

MULTISPECIFIC BINDING CONSTRUCTS AGAINST CHECKPOINT MOLECULES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority of U.S. Provisional Application No. 62/760,801, filed on November 13, 2018; U.S. Provisional Application No. 62/855,580, filed on May 31, 2019; U.S. Application No. 62/898,991, filed on September 11, 2019; and U.S. Application No. 62/931,478, filed on November 6, 2019. The foregoing applications are incorporated herein by reference in their entireties.

BACKGROUND

Cancer is one of the leading causes of death in both the United States and worldwide. While common treatments such as surgery, radiation, chemotherapy, hormone therapy, targeted therapies, and immunotherapy have decreased the rates of cancer-related deaths during the 20th century, there were 14.1 million new cancer cases diagnosed and 8.2 million cancer deaths worldwide as of 2012. Even with improved overall cancer survival rates during the 20th century, cancer is still responsible for one in seven deaths worldwide. See American Cancer Society, Global Cancer Facts & Figures 3rd Edition, Atlanta: American Cancer Society; 2015.

In recent years, an increasing body of evidence suggests that the immune system operates as a significant barrier to tumor formation and progression. The principle that naturally-occurring T cells with anti-tumor potential or activity exist in a patient with cancer has rationalized the development of immunotherapeutic approaches in oncology. Immune cells, such as T cells, macrophages, and natural killer cells, can exhibit anti-tumor activity and effectively control the occurrence and growth of malignant tumors. Tumor-specific or -associated antigens can induce immune cells to recognize and eliminate malignancies (Chen & Mellman, (2013) *Immunity* 39(1):1-10). In spite of the existence of tumor-specific immune responses, malignant tumors often evade or avoid immune attack through a variety of immunomodulatory mechanisms resulting in the failure to control tumor occurrence and progression (Motz & Coukos, (2013) *Immunity* 39(1):61-730). Indeed, an emerging hallmark of cancer is the exploitation of these immunomodulatory mechanisms and the disablement of anti-tumor immune responses, resulting in tumor evasion and escape from immunological killing (Hanahan and Weinberg (2011) *Cell* 144(5):646-674).

Novel approaches in the immunotherapy of cancer involve counteracting these immune evasion and escape mechanisms and inducing the endogenous immune system to reject tumors. However, there remains a need for novel therapeutics that effectively counteract immune evasion, particularly in cancer therapeutics.

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SUMMARY OF THE DISCLOSURE

The present disclosure is based, in part, on novel multispecific and multivalent constructs targeting both PD-1 and PD-L1, such as a bispecific and tetravalent construct. As demonstrated herein, these multispecific constructs have improved *in vitro* and *in vivo* potency as compared to combinations of individual antibodies, as well as compared to clinical checkpoint blockade agents. Also provided herein are novel monoclonal anti-PD-1 antibodies and antigen-binding fragments thereof, and novel monoclonal anti-PD-L1 antibodies and antigen-binding fragments thereof, for use in such multispecific and multivalent constructs. Some of these novel monoclonal anti-PD-1 antibodies and novel monoclonal anti-PD-L1 antibodies share a common light chain, thereby allowing for the generation of multispecific and multivalent constructs having surprising excellent drug-like properties (DLPs) and ease of manufacturability, as well as affinities similar to their parental antibodies. The present disclosure is also based, in part, on the discovery that blocking the interaction between PD-1 expressed by an immune cell and its ligand (*e.g.*, PD-L1 or PD-L2) expressed on a second cell, while bridging the immune cell and the second cell (*e.g.*, another immune cell, or a tumor cell that expresses a PD-1 ligand) strongly enhance, for example, T cell proliferation, IFN γ production and secretion, and the cytolytic activity of T cells. Thus, provided herein are compositions that block the interaction between PD-1 and its ligand while promoting the interaction of (bridging) the cells on which PD-1 and its ligand (PD-L1 or PD-L2) are expressed. As exemplified herein, such compositions of the present disclosure with the capacity to “block and bridge” provide superior anti-tumor efficacy (as measured, *e.g.*, by IFN γ production and secretion and *in vivo* activity) as compared to, *e.g.*, a cocktail having a stoichiometric amount of agents that separately bind the receptor and ligand; or a single agent that binds either the receptor or the ligand. It was also found that the multispecific and multivalent constructs targeting both PD-1 and PD-L1 described herein cause loss of expression of PD-1 on the cell surface, in a valency-dependent fashion. This loss of PD-1 expression was not observed when a combination of the parental antibodies were used in stoichiometrically equivalent amounts. Accordingly, the multispecific and multivalent constructs targeting both PD-1 and PD-L1 described herein provide novel

immunotherapeutic agents with increased potency and efficacy for use in the treatment of cancer.

In one aspect, the present disclosure provides a multispecific antigen-binding construct comprising at least two antigen-binding arms, wherein: a first arm binds PD-1 and comprises a heavy chain variable region and a light chain variable region, the heavy chain variable region of the first arm comprising a CDRH1 of SEQ ID NO: 77, a CDRH2 of SEQ ID NO: 71, and a CDRH3 of SEQ ID NO: 75, and a second arm binds a PD-1 ligand and comprises a heavy chain variable region and a light chain variable region, the heavy chain variable region of the second arm comprising a CDRH1 of SEQ ID NO: 1, a CDRH2 of SEQ ID NO: 7, and a CDRH3 of SEQ ID NO: 8; wherein the light chain variable region of the first arm and the light chain variable region of the second arm each comprise a CDRL1 of SEQ ID NO: 9, a CDRL2 of SEQ ID NO: 5, and a CDRL3 of SEQ ID NO: 10; and wherein the multispecific antigen-binding construct blocks the interaction of PD-1 and the PD-1 ligand.

In some examples, any of the multispecific antigen-binding constructs disclosed herein binds to at least two different receptors or epitopes (*e.g.*, PD-1 and PD-L1), wherein the two different receptors or epitopes bound by the multispecific antigen-binding construct are expressed on the surface of the same cell. For example, in some examples, the multispecific antigen-binding construct simultaneously binds to PD-1 and PD-L1, wherein the PD-1 and PD-L1 are expressed on the surface of the same cell. In some examples, any of the multispecific antigen-binding constructs disclosed herein binds to at least two different receptors or epitopes (*e.g.*, PD-1 and PD-L1), wherein the two different receptors or epitopes bound by the multispecific antigen-binding construct are expressed on the surface of two different cells. For example, in some examples, the multispecific antigen-binding construct simultaneously binds to PD-1 expressed on the surface of a first cell and to a PD-1 ligand, *e.g.*, PD-L1 or PD-L2, expressed on the surface of a second cell.

In some examples, the disclosure provides for a multispecific antigen-binding construct comprising at least two antigen-binding arms, wherein a first arm binds PD-1 expressed by an immune cell, and a second arm binds a PD-1 ligand which is expressed by a second cell, wherein the multispecific antigen-binding construct blocks the interaction of PD-1 and PD-1 ligand. In some examples, the PD-1 ligand is PD-L2. In some examples, the PD-1 ligand is PD-L1. In some examples, the immune cell is a T cell. In some examples, the T cell is a CD8⁺ T cell. In some examples, the immune cell is a natural killer (NK) cell. In

some examples, the immune cell is a macrophage. In some examples, the second cell is a second immune cell. In some examples, the second immune cell is any one or more of a T cell, a B cell, a macrophage, a myeloid-derived suppressor cell, a dendritic cell, or a mesenchymal stromal cell. In some examples, the second immune cell is a regulatory T cell.

