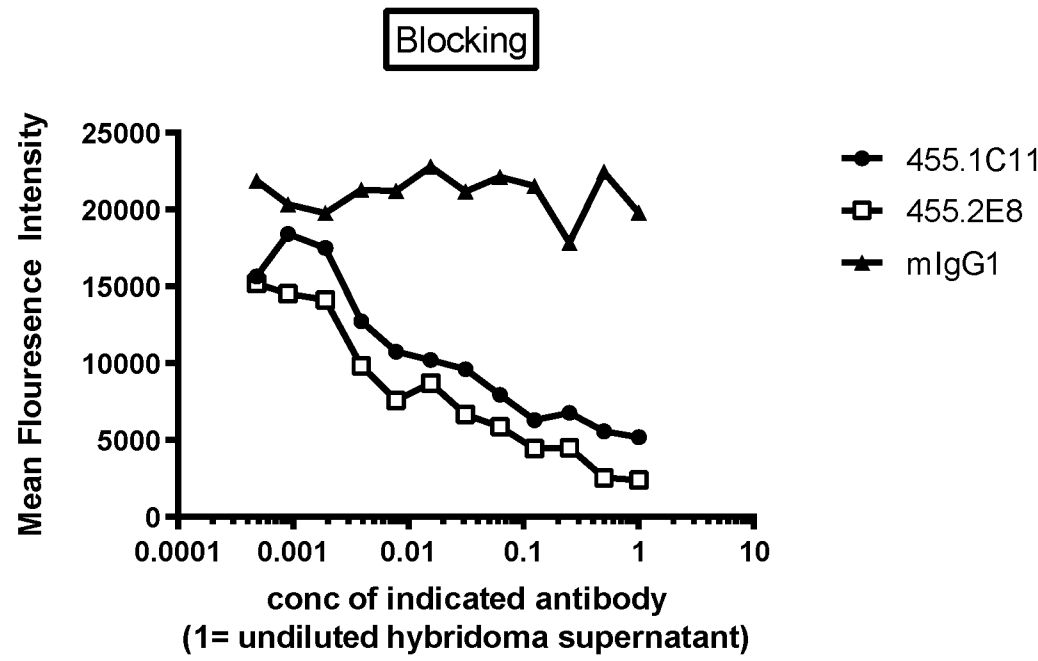


FIG. 3A

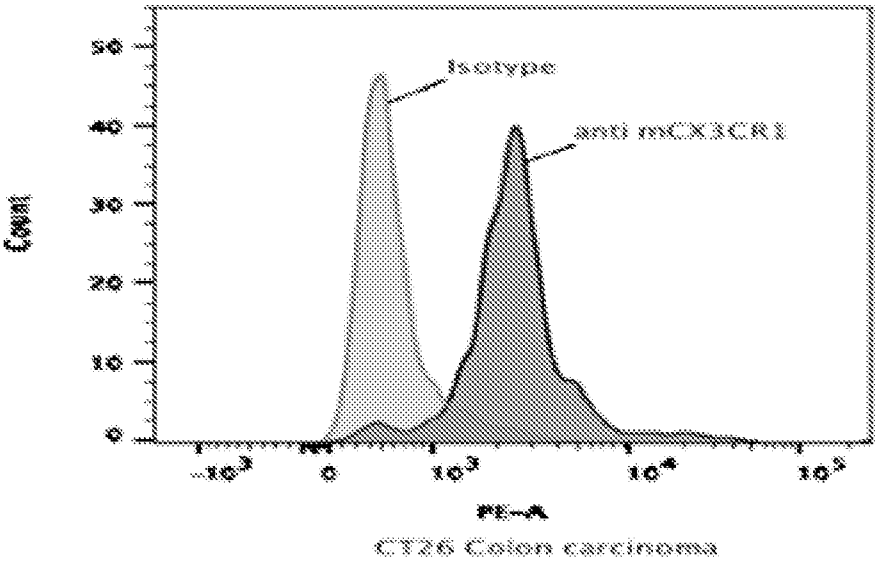


FIG. 3B

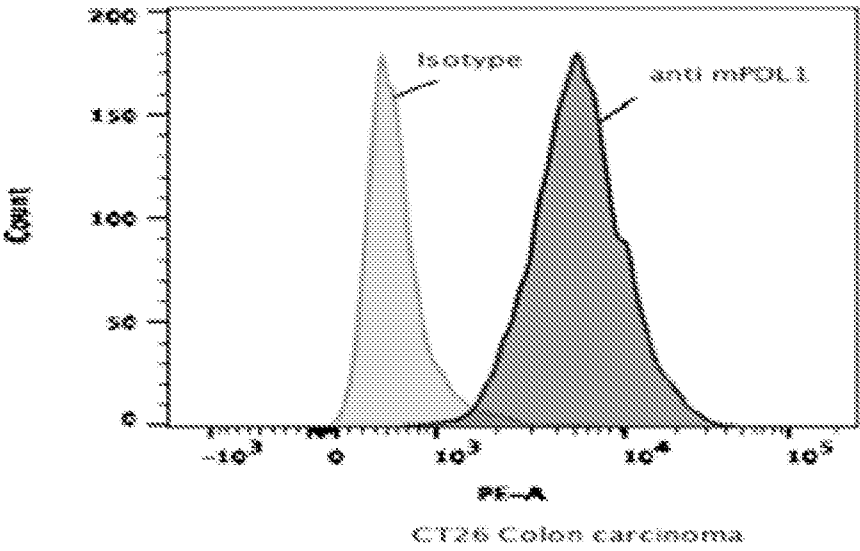


