

endocrinopathies, and inflammatory myopathy. These unique side effects are reported to arise due to breaking immune tolerance upon CTLA-4 blockade (Di Giacomo, A.M., *et al.* (2010) “*The Emerging Toxicity Profiles Of Anti-CTLA-4 Antibodies Across Clinical Indications*,” Semin Oncol. 37:499-507). Accordingly, therapies which overcome these limitations would be of great benefit.

**[0021]** As described in detail below, the present invention addresses this need by providing PD-1 x CTLA-4 bispecific molecules. Such bispecific molecules are capable of binding to PD-1 and CTLA-4 molecules that are present on the surfaces of exhausted and tolerant tumor-infiltrating lymphocytes and other cell types, and of thereby impairing the ability of such cell-surface molecules to respond to their respective ligands. As such, the PD-1 x CTLA-4 bispecific molecules of the present invention act to block PD-1- and CTLA-4-mediated immune system inhibition, so as to promote the activation or continued activation of the immune system. These attributes permit such bispecific molecules to have utility in stimulating the immune system and particularly in the treatment of cancer and pathogen-associated diseases and conditions. The present invention is directed to these and other goals.

## SUMMARY

**[0022]** The present disclosure is directed to bispecific molecules (*e.g.*, diabodies, bispecific antibodies, trivalent binding molecules, *etc.*) that possess at least one epitope-binding site that is immunospecific for an epitope of PD-1 and at least one epitope-binding site that is immunospecific for an epitope of CTLA-4 (*i.e.*, a “PD-1 x CTLA-4 bispecific molecule”). The present disclosure concerns such PD-1 x CTLA-4 bispecific molecules that possess two epitope-binding sites that are immunospecific for one (or two) epitope(s) of PD-1 and two epitope-binding sites that are immunospecific for one (or two) epitope(s) of CTLA-4. The present disclosure also is directed to such PD-1 x CTLA-4 bispecific molecules that additionally comprise an immunoglobulin Fc Region. The PD-1 x CTLA-4 bispecific molecules of the present disclosure are capable of simultaneously binding to PD-1 and to CTLA-4, particularly as such molecules are arrayed on the surfaces of human cells. The disclosure is directed to pharmaceutical compositions that contain such PD-1 x CTLA-4 bispecific molecules, and to methods involving the use of such bispecific molecules in the treatment of cancer and other diseases and conditions. The present disclosure also pertains to methods of using such PD-1 x CTLA-4 bispecific molecules to stimulate an immune response.

**[0023]** In one aspect, there is provided a bispecific molecule possessing both one or more epitope-binding sites capable of immunospecific binding to (an) epitope(s) of PD-1 and one or more epitope-binding sites capable of immunospecific binding to (an) epitope(s) of CTLA-4, wherein the molecule comprises:

- (A) a Heavy Chain Variable Domain and a Light Chain Variable Domain of an antibody that binds PD-1; and
- (B) a Heavy Chain Variable Domain and a Light Chain Variable Domain of an antibody that binds CTLA-4;

wherein the molecule is:

- (i) a diabody, the diabody being a covalently bonded complex that comprises two, three, four or five polypeptide chains; or
- (ii) a trivalent binding molecule, the trivalent binding molecule being a covalently bonded complex that comprises three, four, five, or more polypeptide chains.

**[0024]** The disclosure concerns the embodiment of such bispecific molecules, wherein the bispecific binding molecule exhibits an activity that is enhanced relative to such activity exhibited by two monospecific molecules one of which possesses the Heavy Chain Variable Domain and the Light Chain Variable Domain of the antibody that binds PD-1 and the other of which possesses the Heavy Chain Variable Domain and the Light Chain Variable Domain of the antibody that binds CTLA-4.

**[0025]** The disclosure concerns the embodiment of all such bispecific molecules, wherein the molecule elicits fewer immune-related adverse events (irAEs) when administered to a subject in need thereof relative to such iREs elicited by the administration of a monospecific antibody that binds CTLA-4 such as ipilimumab.