- 5 In some examples, the second cell is a tumor cell. In some examples, the tumor cell is selected from the group consisting of a hematological cancer, a lymphoma, a myeloma, a leukemia, a neurological cancer, melanoma, breast cancer, a prostate cancer, a colorectal cancer, lung cancer, head and neck cancer, a gastrointestinal cancer, liver cancer, pancreatic cancer, a genitourinary cancer, a bone cancer, renal cancer, and a vascular cancer. In some
- 10 examples, both arms have a K_D of at least 1×10^{-7} M, at least 1×10^{-8} M, at least 1×10^{-9} M, or at least 1×10^{-10} M. In some examples, the binding of one arm to its target does not block the binding of the other arm to its target. In some examples, the first arm and second arm bind to their respective

targets and both arms remain bound concurrently. In some examples, binding of the first arm and the second arm to their respective targets can bridge the immune cell and the second cell together. In some examples, the bridging of the immune cell and the second cell is determined by flow cytometry. In some examples, the first arm is an antagonist of PD-1.

5 In some examples of the multispecific antigen-binding constructs, the first arm binds to PD-1 and comprises: (a) a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 70 (FTFX₁X₂YAX₃X₄, wherein X₁=S, R, G, or N; X₂=D, S, N, A, R, or G; X₃=M or L; X₄=S, L, or N); (ii) a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA); and (iii) a CDRH3 comprising SEQ ID NO: 72 (ARGLDFIVGX₅TGNDY, wherein X₅=A, Y, or R); and (b) a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 73 (FTFSDYAMS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 74 (ARGLDFIVGATGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 73 (FTFSDYAMS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 76 (FTFSSYAMS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 77 (FTFSSYAML), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 78 (FTFSNYALS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 79 (FTFSAYAMN), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 80 (FTFRSYAMS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some examples, the CDRH1 of the first arm comprises

SEQ ID NO: 81 (FTFGRYAMS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 82 (FTFNSYAMS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 83 (FTFSNYAMS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 74 (ARGLDFIVGATGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 84 (FTFSGYAMS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 85 (ARGLDFIVGRTGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 86 (FTFSSYAMN), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 85 (ARGLDFIVGRTGNDY). In some examples, the CDRH1 of the first arm comprises SEQ ID NO: 80 (FTFRSYAMS), CDRH2 of the first arm comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 of the first arm comprises SEQ ID NO: 85 (ARGLDFIVGRTGNDY).

In some examples, the CDRL1 of the first arm comprises SEQ ID NO: 9 (RASQSISSYLN), CDRL2 of the first arm comprises SEQ ID NO: 5 (AASSLQS) and CDRL3 of the first arm comprises SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 87. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 88. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 89. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 90. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 91. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 92. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 93. In some examples, the heavy chain variable region of the first arm comprises an amino

acid sequence that is at least 90% identical to SEQ ID NO: 94. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 95. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 96. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 97. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 98. In some examples, the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 99. In some examples, the light chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 59.

In some examples, the second arm is an antagonist of the PD-1 ligand. In some examples, the second arm is an antagonist of PD-L2. In some examples, the second arm is an antagonist of PD-L1. In some examples, the second arm binds to PD-L1 and comprises: a. a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN); (ii) a CDRH2 comprising SEQ ID NO: 2 (GGIIPX₁X₂GX₃ATYA, wherein X₁ is V or I; X₂ is F, L, or V; and X₃ is T or A); and (iii) a CDRH3 comprising SEQ ID NO: 3 (ARLKX₁ELKDAFDI, wherein X₁ is G, F, or N); and b. a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 4 (RASQX₁ISSYLN, wherein X₁ is S, W, or Q); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 6 (X₁QSYSTPLT, wherein X₁ is Q or F).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 7 (GGIIPILGAATYA) and CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 7 (GGIIPILGAATYA), CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm

comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QSYSTPLT). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA) and CDRH3 of the second arm comprises SEQ ID NO: 8

5 (ARLKGELKDAFDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 of the second arm comprises SEQ ID NO: 12 (RASQWISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QSYSTPLT). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 of the second arm comprises SEQ ID NO: 13 (RASQISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QSYSTPLT). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 15 (GGIIPFGIANYA) and CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 15 (GGIIPFGIANYA), CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QSYSTPLT).

30 In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 16 (GGIIPNFGTATYA) and CDRH3 of the second arm comprises SEQ ID NO: 17 (ARLKGELKGAGDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN),

CDRH2 of the second arm comprises SEQ ID NO: 16 (GGIIPNFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 17 (ARLKGELKGAGDI); CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10

5 (QQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 of the second arm comprises SEQ ID NO: 18 (ARLKFELKDAFDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2
10 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 18 (ARLKFELKDAFDI), CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 of the second arm comprises SEQ ID NO: 19 (ARLKGELKDAFDE). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2
15 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 19 (ARLKGELKDAFDE), CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 of the second arm comprises SEQ ID NO: 20 (ARLKNELKDAFDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2
25 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 20 (ARLKNELKDAFDI), CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QQSYSTPLT).

30 In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 21

(GGVIPFLGTANYA), and CDRH3 of the second arm comprises SEQ ID NO: 22 (ARLKGILKDALDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 21 (GGVIPFLGTANYA), CDRH3 of the second arm comprises SEQ ID NO: 22 (ARLKGILKDALDI), CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 29 (GRIPIFGTADYA), and CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 29 (GRIPIFGTADYA), CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI), CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 31 (GGIIPILGTATYA), and CDRH3 of the second arm comprises SEQ ID NO: 32 (ARRKGELKDAFDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 31 (GGIIPILGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 32 (ARRKGELKDAFDI), CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 33 (GGIPIVATANYA), and CDRH3 of the second arm comprises SEQ ID NO: 32 (ARRKGELKDAFDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 33 (GGIPIVATANYA), CDRH3 of the second arm comprises SEQ ID NO: 32 (ARRKGELKDAFDI), CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 34 (GGIPIFGKATYA), and CDRH3 of the second arm comprises SEQ ID NO: 32 (ARRKGELKDAFDI). In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 34 (GGIPIFGKATYA), CDRH3 of the second arm comprises SEQ ID NO: 32 (ARRKGELKDAFDI), CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 38 (FQSYSTPLT).

In some examples, the CDRH1 of the second arm comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 of the second arm comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 of the second arm comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 of the second arm comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 of the second arm comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 of the second arm comprises SEQ ID NO: 39 (QQSYSTILT).

In some examples, the second arm comprises: a. a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 14 (GTFSSYAFS), (ii) a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA) and (iii) a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); and b. a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the second arm comprises a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), (ii) a CDRH2 comprising SEQ ID NO: 24 (GGIPIVGLIANYA), and (iii) a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI). In some examples, the second arm comprises a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the second arm comprises a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), (ii) a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and (iii) a CDRH3 comprising SEQ ID NO: 25 (ARLKGEFKDAFDI). In some examples, the second arm comprises a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the second arm comprises a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), (ii) a CDRH2 comprising SEQ ID NO: 26 (GRIPLFGTAHYA), and (iii) a CDRH3 comprising SEQ ID NO: 8 (ARLKGEFKDAFDI). In some examples, the second arm comprises a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the second arm comprises a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), (ii) a CDRH2 comprising SEQ ID NO: 27 (GRINPILGTANYA), and (iii) a CDRH3 comprising SEQ ID NO: 28 (ARLKGEFKDAFSI). In some examples, the second arm comprises a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the second arm comprises a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), (ii) a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and (iii) a CDRH3 comprising SEQ ID NO: 30 (ARLKGEFKCAFDI). In some examples, the second arm comprises a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the second arm comprises a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 122 (GTKSSY AIS), (ii) CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and (iii) a CDRH3 comprising SEQ ID NO: 30 (ARLKGEFKCAFDI). In some examples, the second arm further comprises a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9

(RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some examples, the second arm comprises a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 36 (GPFRSHAVS), (ii) a CDRH2
5 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and (iii) a CDRH3 comprising SEQ ID NO: 37 (ARLKSELKDAFDI). In some examples, the second arm comprises a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

10 In some examples, the second arm that binds PD-L1 comprises a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58 and a light chain variable region comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 59, 60, 61, 62, or 63.