FIG. 4A

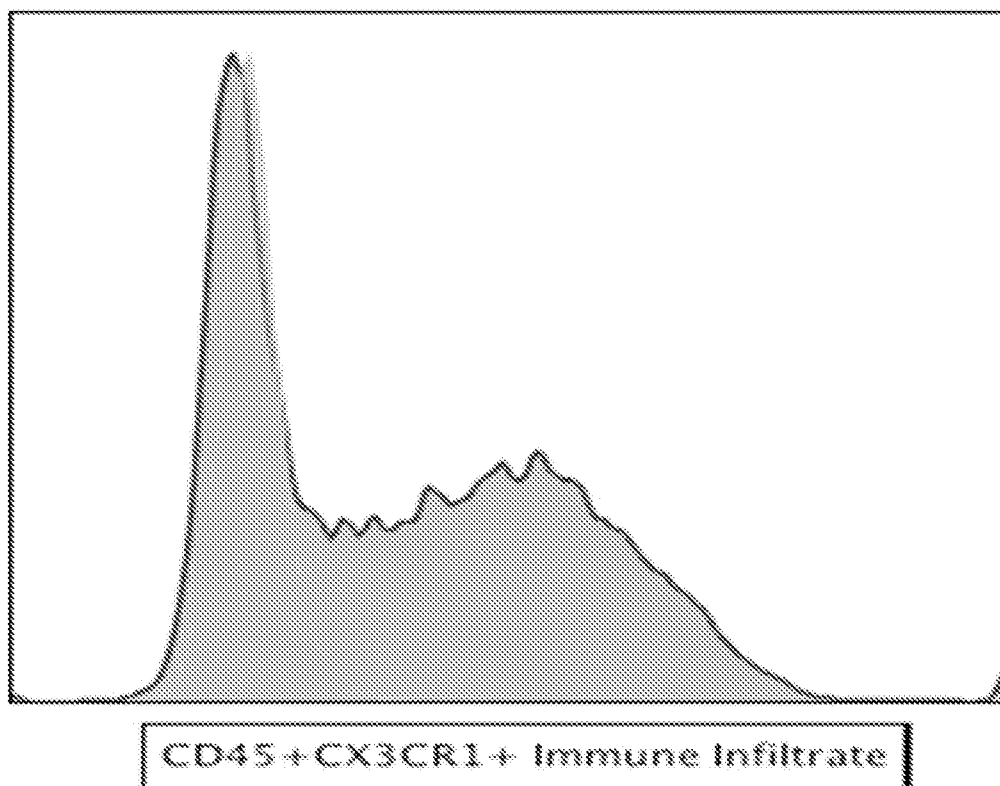


FIG. 4B

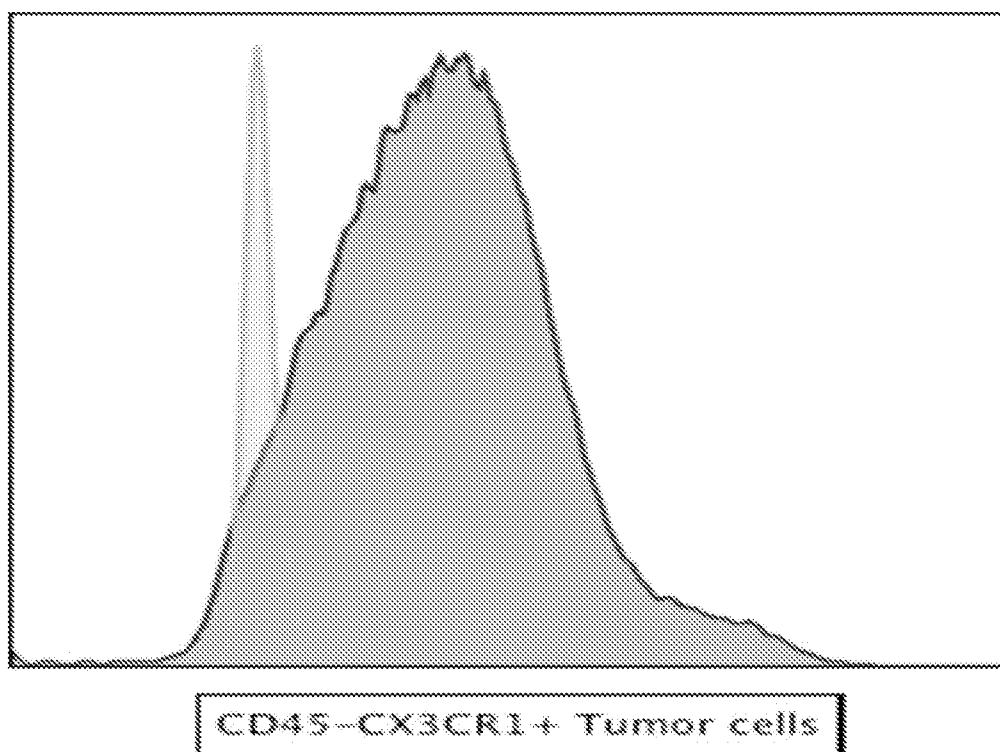