**[0026]** The disclosure additionally concerns the embodiment of such bispecific molecules in which the molecule comprises an Fc Region. The disclosure additionally concerns the embodiment of such bispecific molecules wherein the Fc Region is a variant Fc Region that comprises:

- (A) one or more amino acid modifications that reduces the affinity of the variant Fc Region for an FcγR; and/or
- (B) one or more amino acid modifications that enhances the serum half-life of the variant Fc Region.

**[0027]** The disclosure additionally concerns the embodiment of such bispecific molecules wherein the modifications that reduces the affinity of the variant Fc Region for an FcγR comprise the substitution of L234A; L235A; or L234A and L235A, wherein the numbering is that of the EU index as in Kabat.

**[0028]** The disclosure additionally concerns the embodiment of such bispecific molecules wherein the modifications that enhances the serum half-life of the variant Fc Region comprise the substitution of M252Y; M252Y and S254T; M252Y and T256E; M252Y, S254T and T256E; or K288D and H435K, wherein the numbering is that of the EU index as in Kabat.

**[0029]** The disclosure additionally concerns the embodiment of all such bispecific molecules wherein the molecule is the diabody and comprises two epitope-binding sites capable of immunospecific binding to an epitope of PD-1 and two epitope-binding sites capable of immunospecific binding to an epitope of CTLA-4.

**[0030]** The disclosure additionally concerns the embodiment of all such bispecific molecules wherein the molecule is the trivalent binding molecule and comprises two epitope-binding sites capable of immunospecific binding to an epitope of PD-1 and one epitope-binding site capable of immunospecific binding to an epitope of CTLA-4.

**[0031]** The disclosure additionally concerns the embodiment of all such bispecific molecules wherein the molecule is capable of binding to PD-1 and CTLA-4 molecules present on the cell surface.

**[0032]** The disclosure additionally concerns the embodiment of all such bispecific molecules wherein the molecule is capable of simultaneously binding to PD-1 and CTLA-4.

**[0033]** The disclosure additionally concerns the embodiment of all such bispecific molecules wherein the molecule promotes the stimulation of immune cells, and particularly wherein the stimulation of immune cells results in:

- (A) immune cell proliferation; and/or
- (B) immune cell production and/or release of at least one cytokine; and/or
- (C) immune cell production and/or release of at least one lytic molecule; and/or
- (D) immune cell expression of at least one activation marker.

**[0034]** The disclosure additionally concerns the embodiment of all such bispecific molecules wherein the immune cell is a T-lymphocyte or an NK-cell.

**[0035]** The disclosure additionally concerns the embodiment of all such bispecific molecules wherein the epitope-binding sites capable of immunospecific binding to an epitope of PD-1 comprise:

- (A) the VH Domain of PD-1 mAb 1 (**SEQ ID NO:47**) and the VL Domain of PD-1 mAb 1 (**SEQ ID NO:48**); or
- (B) the VH Domain of PD-1 mAb 2 (**SEQ ID NO:49**) and the VL Domain of PD-1 mAb 2 (**SEQ ID NO:50**); or
- (C) the VH Domain of PD-1 mAb 3 (**SEQ ID NO:51**) and the VL Domain of PD-1 mAb 3 (**SEQ ID NO:52**); or
- (D) the VH Domain of PD-1 mAb 4 (**SEQ ID NO:53**) and the VL Domain of PD-1 mAb 4 (**SEQ ID NO:54**); or
- (E) the VH Domain of PD-1 mAb 5 (**SEQ ID NO:55**) and the VL Domain of PD-1 mAb 5 (**SEQ ID NO:56**); or
- (F) the VH Domain of PD-1 mAb 6 (**SEQ ID NO:57**) and the VL Domain of PD-1 mAb 6 (**SEQ ID NO:58**); or
- (G) the VH Domain of PD-1 mAb 6-I VH (**SEQ ID NO:86**) and the VL Domain of PD-1 mAb 6-SQ VL (**SEQ ID NO:87**); or
- (H) the VH Domain of PD-1 mAb 7 (**SEQ ID NO:59**) and the VL Domain of PD-1 mAb 7 (**SEQ ID NO:60**); or
- (I) the VH Domain of PD-1 mAb 8 (**SEQ ID NO:61**) and the VL Domain of PD-1 mAb 8 (**SEQ ID NO:62**).