15 In some examples, the heavy chain variable region of the second arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 35
(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPVFG
TATYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARLKGELKDAFDIWGQ
GTMVTVSS). In some examples, the heavy chain variable region of the second arm

20 comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 40
(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAFSWVRQAPGQGLEWMGGIIPVF
GTATYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARLKGELKDAFDIWG
QGTLVTVSS). In some examples, the heavy chain variable region of the second arm
comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 41

25 (QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPFGI
ANYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARLKGELKDAFDIWGQG
TLVTVSS). In some examples, the heavy chain variable region of the second arm comprises
an amino acid sequence that is at least 90% identical to SEQ ID NO: 42

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPNFG
30 TATYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARLKGELKGAGDIWGQ
GTLVTVSS). In some examples, the heavy chain variable region of the second arm
comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 43

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPVFG
TATYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARLKFELKDAFDIWGQG

TLVTVSS). In some examples, the heavy chain variable region of the second arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 44

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPVFG
TATYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDEWGQ

5 GTLVTVSS). In some examples, the heavy chain variable region of the second arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 45

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPVFG
TATYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQ
GTLVTAST). In some examples, the heavy chain variable region of the second arm

10 comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 46

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPVFG
TATYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKNELKDAFDIWGQ
GTLVTVSS). In some examples, the heavy chain variable region of the second arm

comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 47

15 (QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGVIPFL
GTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGILKDALDIWG
QGTLVTVSS). In some examples, the heavy chain variable region of the second arm

comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 48

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAISWVRQAPGQDLEWMGGIIPVGI

20 ANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQ
TLVTVSS). In some examples, the heavy chain variable region of the second arm comprises
an amino acid sequence that is at least 90% identical to SEQ ID NO: 49

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAISWVRQAPGQGLEWMGGIIPVFG
TATYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGFEKDAFDIWGQ

25 GTLVTVSS). In some examples, the heavy chain variable region of the second arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 50

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAISWVRQAPGQGLEWMGRIPLFG
TAHYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQ
GTLVTVSS). In some examples, the heavy chain variable region of the second arm

30 comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 51

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAISWVRQAPGQGLEWMGRINPILG
TANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFSIWGQ
GTLVTVSS). In some examples, the heavy chain variable region of the second arm

comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 52

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGRIPIFG
TADYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARLKGELKDAFDIWGQ
GTLVTVSS). In some examples, the heavy chain variable region of the second arm
comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 53

5 (QVQLVQSGAEVKKPGSSVKVSCASGGKFSSYAISWVRQAPGQGLEWMGGIIPVFG
TATYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARLKGELKCAFDIWGQ
GTLVTVSS). In some examples, the heavy chain variable region of the second arm
comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 54

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPILG
10 TATYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARRKGELKDAFDIWGQ
GTLVTVSS). In some examples, the heavy chain variable region of the second arm
comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 55

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPILG
AATYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARLKGELKDAFDIWGQ
15 GTLVTVSS). In some examples, the heavy chain variable region of the second arm
comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 56

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIPIVA
TANYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARRKGELKDAFDIWGQ
GTLVTVSS). In some examples, the heavy chain variable region of the second arm
20 comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 57

(QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAINWVRQAPGQGLEWMGGIPIFG
KATYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARRKGELKDAFDIWGQ
GTLVTVSS). In some examples, the heavy chain variable region of the second arm
comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 58

25 (QVQLVQSGAEVKKPGSSVKVSCASGGPFRSHAVSWVRQAPGQGLEWMGGIIPVF
GTATYAQKFQGRVTITADESTSTAYMELSSLRSEDVAVYYCARLKSELKDAFDIWGQ
GTLVTVSS). In some examples, the light chain variable region of the second arm comprises
an amino acid sequence that is at least 90% identical to SEQ ID NO: 59

(DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGV
30 PSRFGSGSGTDFTLTISLQPEDFATYYCQQSYSTPLTFGGGTKVEIK). In some
examples, the light chain variable region of the second arm comprises an amino acid
sequence that is at least 90% identical to SEQ ID NO: 60

(DIQMTQSPSSLSASVGDRVTITCRASQWISSYLNWYQQKPGKAPKLLIYAASSLQSG
VPSRFGSGSGTDFTLTISLQPEDFATYYCQQSYSTPLTFGGGTKVEIK). In some

examples, the light chain variable region of the second arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 61

(DIQMTQSPSSLSASVGDRVTITCRASQQISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQSYSTPLTFGGGTKVEIK). In some

examples, the light chain variable region of the second arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 62

(DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCFQSYSTPLTFGGGTKVEIK). In some

examples, the light chain variable region of the second arm comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 63

(DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQSYSTILTFGGGTKVEIK).

In some examples of any of the examples described herein, the construct is a bispecific antibody. In some examples, the bispecific antibody is an antagonist of both PD-1 and PD-1 ligand. In some examples, the construct comprises a common light chain. In some examples, one or both of the arms is an aptamer. In some examples, one or both of the arms is a protein other than an antibody. In some examples, the construct comprises at least two bispecific antibodies. In some examples, one of the at least two bispecific antibodies is monovalent for PD-1. In some examples, one of the at least two bispecific antibodies is monovalent for PD-1 ligand. In some examples, at least one of the arms is a bivalent antibody specific for PD-1. In some examples, at least one of the arms is a bivalent antibody specific for PD-L1. In some examples, at least one of the arms is a bivalent antibody specific for PD-1, and at least one of the arms is a bivalent antibody specific for PD-L1. In some examples, at least one of the arms is bivalent for PD-1. In some examples, at least one of the arms is bivalent for PD-L1. In some examples, at least one of the arms is bivalent for PD-1, and at least one of the arms is bivalent for PD-L1. In some examples, the bispecific antibody binds two different epitopes on PD-1. In some examples, the bispecific antibody binds two different epitopes on the PD-1 ligand.

In some examples, any of the multispecific antigen-binding constructs disclosed herein comprises at least two monospecific antibodies. In some examples, at least one of the monospecific antibodies is an anti-PD-1 antibody. In some examples, the anti-PD-1 antibody is a bivalent anti-PD-1 antibody. In some examples, at least one of the monospecific antibodies is an anti-PD-L1 antibody. In some examples, the anti-PD-1

antibody is a bivalent anti-PD-L1 antibody. In some examples, the construct comprises a bivalent anti-PD-1 antibody and a bivalent anti-PD-L1 antibody. In some examples, the construct is a fusion construct in which a polypeptide comprising the variable heavy chain of the anti-PD-1 antibody is fused to a polypeptide comprising the variable heavy chain of the anti-PD-L1 antibody. In some examples, the polypeptide comprising the variable heavy chain of the anti-PD-1 antibody is fused to the polypeptide comprising the variable heavy chain of the anti-PD-L1 antibody by means of a linker. In some examples, the fusion construct comprises a common light chain. In some examples, the N-terminal variable heavy chain of the fusion construct binds to PD-1 in the presence of the common light chain, and the C-terminal variable heavy chain of the fusion construct binds to PD-L1 in the presence of the common light chain. In some examples, the N-terminal variable heavy chain of the fusion construct binds to PD-L1 in the presence of the common light chain, and the C-terminal variable heavy chain of the fusion construct binds to PD-1 in the presence of the common light chain.