FIG. 5B

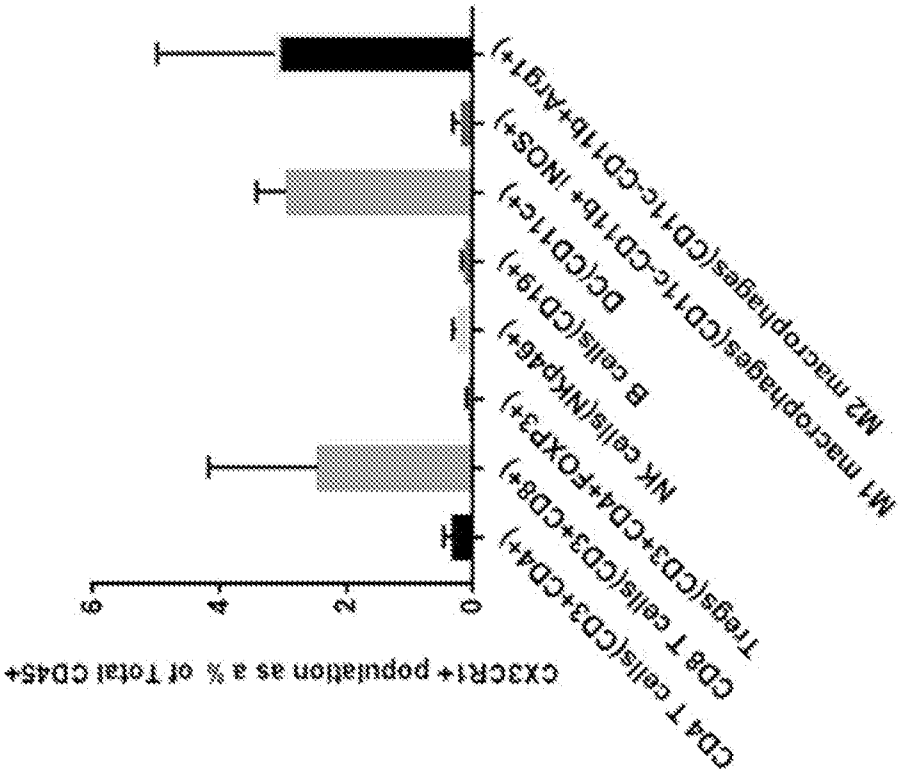


FIG. 5A

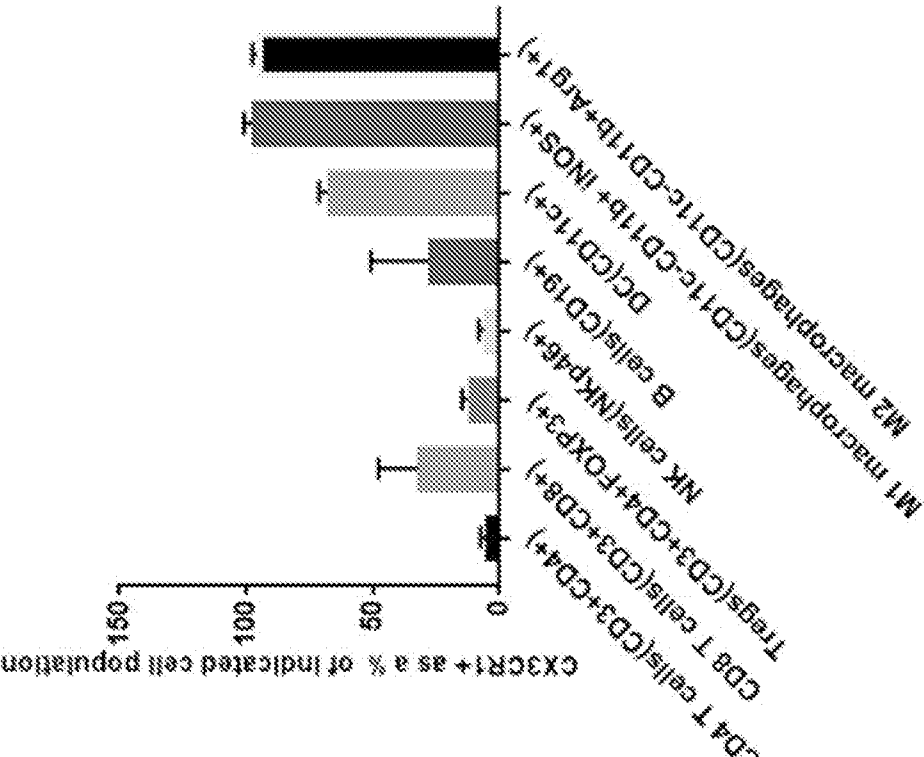


FIG. 6