**[0036]** The disclosure additionally concerns the embodiment of all such bispecific molecules wherein the epitope-binding site(s) capable of immunospecific binding to an epitope of CTLA-4 comprise:

- (A) the VH Domain of CTLA-4 mAb 1 (**SEQ ID NO:76**) and the VL Domain of CTLA-4 mAb 1 (**SEQ ID NO:77**); or
- (B) the VH Domain of CTLA-4 mAb 2 (**SEQ ID NO:78**) and the VL Domain of CTLA-4 mAb 2 (**SEQ ID NO:79**); or
- (C) the VH Domain of CTLA-4 mAb 3 (**SEQ ID NO:90**) and the VL Domain of CTLA-4 mAb 3 (**SEQ ID NO:91**).

[0037] The disclosure additionally concerns the embodiment of such bispecific molecules wherein:

- (A) the epitope-binding sites capable of immunospecific binding to an epitope of PD-1 comprise the VH Domain of PD-1 mAb 6-I VH (**SEQ ID NO:86**) and the VL Domain of PD-1 mAb 6-SQ (**SEQ ID NO:87**); and
- (B) the epitope-binding site(s) capable of immunospecific binding to an epitope of CTLA-4 comprise(s) the VH Domain of CTLA-4 mAb 3 (**SEQ ID NO:90**) and the VL Domain of CTLA-4 mAb 3 (**SEQ ID NO:91**).

[0038] The disclosure additionally concerns the embodiment of all such bispecific molecules wherein the molecule comprises:

- (A) two polypeptide chains having **SEQ ID NO:95**, and two polypeptide chain having **SEQ ID NO:96**; or
- (B) two polypeptide chains having **SEQ ID NO:97**, and two polypeptide chain having **SEQ ID NO:98**; or
- (C) two polypeptide chains having **SEQ ID NO:99**, and two polypeptide chain having **SEQ ID NO:100**; or
- (D) two polypeptide chains having **SEQ ID NO:102**, and two polypeptide chain having **SEQ ID NO:103**; or
- (E) two polypeptide chains having **SEQ ID NO:101**, and two polypeptide chain having **SEQ ID NO:100**; or
- (F) one polypeptide chains having **SEQ ID NO:104**, one polypeptide chain having **SEQ ID NO:105**, one polypeptide chain having **SEQ ID NO:106**, and one polypeptide chain having **SEQ ID NO:107**; or
- (G) one polypeptide chains having **SEQ ID NO:108**, one polypeptide chain having **SEQ ID NO:105**, one polypeptide chain having **SEQ ID NO:109**, and one polypeptide chain having **SEQ ID NO:107**.

[0039] The disclosure additionally concerns the embodiment of such bispecific molecules in which the molecule comprises an Albumin-Binding Domain, and especially a deimmunized Albumin-Binding Domain.

[0040] In another aspect, there is provided a pharmaceutical composition that comprises an effective amount of any of such bispecific molecules and a pharmaceutically acceptable carrier.