In some examples and examples, the disclosure provides for a multispecific antigen-binding construct comprising at least two units of antigen-binding, wherein a first unit of antigen-binding binds PD-1, and a second unit of antigen-binding binds a PD-1 ligand. In some examples, the first unit of antigen-binding binds PD-1 expressed by an immune cell. In some examples, the second unit of antigen-binding binds PD-1 expressed by a second cell. In some examples, the multispecific antigen-binding construct blocks the interaction of PD-1 and a PD-1 ligand, such as PD-L1 or PD-L2. In some examples, the multispecific antigen-binding construct blocks the interaction of PD-1 and a PD-1 ligand, such as PD-L1 or PD-L2. In some examples, the multispecific antigen-binding construct comprises at least two units of antigen-binding that bind PD-1. In some examples, the multispecific antigen-binding construct comprises two units of antigen-binding that bind PD-1. In some examples, the multispecific antigen-binding construct comprises at least two units of antigen-binding that bind a PD-1 ligand, such as PD-L1 or PD-L2. In some examples, the multispecific antigen-binding construct comprises two units of antigen-binding that bind a PD-1 ligand, such as PD-L1 or PD-L2. In some examples, the multispecific antigen-binding construct comprises at least four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind a PD-1 ligand, such as PD-L1 or PD-L2. In some examples, the multispecific antigen-binding construct comprises four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind a PD-1 ligand, such as PD-L1 or PD-L2. In some

examples, each unit of antigen-binding is capable of binding independently to its cognate antigen, *i.e.*, PD-1 or a PD-1 ligand, such as PD-L1 or PD-L2. In some examples, the multispecific antigen-binding construct promotes loss of PD-1 expression from a cell. In some examples, the loss of PD-1 expression is due to PD-1 shedding. In some examples, the multispecific antigen-binding construct blocks interaction of PD-1 and the PD-1 ligand, such as PD-L1 or PD-L2. In some examples, the multispecific antigen-binding construct comprises a common light chain. For example, at least two units of antigen-binding comprise a common light chain.

In some examples, the first unit of antigen-binding binds PD-1 and comprises:

- (a) a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 70 (FTFX₁X₂YAX₃X₄, wherein X₁=S, R, G, or N; X₂=D, S, N, A, R, or G; X₃= M or L; X₄= S, L, or N); (ii) a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA); and (iii) a CDRH3 comprising SEQ ID NO: 72 (ARGLDFIVGX₅TGNDY, wherein X₅=A, Y, or R); and
- (b) a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some such examples, the first unit of antigen-binding binds PD-1 and comprises:

- (a) a CDRH1 comprising SEQ ID NO: 73 (FTFSDYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 74 (ARGLDFIVGATGNDY);
- (b) a CDRH1 comprising SEQ ID NO: 73 (FTFSDYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- (c) a CDRH1 comprising SEQ ID NO: 76 (FTFSSYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- (d) a CDRH1 comprising SEQ ID NO: 77 (FTFSSYAML), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- (e) a CDRH1 comprising SEQ ID NO: 78 (FTFSNYALS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);

- (f) a CDRH1 comprising SEQ ID NO: 79 (FTFSAYAMN), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- 5 (g) a CDRH1 comprising SEQ ID NO: 80 (FTFRSYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- (h) a CDRH1 comprising SEQ ID NO: 81 (FTFGRYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- 10 (i) a CDRH1 comprising SEQ ID NO: 82 (FTFNSYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- (j) a CDRH1 comprising SEQ ID NO: 83 (FTFSNYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 74 (ARGLDFIVGATGNDY);
- 15 (k) a CDRH1 comprising SEQ ID NO: 84 (FTFSGYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 85 (ARGLDFIVGRTGNDY);
- (l) a CDRH1 comprising SEQ ID NO: 86 (FTFSSYAMN), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA) and a CDRH3 comprising SEQ ID NO: 85 (ARGLDFIVGRTGNDY); or
- 20 (m) a CDRH1 comprising SEQ ID NO: 80 (FTFRSYAMS), a comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 85 (ARGLDFIVGRTGNDY).
- 25 In some examples, the first unit of antigen-binding binds PD-1 and comprises:
- (a) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 87;
- (b) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 88;
- 30 (c) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 89;
- (d) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 90;

- (e) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 91;
- (f) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 92;
- 5 (g) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 93;
- (h) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 94;
- 10 (i) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 95;
- (j) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 96;
- (k) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 97;
- 15 (l) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 98; or
- (m) a heavy chain variable region comprising amino acid sequence that is at least 90% identical to SEQ ID NO: 99.

20 In some examples, the first unit of antigen-binding binds PD-1 and comprises a light chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 59.

In some examples, the second unit of antigen-binding binds PD-L2. In some examples, the second unit of antigen-binding binds PD-L1. In some examples, the second unit of antigen-binding binds PD-L1 and comprises:

- 25 a. a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN); (ii) a CDRH2 comprising SEQ ID NO: 2 (GGIIPX₁X₂GX₃ATYA, wherein X₁ is V or I; X₂ is F, L, or V; and X₃ is T or A); and (iii) a CDRH3 comprising SEQ ID NO: 3 (ARLKX₁ELKDAFDI, wherein X₁ is G, F, or N); and
- b. a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 4 (RASQX₁ISSYLN, wherein X₁ is S, W, or Q); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 6 (X₁QSYSTPLT, wherein X₁ is Q or F).
- 30

In some such examples, the second unit of antigen-binding binds PD-L1 and comprises:

- (a) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 7 (GGIIPILGAATYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);
- 5 (b) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 7 (GGIIPILGAATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 10 (c) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 15 (d) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA) and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);
- (e) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 12 (RASQWISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 20 (f) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 13 (RASQQISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 25 (g) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 30 (h) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 15 (GGIIPIFGIANYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);

- 5 (i) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 15 (GGIPIFGIANYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (CQQSYSTPLTF);
- (j) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 16 (GGIIPNFGTATYA), and a CDRH3 comprising SEQ ID NO: 17 (ARLKGELKGAGDI);
- 10 (k) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 16 (GGIIPNFGTATYA), a CDRH3 comprising SEQ ID NO: 17 (ARLKGELKGAGDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 15 (l) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 18 (ARLKFELKDAFDI);
- (m) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 18 (ARLKFELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 20 (n) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 19 (ARLKGELKDAFDE);
- 25 (o) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 19 (ARLKGELKDAFDE), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 30 (p) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 20 (ARLKNELKDAFDI);
- (q) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 20

- (ARLKNELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 5 (r) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 21 (GGVIPFLGTANYA), and a CDRH3 comprising SEQ ID NO: 22 (ARLKGILKDALDI);
- (s) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 21 (GGVIPFLGTANYA), a CDRH3 comprising SEQ ID NO: 22 (ARLKGILKDALDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a
- 10 CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- (t) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising EQ ID NO: 29 (GRIPIFGTADYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);
- 15 (u) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 29 (GRIPIFGTADYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 20 (v) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 31 (GGIIPILGTATYA), and a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI);
- (w) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 31 (GGIIPILGTATYA), a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a
- 25 CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- (x) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 33 (GGIPIVATANYA), and a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI);
- 30 (y) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 33 (GGIPIVATANYA), a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a

CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);

(z) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 34 (GGIPIFGKATYA), and a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI);

5 (aa) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 34 (GGIPIFGKATYA), a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);

10 (bb) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 38 (FQSYSTPLT);

15 (cc) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 39 (QQSYSTILT);

20 (dd) a CDRH1 comprising SEQ ID NO: 14 (GTFSSYAFS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA) and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);

25 (ee) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), a CDRH2 comprising SEQ ID NO: 24 (GGIPIVGLIANYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);

(ff) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), a CDRH2 comprising SEQ ID NO: 24 (GGIPIVGLIANYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);

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- (gg) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 25 (ARLKGEFKDAFDI);
- 5 (hh) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 25 (ARLKGEFKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 10 (ii) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 26 (GRIIPLFGTAHYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGEFKDAFDI);
- (jj) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 26 (GRIIPLFGTAHYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGEFKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a
- 15 CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- (kk) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 27 (GRINPILGTANYA), and a CDRH3 comprising SEQ ID NO: 28 (ARLKGEFKDAFSI);
- 20 (ll) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 27 (GRINPILGTANYA), and a CDRH3 comprising SEQ ID NO: 28 (ARLKGEFKDAFSI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 25 (mm) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 30 (ARLKGEFKCAFDI);
- (nn) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a comprising SEQ ID NO: 30
- 30 (ARLKGEFKCAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);

(oo) a CDRH1 comprising SEQ ID NO: 36 (GPFRSHAVS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 37 (ARLKSELKDAFDI); or

(pp) a CDRH1 comprising SEQ ID NO: 36 (GPFRSHAVS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 37 (ARLKSELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some such examples, the second unit of antigen-binding binds PD-L1 and comprises and comprises a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58, and a light chain variable region comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 59, 60, 61, 62, or 63.