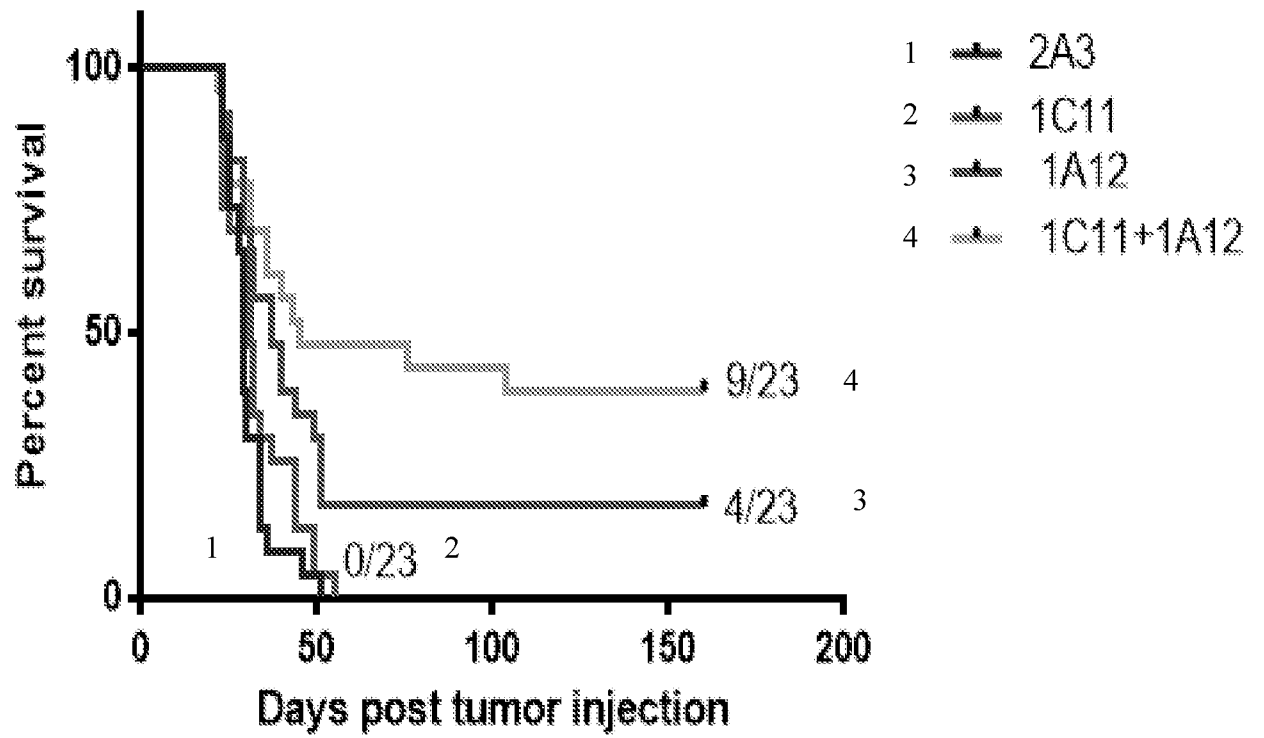


FIG. 7

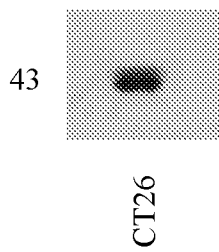


FIG. 8

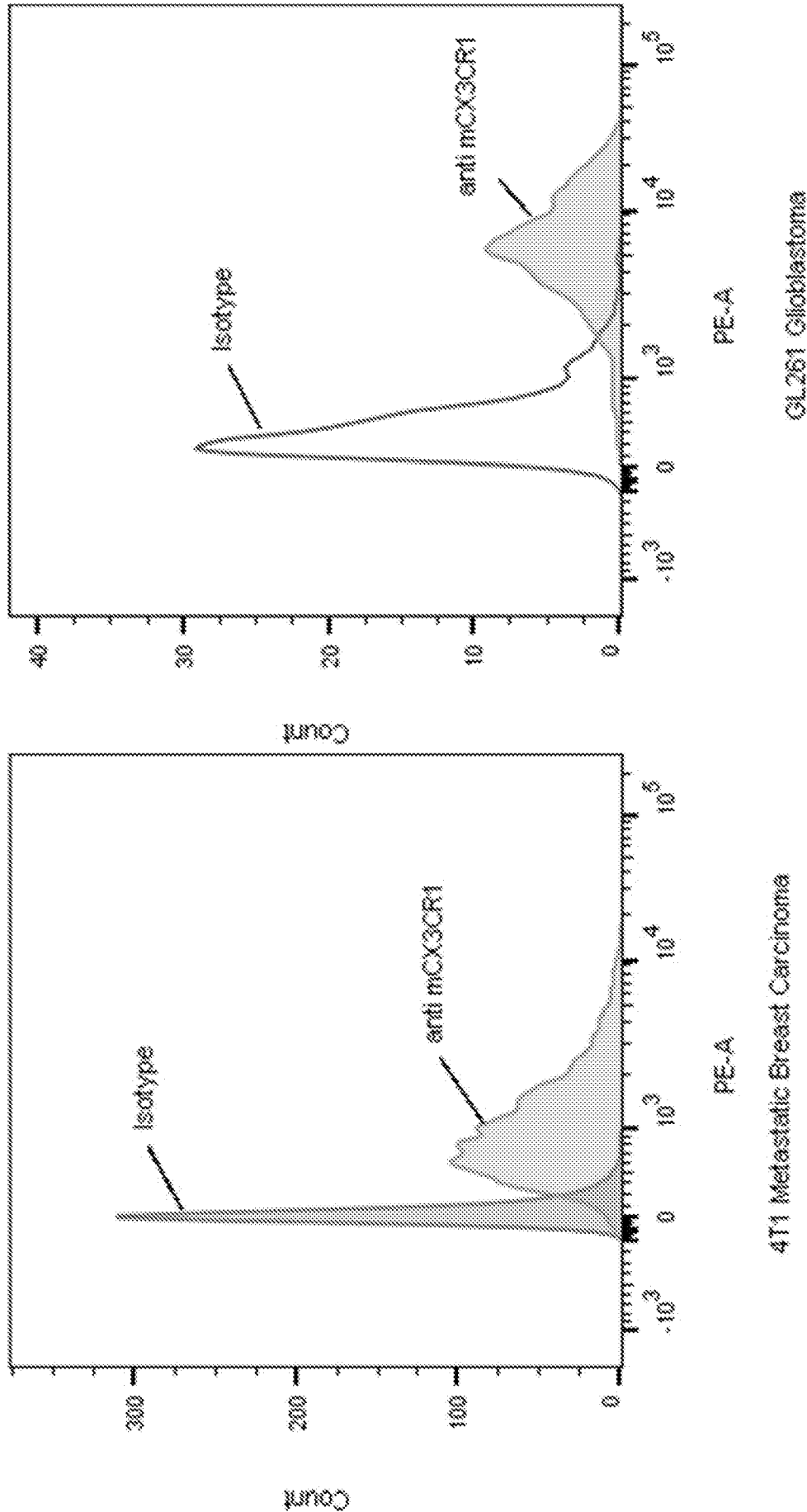
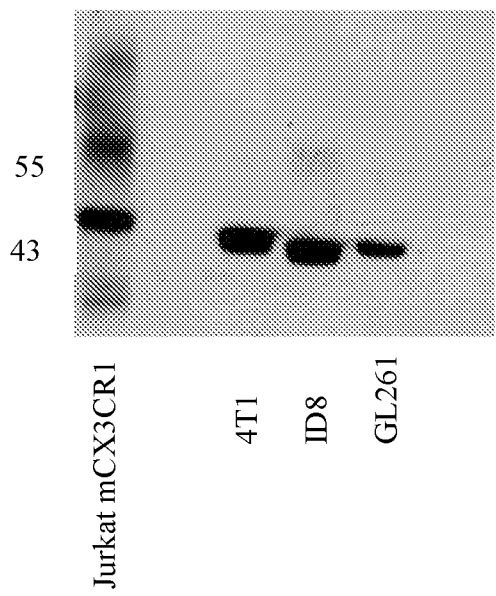


FIG. 9



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/29772

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 39/395; C07K 16/28; C07K 16/30; C07K 16/24; A61P 35/02; A61P 35/02 (2020.01)