**[0041]** The disclosure additionally concerns the use of such pharmaceutical composition or the use of any of the above-described bispecific molecules to promote stimulation of an immune-mediated response of a subject in need thereof, and in particular, wherein such molecule promotes the stimulation of immune cells, and in particular, stimulation of NK-cells and/or T-lymphocytes. The disclosure particularly concerns the embodiments wherein such stimulation results in immune cell proliferation, immune cell production and/or release of cytokines (*e.g.*, IFN $\gamma$ , IL-2, TNF $\alpha$ , *etc.*), immune cell production and/or release of lytic molecules (*e.g.*, granzyme, perforin, *etc.*), and/or immune cell expression of activation markers (*e.g.*, CD69, CD25, CD107a, *etc.*). The disclosure further concerns methods of treating cancer or other diseases that involve the use or administration of any of the above-described PD-1 x CTLA-4 bispecific molecules to stimulate an immune mediated response. The disclosure particularly concerns the embodiments in which the immune stimulatory activity of any of the above-described PD-1 x CTLA-4 bispecific molecules is more potent than the joint or combined administration of a separate anti-PD-1 antibody and a separate anti-CTLA-4 antibody (especially, wherein such antibodies are monospecific for such molecules). The disclosure also concerns embodiments in which immune cells, particularly NK-cells and/or T-lymphocytes, stimulated by the above-described PD-1 x CTLA-4 bispecific molecules exhibit enhanced proliferation, altered production and/or release of cytokines (*e.g.*, IFN $\gamma$ , IL-2, TNF $\alpha$ , *etc.*), altered production and/or release of lytic molecules, and/or altered expression of activation markers relative to that exhibited by such cells stimulated by the joint or combined administration of a separate anti-PD-1 antibody and a separate anti-CTLA-4 antibody. The disclosure also concerns embodiments in which the above-described PD-1 x CTLA-4 bispecific molecules have a reduced incidence of irAEs. The disclosure additionally concerns the embodiments in which any of the above-described PD-1 x CTLA-4 bispecific molecules are used in the treatment of a disease or condition associated with a suppressed immune system, especially cancer or an infection.

**[0042]** The disclosure additionally concerns such a use to treat a disease or condition associated with a suppressed immune system, or in the treatment of such a disease or condition. The disclosure particularly concerns such a use in in the treatment of a disease or condition associated with a suppressed immune system, or wherein the disease or condition is cancer or an infection (particularly, an infection characterized by the presence of a bacterial, fungal, viral or protozoan pathogen).

[0043] The disclosure particularly concerns such a use wherein:

- (A) the use is in the treatment of cancer, and the cancer is characterized by the presence of a cancer cell selected from the group consisting of a cell of: an adrenal gland tumor, an AIDS-associated cancer, an alveolar soft part sarcoma, an astrocytic tumor, bladder cancer, bone cancer, a brain and spinal cord cancer, a metastatic brain tumor, a breast cancer, a carotid body tumors, a cervical cancer, a chondrosarcoma, a chordoma, a chromophobe renal cell carcinoma, a clear cell carcinoma, a colon cancer, a colorectal cancer, a cutaneous benign fibrous histiocytoma, a desmoplastic small round cell tumor, an ependymoma, a Ewing's tumor, an extraskeletal myxoid chondrosarcoma, a fibrogenesis imperfecta ossium, a fibrous dysplasia of the bone, a gallbladder or bile duct cancer, gastric cancer, a gestational trophoblastic disease, a germ cell tumor, a head and neck cancer, hepatocellular carcinoma, an islet cell tumor, a Kaposi's Sarcoma, a kidney cancer, a leukemia, a lipoma/benign lipomatous tumor, a liposarcoma/malignant lipomatous tumor, a liver cancer, a lymphoma, a lung cancer, a medulloblastoma, a melanoma, a meningioma, a multiple endocrine neoplasia, a multiple myeloma, a myelodysplastic syndrome, a neuroblastoma, a neuroendocrine tumors, an ovarian cancer, a pancreatic cancer, a papillary thyroid carcinoma, a parathyroid tumor, a pediatric cancer, a peripheral nerve sheath tumor, a pheochromocytoma, a pituitary tumor, a prostate cancer, a posterior uveal melanoma, a rare hematologic disorder, a renal metastatic cancer, a rhabdoid tumor, a rhabdomyosarcoma, a sarcoma, a skin cancer, a soft-tissue sarcoma, a squamous cell cancer, a stomach cancer, a synovial sarcoma, a testicular cancer, a thymic carcinoma, a thymoma, a thyroid metastatic cancer, and a uterine cancer; or
- (B) the use is in the treatment of infection, and the infection is a chronic viral, bacterial, fungal and parasitic infection, characterized the presence of Epstein Barr virus, Hepatitis A Virus (HAV); Hepatitis B Virus (HBV); Hepatitis C Virus (HCV); herpes viruses (*e.g.* HSV-1, HSV-2, HHV-6, CMV), Human Immunodeficiency Virus (HIV), Vesicular Stomatitis Virus (VSV), *Bacilli*, *Citrobacter*, *Cholera*, *Diphtheria*, *Enterobacter*, *Gonococci*, *Helicobacter pylori*, *Klebsiella*, *Legionella*, *Meningococci*, mycobacteria, *Pseudomonas*, *Pneumococci*, rickettsia bacteria, *Salmonella*, *Serratia*, *Staphylococci*, *Streptococci*, *Tetanus*, *Aspergillus* (*A. fumigatus*, *A. niger*, *etc.*), *Blastomyces dermatitidis*, *Candida* (*C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis*, *etc.*), *Cryptococcus neoformans*, Genus *Mucorales* (*mucor*, *absidia*, *rhizopus*), *Sporothrix schenckii*, *Paracoccidioides brasiliensis*, *Coccidioides immitis*, *Histoplasma*

*capsulatum*, *Leptospirosis*, *Borrelia burgdorferi*, helminth parasite (hookworm, tapeworms, flukes, flatworms (e.g. *Schistosomia*), *Giardia lambia*, *trichinella*, *Dientamoeba Fragilis*, *Trypanosoma brucei*, *Trypanosoma cruzi*, or *Leishmania donovani*.

[0044] The disclosure particularly concerns such use in the treatment of cancer, wherein the cancer is colorectal cancer, hepatocellular carcinoma, glioma, kidney cancer, breast cancer, multiple myeloma, bladder cancer, neuroblastoma; sarcoma, non-Hodgkin's lymphoma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, a rectal cancer, acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), acute B lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm (BPDCN), non-Hodgkin's lymphomas (NHL), including mantel cell leukemia (MCL), and small lymphocytic lymphoma (SLL), Hodgkin's lymphoma, systemic mastocytosis, or Burkitt's lymphoma.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0045] **Figure 1** provides a schematic of a representative covalently bonded diabody having two epitope-binding sites composed of two polypeptide chains, each having an E-coil or K-coil Heterodimer-Promoting Domain (alternative Heterodimer-Promoting Domains are provided below). A cysteine residue may be present in a linker and/or in the Heterodimer-Promoting Domain as shown in **Figure 3B**. VL and VH Domains that recognize the same epitope are shown using the same shading or fill pattern.

[0046] **Figure 2** provides a schematic of a representative covalently bonded diabody molecule having two epitope-binding sites composed of two polypeptide chains, each having a CH2 and CH3 Domain, such that the associated chains form all or part of an Fc Region. VL and VH Domains that recognize the same epitope are shown using the same shading or fill pattern.

[0047] **Figures 3A-3C** provide schematics showing representative covalently bonded tetravalent diabodies having four epitope-binding sites composed of two pairs of polypeptide chains (*i.e.*, four polypeptide chains in all). One polypeptide of each pair possesses a CH2 and CH3 Domain, such that the associated chains form all or part of an Fc Region. VL and VH Domains that recognize the same epitope are shown using the same shading or fill pattern. The two pairs of polypeptide chains may be same. In such embodiments wherein the two pairs of