In some examples, the second unit of antigen-binding binds PD-L1 and comprises a heavy chain variable region comprising:

- (a) an amino acid sequence that is at least 90% identical to SEQ ID NO: 35;
- (b) an amino acid sequence that is at least 90% identical to SEQ ID NO: 40;
- (c) an amino acid sequence that is at least 90% identical to SEQ ID NO: 41;
- (d) an amino acid sequence that is at least 90% identical to SEQ ID NO: 42;
- (e) an amino acid sequence that is at least 90% identical to SEQ ID NO: 43;
- (f) an amino acid sequence that is at least 90% identical to SEQ ID NO: 44;
- (g) an amino acid sequence that is at least 90% identical to SEQ ID NO: 45;
- (h) an amino acid sequence that is at least 90% identical to SEQ ID NO: 46;
- (i) an amino acid sequence that is at least 90% identical to SEQ ID NO: 47;
- (j) an amino acid sequence that is at least 90% identical to SEQ ID NO: 48;
- (k) an amino acid sequence that is at least 90% identical to SEQ ID NO: 49;
- (l) an amino acid sequence that is at least 90% identical to SEQ ID NO: 50;
- (m) an amino acid sequence that is at least 90% identical to SEQ ID NO: 51;
- (n) an amino acid sequence that is at least 90% identical to SEQ ID NO: 52;
- (o) an amino acid sequence that is at least 90% identical to SEQ ID NO: 53;
- (p) an amino acid sequence that is at least 90% identical to SEQ ID NO: 54;
- (q) an amino acid sequence that is at least 90% identical to SEQ ID NO: 55;
- (r) an amino acid sequence that is at least 90% identical to SEQ ID NO: 56;

- (s) an amino acid sequence that is at least 90% identical to SEQ ID NO: 57; or
- (t) an amino acid sequence that is at least 90% identical to SEQ ID NO: 58;

In some examples, the second unit of antigen-binding binds PD-L1 and comprises a light chain variable region comprising:

- 5 (a) an amino acid sequence that is at least 90% identical to SEQ ID NO: 59;
- (b) an amino acid sequence that is at least 90% identical to SEQ ID NO: 60;
- (c) an amino acid sequence that is at least 90% identical to SEQ ID NO: 61;
- (d) an amino acid sequence that is at least 90% identical to SEQ ID NO: 62; or
- (e) an amino acid sequence that is at least 90% identical to SEQ ID NO: 63.

10 Also provided herein, in some examples and examples, is a multispecific antigen-binding construct comprising four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind PD-L1, and wherein the construct comprises a heavy chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 100 or 102, and a light chain amino acid sequence that is at least
15 85%, identical to the amino acid sequence of SEQ ID NO: 101 or 103.

Also provided herein, in some examples and examples, is a multispecific antigen-binding construct comprising four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind PD-L1, and wherein the construct comprises a heavy chain amino acid sequence that is at least 85%, identical to the amino acid
20 sequence of SEQ ID NO: 100 and a light chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 101.

Also provided herein, in some examples and examples, is a multispecific antigen-binding construct comprising four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind PD-L1, and wherein the construct
25 comprises a heavy chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 102 and a light chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 103.

In some examples, the construct does not comprise an Fc domain. In some examples, the first arm or second arm, or both, comprises a heavy chain comprising one or more
30 immunoglobulin Fc modifications. In some examples, the immunoglobulin Fc domain of the heavy chain comprises one or more amino acid mutations that promote heterodimerization of the first and second arms. In some examples, the mutation is present in a CH3 domain of the heavy chain. In some examples, the multispecific antigen-binding construct is produced in a quadroma cell. In some examples, the

construct comprises one or more immunoglobulin constant region modifications. In some examples, the immunoglobulin constant region comprises one or more amino acid mutations that promote heterodimerization of antibodies. In some examples, one or more mutations is present in the light chain constant region of one arm and one or more mutations is present in the heavy chain constant region of another arm. In some examples, the bispecific antibody is of a format selected from the group consisting of a bispecific IgG, bispecific antibody fragment, bispecific fusion protein, appended IgG, and bispecific antibody conjugate. In some examples, the Fc region has reduced effector function. In some examples, the Fc region enhances half-life of the construct.

In some examples, the construct comprises a heavy chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 100 or 102. In some examples, the construct comprises a light chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 101 or 103. In some examples, the construct comprises a heavy chain amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO: 100, and wherein the construct comprises a light chain amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO: 101. In some examples, the construct comprises a heavy chain amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO: 102, and wherein the construct comprises a light chain amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO: 103.

In some examples, any of the multispecific antigen-binding constructs disclosed herein are aglycosylated. In some examples, the multispecific antigen-binding construct is capable of binding human PD-1. In some examples, the multispecific antigen-binding construct is capable of binding murine PD-1. In some examples, the multispecific antigen-binding construct is capable of binding cynomolgus monkey PD-1. In some examples, the multispecific antigen-binding construct is capable of binding human, murine and cynomolgus monkey PD-1 with similar affinity.

In some examples, any of the multispecific antigen-binding constructs disclosed herein is capable of reducing PD-1 levels on a cell. In some examples, the multispecific antigen-binding construct is capable of inducing PD-1 degradation. In some examples, the multispecific antigen-binding construct is capable of reducing PD-1 expression. In some examples, the multispecific antigen-binding construct is capable of reducing PD-1 cell surface expression. In some examples, the multispecific antigen-binding construct is capable of reducing PD-1 cell surface expression by inducing shedding of PD-1 from the cell

surface. In some examples, the multispecific antigen-binding construct is capable of binding both PD-1 and PD-L1 and reducing PD-1 levels on a cell. In some examples, the multispecific antigen-binding construct is capable of binding both PD-1 and PD-L1 and inducing PD-1 degradation. In some examples, the multispecific antigen-binding construct is capable of binding both PD-1 and PD-L1 and reducing PD-1 expression. In some examples, the multispecific antigen-binding construct is capable of inducing shedding of PD-1 from an immune cell. In some examples, the multispecific antigen-binding construct is capable of binding both PD-1 and PD-L1 and inducing PD-1 shedding from an immune cell. In some examples, the multispecific antigen-binding construct is capable of sequestering PD-L1, such that PD-L1 cannot bind CD80. In some examples, the multispecific antigen-binding construct is capable of sequestering PD-L1, such that PD-L1 cannot bind CD80, and wherein CD80 is free to bind CD28. In some examples, the cell is an immune cell, such as a T cell. In some examples, the immune cell (*e.g.*, T cell) is a tumor infiltrating lymphocyte (TIL). In some examples, engagement of a multispecific antigen-binding molecule described herein to PD-1 expressed by an immune cell in the tumor microenvironment results in the downregulation of PD-1 by the immune cell. In some examples, the immune cell is a T cell. In some examples, the immune cell (*e.g.*, T cell) is a tumor infiltrating lymphocyte (TIL).

In some examples, any of the multispecific antigen-binding constructs disclosed herein is capable of inducing at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% greater interferon-gamma levels (*e.g.*, as measured in a *Staphylococcus aureus* Enterotoxin A (“SEA”) assay) as compared to a reference antigen-binding construct (*e.g.*, pembrolizumab or atezolizumab) or to a reference combination of antigen-binding constructs (*e.g.*, a composition comprising the PD-1 and PD-L1 arms of the multispecific antigen-binding construct, wherein the PD-1 and PD-L1 arms in the composition are not conjugated to one another). In some examples, the multispecific antigen-binding construct is capable of inducing at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% greater interleukin-2 levels (*e.g.*, as measured in a SEA assay) as compared to a reference antigen-binding construct (*e.g.*, pembrolizumab or atezolizumab) or to a reference combination of antigen-binding constructs (*e.g.*, a composition comprising the PD-1 and PD-L1 arms of the multispecific antigen-binding construct, wherein the PD-1 and PD-L1 arms in the composition are not conjugated to one another).