CPC - A61K 39/395; C07K 16/28; C07K 16/30; C07K 16/24; A61K 39/001129; C07K 2317/31; C07K 2317/35; A61P 35/02; A61P 35/02

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2018/0303922 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 25 October 2018 (25.10.2018) claims 120, 131.	1-2
A	US 7,390,490 B1 (IMAI et al.) 24 June 2008 (24.06.2008) claim 1	1-2
A	WO 2017/019896 A1 (NOVARTIS AG) 2 February 2017 (02.02.2017) Especially claim 1	1-2
A	YAN et al. CX3CR1 identifies PD-1 therapy-responsive CD8+ T cells that withstand chemotherapy during cancer chemioimmunotherapy. JCI Insight. 19 April 2018, Vol 3, No 8, Pages e97828 1-14. Especially abstract	1-2
A	WO 2017/181111 A2 (GENENTECH, INC.) 19 October 2017 (19.10.2017) claim 31	1-2

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 July 2020

Date of mailing of the international search report

28 SEP 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Lee Young

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/29772

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

☒ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a))

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/29772

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 36-40, 46, 47
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 36-40, 46, 47 are held unsearchable as they refer to sequences in instant application pg 69-70 Table 2, where none of these sequences are indicated to have a SEQ ID NO:, nor are they found in C/ST.25 text file. It would be impossible to conduct a sequence search of the prior art using the system sequence search tool.
3. ☒ Claims Nos.: 12-35, 41-45, 48-65
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
-----Go to Extra Sheet for continuation-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-2, limited to a single agent that that inhibits or blocks both CX3CR1 and the immune checkpoint.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US 20/29772

Continuation of Box III: Observations where Unity of Invention is lacking

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

Group I+: Claims 1-11 drawn to administering at least one agent that inhibits or blocks CX3CR1 and immune checkpoint. The method of treating a plant will be searched to the extent that the at least one agent is the first named, a single agent that inhibits or blocks both CX3CR1 and an immune checkpoint. It is believed that claims 1-2 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass a single agent that inhibits or blocks both CX3CR1 and an immune checkpoint. Additional first agents, first agent targets and second agents will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional first agents, first agent targets and second agents. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: first agent antibody directed against CX3CL1, and second agent antibody against immune checkpoint (claims 1, 3, 10, 11).

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

Special Technical Features:

Among the inventions listed as Groups I+ are the specific target of the first agent (e.g., CX3CR1, CX3CL1) and types of agent (e.g., antibody, small molecule, RNAi) recited therein. Each invention requires a specific target of the first agent and the type of agent not required by any other inventions.

Common Technical Feature:

Group I+ inventions share claim 1.

However, said common technical feature do not represent a contribution over the prior art, and is disclosed by US 2018/0303922 A1 to President and Fellows of Harvard College (hereinafter "Harvard").

As to claim 1, Harvard discloses a method of treating a subject afflicted with a cancer comprising administering to the subject a therapeutically effective amount of at least one agent that inhibits or blocks both chemokine (C-X3-C motif) receptor 1 (CX3CR1) and an immune checkpoint (claims 120 and 131; "120. A method of increasing the effector function of effector T cells (Teffs) comprising contacting Teffs with a bispecific antibody selective for both PD-1 [i.e. would function as an immune checkpoint inhibitor] and a Teff cell surface protein, thereby increasing the effector function of the Teffs"; "131. The method of any one of claims 120-130, wherein the Teff cell surface protein is selected from the group consisting of ... CX3CR1").

As the common technical feature was known in the art at the time of the invention, this cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Group I+ inventions lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

item 4 (cont.): Claims 12-35, 41-45, 48-65 are multiple dependent claims and are not drafted according to the second and third sentences of PCT Rule 6.4(a).

Claims 36-40, 46, 47 are held unsearchable as they refer to sequences in instant application pg 69-70 Table 2, where none of these sequences are indicated to have a SEQ ID NO.; nor are they found in C/ST.25 text file. It would be impossible to conduct a sequence search of the prior art using the system sequence search tool.