In some examples, the multispecific antigen-binding construct induces at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 200%, 300%, 400%, or 500% more killing of tumor cells (*e.g.*, leukemia cells, lymphoma cells, melanoma, or breast cancer cells), as compared to a reference antigen-binding construct (*e.g.*, pembrolizumab or atezolizumab) or to a reference combination of antigen-binding constructs (*e.g.*, a composition comprising the PD-1 and PD-L1 arms of the multispecific antigen-binding construct, wherein the PD-1 and PD-L1 arms in the composition are not conjugated to one another).

In some examples, the multispecific antigen-binding construct is capable of extending the survival of a subject suffering from a cancer (*e.g.*, leukemia, lymphoma, melanoma, and/or breast cancer) by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 100%, 200%, 300%, 400%, or 500% longer than a subject administered a reference antigen-binding construct (*e.g.*, pembrolizumab or atezolizumab) or a reference combination of antigen-binding constructs (*e.g.*, a composition comprising the PD-1 and PD-L1 arms of the multispecific antigen-binding construct, wherein the PD-1 and PD-L1 arms in the composition are not conjugated to one another). In some examples, the multispecific antigen-binding construct is capable of inducing at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 100%, 200%, 300%, 400%, or 500% more shedding of PD-1 from an immune cell as compared to an untreated immune cell or as compared to an immune cell treated with reference antigen-binding construct (*e.g.*, pembrolizumab or atezolizumab) or a reference combination of antigen-binding constructs (*e.g.*, a composition comprising the PD-1 and PD-L1 arms of the multispecific antigen-binding construct, wherein the PD-1 and PD-L1 arms in the composition are not conjugated to one another). In some examples, the multispecific antigen-binding construct is capable of reducing PD-1 levels at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 100%, 200%, 300%, 400%, or 500% (*e.g.*, by causing PD-1 shedding from the cell surface and/or inducing PD-1 degradation and/or reducing PD-1 expression) as compared to an untreated immune cell or to an immune cell treated with a reference antigen-binding construct (*e.g.*, pembrolizumab or atezolizumab) or a reference combination of antigen-binding constructs (*e.g.*, a composition comprising the PD-1 and PD-L1 arms of the multispecific antigen-binding construct, wherein the PD-1 and PD-L1 arms in the composition are not conjugated to one another).

In some examples, engagement of a multispecific antigen-binding molecule described herein to PD-1 expressed by a cell results in the downregulation and/or loss of cell-surface expression of PD-1 by the cell. Such down-regulation or loss of cell-surface expression can be due to, in part, for example, shedding of extracellular PD-1 from the surface of the immune cell. In some examples, the cell is an immune cell, such as a T cell. In some examples, the immune cell (e.g., T cell) is a tumor infiltrating lymphocyte (TIL). In some examples, engagement of a multispecific antigen-binding molecule described herein to PD-1 expressed by an immune cell in the tumor microenvironment results in the downregulation of PD-1 by the immune cell. In some examples, the immune cell is a T cell. In some examples, the immune cell (e.g., T cell) is a tumor infiltrating lymphocyte (TIL).

In some examples, the disclosure provides for a method for treating a proliferative disorder in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of any of the multispecific antigen-binding constructs disclosed herein, thereby treating the proliferative disorder in the subject. In some examples, the proliferative disorder is cancer. In some examples, the cancer is selected from the group consisting of a hematological cancer, a neurological cancer, melanoma, breast cancer, lung cancer, head and neck cancer, a gastrointestinal cancer, liver cancer, pancreatic cancer, a genitourinary cancer, a bone cancer, and a vascular cancer. In some examples, the disclosure provides for a method of enhancing an immune response in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of any of the multispecific antigen-binding constructs disclosed herein, thereby enhancing the immune response in the subject. In some examples, the enhanced immune response includes any one or more of enhanced T cell function, enhanced NK cell function, or enhanced macrophage function. In some examples, the enhancement of T cell function is greater upon administration of the multispecific antigen-binding construct, as compared to an agent that binds either PD-1 or a PD-1 ligand, or a cocktail comprising an agent that binds PD-1 and an agent that binds a PD-1 ligand. In some examples, the T cell function is any one or more of increased IFN γ production from T cells, enhanced T cell survival, increased T cell proliferation, or rescue from an exhausted T cell phenotype. In some examples, the enhanced T cell function is greater upon administration of the multispecific antigen-binding construct, as compared to an agent that binds either PD-1 or a PD-1 ligand, or a cocktail comprising an agent that binds PD-1 and an agent that binds a PD-1 ligand. In some

examples, the multispecific antigen-binding construct is administered subcutaneously, intravenously, intradermally, intraperitoneally, orally, intramuscularly or intracranially. In some examples, the multispecific antigen-binding construct binds to PD-1 and to PD-L1 expressed on the surface of the same cell in the subject. In some examples, the multispecific antigen-binding construct binds to PD-1 expressed on the surface of a first cell in the subject, and wherein the multispecific antigen-binding construct binds to PD-L1 expressed on the surface of a second cell in the subject.

In some examples, the disclosure provides for an anti-PD1 antibody or antigen-binding fragment thereof, wherein the anti-PD-1 antibody or antigen-binding fragment comprises: (a) a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 70 (FTFX₁X₂YAX₃X₄, wherein X₁=S, R, G, or N; X₂=D, S, N, A, R, or G; X₃=M or L; X₄=S, L, or N); (ii) a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA); and (iii) a CDRH3 comprising SEQ ID NO: 72 (ARGLDFIVGX₅TGNDY, wherein X₅=A, Y, or R); and (b) a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT). In some examples, the anti-PD-1 antibody or antigen-binding fragment comprises: (a) a heavy chain variable region comprising (i) a CDRH1 comprising the amino acid sequence of any one of SEQ ID NOs: 73, 76, 77, 78, 79, 80, 81, 82, 83, 84, or 86; (ii) a CDRH2 comprising the amino acid sequence of SEQ ID NO: 71; and (iii) a CDRH3 comprising the amino acid sequence of any one of SEQ ID NOs: 74, 75, or 85; and (b) a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT). In some examples, the heavy chain variable region comprises an amino acid sequence that is at least 85% identical to the amino acid sequence of any one of SEQ ID NOs: 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99. In some examples, the light chain variable region comprises an amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO: 59.

In some examples, the disclosure provides for an anti-PD-L1 antibody or antigen-binding fragment thereof, wherein the anti-PD-L1 antibody or antigen-binding fragment comprises: a. a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN); (ii) a CDRH2 comprising SEQ ID NO: 2 (GGIIPX₁X₂GX₃ATYA, wherein X₁ is V or I; X₂ is F, L, or V; and X₃ is T or A); and (iii) a CDRH3 comprising SEQ ID NO: 3 (ARLKX₁ELKDAFDI, wherein X₁ is G, F, or N); and b. a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 4 (RASQX₁ISSYLN, wherein X₁ is

S, W, or Q); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 6 (X₁QSYSTPLT, wherein X₁ is Q or F). In some examples, the anti-PD-L1 antibody or antigen-binding fragment comprises: (a) a heavy chain variable region comprising (i) a CDRH1 comprising the amino acid sequence of any one of SEQ ID NOs: 1, 14, 23, 36, or 122; (ii) a CDRH2 comprising the amino acid sequence of any one of SEQ ID NOs: 11, 15, 16, 21, 24, 26, 27, 29, 31, 33, or 34; and (iii) a CDRH3 comprising the amino acid sequence of any one of SEQ ID NOs: 8, 17, 18, 19, 20, 22, 25, 28, 30, 32, or 37; and (b) a light chain variable region comprising: (i) a CDRL1 comprising the amino acid sequence of any one of SEQ ID NOs: 9, 12, or 13; (ii) a CDRL2 comprising the amino acid sequence of SEQ ID NO: 5; and (iii) a CDRL3 comprising the amino acid sequence of any one of SEQ ID NOs: 10, 38 or 39. In some examples, the heavy chain variable region comprises an amino acid sequence that is at least 85% identical to the amino acid sequence of any one of SEQ ID NOs: 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, or 35. In some examples, the light chain variable region comprises an amino acid sequence that is at least 85% identical to the amino acid sequence of any one of SEQ ID NOs: 59, 60, 61, 62, or 63.

In some examples, the disclosure provides for a method for treating a proliferative disorder in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of any of the antibodies or antigen-binding constructs disclosed herein, thereby treating the proliferative disorder in the subject. In some examples, the proliferative disorder is cancer. In some examples, the cancer is selected from the group consisting of a hematological cancer, a neurological cancer, melanoma, breast cancer, lung cancer, head and neck cancer, a gastrointestinal cancer, liver cancer, pancreatic cancer, a genitourinary cancer, a bone cancer, and a vascular cancer.

In some examples, the disclosure provides for a method of enhancing an immune response in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of any of the antibodies or antigen-binding constructs disclosed herein, thereby enhancing the immune response in the subject.

In some examples, any of the methods described herein can involve detecting the presence or absence of PD-1 expression by one or more cells (or a population of cells, such as TILs) before and/or after contact with a multispecific antigen-binding molecule described herein. For example, any of the methods described herein can involve detecting the presence or absence of PD-1 expression by one or more cells (or a population of cells, such as TILs) before and/or after administration of a multispecific antigen-binding molecule described

herein to a subject (e.g., a cancer patient). Such methods are useful, e.g., determining a therapeutically effective amount of the molecule for use in treatment of a given patient or patient population. Methods of detecting the presence, reduction in expression, and/or absence of PD-1 expression are known to those of skill in the art, for example, using flow cytometry, Western blotting, ELISA, etc.

In another example, the disclosure features a method comprising measuring the level of PD-1 expression by one or more cells (or a population of cells, such as TILs) before and/or after contact with a multispecific antigen-binding molecule described herein. In some examples, the method comprises measuring the level of PD-1 expression by one or more cells (or a population of cells, such as TILs) before and/or after administration of a multispecific antigen-binding molecule described herein to a subject (e.g., a cancer patient).

In yet another example, the disclosure features a method comprising measuring the level of PD-1 expression by one or more cells (or a population of cells, such as TILs) before and/or after contact with a multispecific antigen-binding molecule described herein. For example, any of the methods described herein can involve measuring the level of PD-1 expression by one or more cells (or a population of cells, such as TILs) before and/or after administration of a multispecific antigen-binding molecule described herein to a subject (e.g., a cancer patient). Such methods are useful for, among other things, detecting or measuring a biological effect of a molecule described herein on the subject. In some examples, a reduction in the level of PD-1 expression by an immune cell (e.g., TILs isolated from a patient) following treatment with a multispecific antigen-binding molecule described herein indicates that the molecule has had a biological effect in the subject. In some examples, a reduction in the level of PD-1 expression by an immune cell (e.g., TILs isolated from a patient) following treatment with a multispecific antigen-binding molecule described herein indicates that the patient should receive one or more doses of the molecule, or otherwise continue on a therapy comprising the molecule.

In yet another example, the disclosure features methods for determining whether a biological effect has occurred in a patient or population of patients treated with a multispecific antigen-binding molecule described herein. The method comprises detecting the presence or amount of PD-1 expression by one or more test immune cells (e.g., effector immune cells, such as those in the tumor microenvironment) obtained from a patient or patients who have been administered a multispecific antigen-binding molecule described herein, wherein a reduced level of PD-1 expression (e.g., cell surface expression) of PD-1 by the one or more immune cells relative to a control expression level (e.g., the expression level

of PD-1 by immune cells of the same histological type as the test immune cells prior to administration of the molecule) indicates that a biological effect has occurred in the patient or population of patients. In some examples, the method comprises administering the multispecific antigen-binding molecule prior to the detecting. In some examples, the method
5 comprises administering a multispecific antigen-binding molecule to the patient or population of patients in whom the occurrence of a biological effect has been determined. In some examples, control PD-1 expression level is approximately the median or average expression level of PD-1 by immune cells of the same histological type in a population of subjects who have not been diagnosed as having a cancer. In some examples, control PD-1 expression
10 level is approximately the median or average expression level of PD-1 by immune cells of the same histological type in a population of subjects who have not been administered a multispecific antigen-binding molecule described herein and/or an agent that binds to and/or inhibits PD-1.

In yet another example, the disclosure features a method for reducing the expression
15 of PD-1 by one or more immune cells in a subject (*e.g.*, a cancer patient), the method comprising administering a multispecific antigen-binding molecule described herein to a subject to thereby reduce the expression of PD-1 by one or more immune cells in the subject. In some examples, the method comprises determining whether a reduction in PD-1 expression by one or more immune cells in the patient has occurred. In some examples, the
20 method comprises obtaining from the subject a biological sample (*e.g.*, a tumor biopsy) comprising one or more immune cells (*e.g.*, after administration of the molecule to the subject). In some examples, the method comprises measuring the level of PD-1 expression by one or more immune cells in the biological sample.

In yet another example, the disclosure features a method for inhibiting binding
25 between PD-L1 and CD80 in a subject (*e.g.*, a cancer patient), the method comprising administering a multispecific antigen-binding molecule described herein to a subject to thereby inhibit binding between PD-L1 and CD80 in the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

30 The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Figure 1 shows the induction of interferon-gamma (IFN γ) in a mixed lymphocyte reaction (MLR) assay treated with various antibody cocktails or bispecific antibodies

including a PembrolizumabxAtezolizumab bispecific, a NivolumabxAtezolizumab bispecific, a cocktail of KEYTRUDA and Atezolizumab, and a cocktail of Nivolumab and Atezolizumab, as compared to KEYTRUDA alone. The results show the concentration of IFN γ as pg/mL at the final concentrations of antibodies tested, as indicated.

5 **Figure 2** shows the induction of interferon-gamma (IFN γ) in mixed lymphocyte reaction (MLR) assay treated with various monoclonal and bispecific antibodies including a PembrolizumabxNivolumab bispecific, AtezolizumabxAtezolizumab tetravalent fusion, Nivolumab, and Atezolizumab, as compared to KEYTRUDA alone. The results show the concentration of IFN γ as pg/mL at the final concentrations of antibodies tested, as indicated.

10 **Figure 3** shows a schematic and amino acid sequence for a PembrolizumabxAtezolizumab bispecific. Separate sequences are given for the Pembrolizumab__aglyco-IgG1-(G4S)₄ heavy chain (H chain; SEQ ID NO: 104) and light chain (L chain; SEQ ID NO: 105) and for the Atezolizumab__FabH-(G4S)₄ heavy chain (H chain; SEQ ID NO: 106) and light chain (L chain; SEQ ID NO: 107).

15 **Figure 4** shows a schematic and amino acid sequence for a NivolumabxAtezolizumab bispecific. Separate sequences are given for the Nivolumab aglyco-IgG1-(G4S)₄ heavy chain (H chain; SEQ ID NO: 108) and light chain (L chain; SEQ ID NO: 109) and for the Atezolizumab FabH-(G4S)₄ heavy chain (H chain; SEQ ID NO: 106) and light chain (L chain; SEQ ID NO: 107).

20 **Figure 5** shows a schematic and amino acid sequence for a 949 aglyco-IgG1xAtezolizumab bispecific. Separate sequences are given for the 949__aglyco-IgG1-(G4S)₄ heavy chain (H chain; SEQ ID NO: 110) and light chain (L chain; SEQ ID NO: 111) and for the Atezolizumab__FabH-(G4S)₄ heavy chain (H chain; SEQ ID NO: 106) and light chain (L chain; SEQ ID NO: 107).

25 **Figure 6** shows a schematic and amino acid sequence for an Atezolizumab xNivolumab bispecific. Separate sequences are given for the Atezolizumab__aglyco-IgG1-(G4S)₄ heavy chain (H chain; SEQ ID NO: 112) and light chain (L chain; SEQ ID NO: 107) and Nivolumab__HC Fab-(G4S)₄ heavy chain (H chain; SEQ ID NO: 113) and light chain (L chain; SEQ ID NO: 109).

30 **Figure 7** shows IFN- γ release, in pg/mL, in a mixed lymphocyte reaction (MLR) assay as a function of antibodies tested at various concentrations (right panel). These results indicate that bispecific antibodies PD-1xPD-L1 (Pembrolizumabx Atezolizumab) or (NivolumabxAtezolizumab) in multispecific format induce a greater IFN- γ response at femtomolar concentrations, as compared to a cocktail of Pembrolizumab and Atezolizumab

or Nivolumab and Atezolizumab. Size exclusion chromatography of the bispecific formats against mAb precursors is shown (left panel).

Figure 8 shows an example of a workflow for identifying multispecific (*e.g.*, bispecific) antibodies that demonstrate synergy. The process includes an unbiased screen of checkpoint blocker combinations in a mixed lymphocyte reaction (MLR) assay, which measures IFN- γ release, in pg/mL, at various concentrations. In the second step of the illustrated workflow, common light chain bispecifics were generated to further test their efficacy; here, various bispecific formats are depicted. The identified bispecific formats outperform known PD-1 blockers in a T-cell activation assay.

Figures 9A-9B show that Bispecific 3 induced higher killing of K562-CD32-PDL1 target cells (**Figure 9A**) and increased IFN γ production (**Figure 9B**) by CD3/CD28 expanded T-cells in an antigen non-specific T cell assay, as compared to both an isotype control antibody and KEYTRUDA. This increased killing by Bispecific 3 was seen even at low concentrations of 0.01 nM.

Figures 10A-10B show an effect of Bispecific 3 on tumor cell killing. **Figure 10A** shows that Bispecific 3 increased the specific killing of K562-A2-CMV-PDL1 tumor antigen target cells by CMV specific T-cells at low concentrations of 0.001 through 0.01 nM, as compared to both KEYTRUDA and the combination of mAb1 and mAb28, indicating that Bispecific 3 can be used to mediate antigen-specific killing of target cells at lower doses.

Figure 10B shows that Bispecific 3 was more effective than either KEYTRUDA or the combination of mAb1 and mAb28 in the specific killing of Raji-A2-CMV-PDL1 cell tumor antigen target cells by CMV specific T-cells at low concentrations of 0.001 nM, again indicating that Bispecific 3 can be used to mediate antigen-specific killing of target cells at lower doses.

Figure 11 shows that Bispecific 3 induced more IL-2 than KEYTRUDA at all tested doses in an SEA stimulation assay. Importantly, Bispecific 3 induced increased IL-2 production starting at lower concentrations of antibody as compared to both KEYTRUDA and mAb1 and mAb28.

Figures 12A-12G demonstrate that Bispecific 3 has a unique ability to cause the internalization and subsequent degradation or loss of expression of PD-1, and that this property is dependent on engagement of both the PD-1 and PD-L1 targeting arms of the molecule. **Figures 12A-12B** demonstrate that only Bispecific 3 results in internalization and subsequent degradation or loss of expression of PD-1, when compared to isotype control, KEYTRUDA, mAb1 and mAb28, Atezolizumab, or Atezolizumab and KEYTRUDA.

Additionally, as shown in **Figure 12C**, when the anti-PD-L1 antibody, mAb1, was added at 50 nM to wells 5 minutes before adding Bispecific 3, the ability of Bispecific 3 to drive PD-1 internalization was lost. This suggests that both arms of Bispecific 3 should be engaged to drive PD-1 loss of expression and/or internalization and/or degradation. **Figure 12D** shows that treatment with Bispecific 3 increases the amount of PD-1 in the supernatant when both binding arms of the bispecific are engaged concurrently. This effect is lost when the PD-L1 targeting arm is blocked by mAb1. This suggests that Bispecific 3 increases shedding of PD-1 into the supernatant. **Figure 12E** demonstrates that the valency of the binding arms influences the degree of the loss of PD-1 expression. Bispecific 5 was made having a first N-terminal Fab binding PD-L1 based on the VH and VL sequences of mAb1 and having a second N-terminal Fab binding PD-1 based on the VH and VL sequences of mAb28. In other words, Bispecific 5 has one monovalent arm binding PD-L1 and one monovalent arm binding PD-1, as compared to Bispecific 3, which has bivalent arms binding PD-L1 and bivalent arms binding PD-1. As shown, loss of PD-1 expression starts to occur at higher doses of Bispecific 5 (bivalent) versus Bispecific 3 (tetravalent), suggesting that the increased valency of Bispecific 3 is responsible for this difference. **Figure 12F** demonstrates that pretreatment with Batimastat, a broad-spectrum inhibitor of multiple MMPs and ADAMs, sheddases or proteases responsible for cleaving proteins off the plasma membrane of cells, greatly reduces the amount of cell-associated PD-1 loss, suggesting that PD-1 loss or shedding is due to cleavage by an MMP or ADAM protease. **Figure 12G** suggests that Bispecific 3 drives loss of cell-surface PD-1 expression primarily when it binds to PD-1 and PD-L1 that are in the trans configuration, *i.e.*, are being expressed by different cells.

Figures 13A-13B shows *in vivo* results using Bispecific 3. **Figure 13A** depicts a schematic of the experimental protocol. **Figure 13B** demonstrates that both Bispecific 3 and the combination mAb1 and mAb28 groups had significant delays in tumor growth as compared to both the isotype and KEYTRUDA groups. In addition, at day 24, there was a significant divergence between the Bispecific 3 group and the group treated with a combination of mAb1 and mAb28, with Bispecific 3 causing a greater delay in tumor growth as compared to the combination. The No T cell transfer group had tumors that grew more aggressively than any group containing T cells. In this model, KEYTRUDA gave no benefit in delaying KACP tumor growth as compared to the isotype control.

Figures 14A-14B shows *in vivo* results using Bispecific 3 in a K562-A2-CMV-PD-L1 tumor mouse model. **Figures 14A and 14B** are graphs illustrating that, while each of the different treatment groups resulted in a delay in average tumor growth as compared to

untreated mice, treatment with T cells and Bispecific 3 resulted in the greatest delay in average tumor growth over time. The No T cell transfer group had tumors that grew more aggressively than any group containing T cells. In this model, KEYTRUDA gave no benefit in delaying K562-A2-CMV-PD-L1 tumor growth as compared to the isotype control.

5 **Figures 15A-15B** illustrate *in vivo* results using Bispecific 3 in several syngeneic tumor models. **Figure 15A** is a graph showing that treatment of an EMT-6 syngeneic tumor model with Bispecific 3 resulted in a greater delay in tumor growth as compared to the control treatment. **Figure 15B** is a graph showing that treatment of an MB49 syngeneic tumor model with Bispecific 3 resulted in a greater delay in tumor growth as compared to the control treatment.

10 **Figures 16A-16B** show *in vivo* results using Bispecific 1 in an MC38-hPD-L1 model in humanized PD-1/PD-L1 transgenic mice. **Figure 16A** shows that both KEYTRUDA and Bispecific 1 treatment effectively controlled tumor growth in the MC38-hPD-L1 tumor mice as compared to control-treated mice. **Figure 16B** is a survival graph and illustrates that Bispecific 1 increased the survival of MC38-PD-L1 tumor mice as compared to control-treated mice.

15 **Figures 17A-17F** illustrate *in vivo* results using Bispecific 3 in a B16F10-hPD-L1 model. **Figure 17A** is a series of graphs each showing the effect of different treatments on tumor growth measured at a 15-day cutoff in B16F10-HuPD-L1 mice. Different groups of mice (n=8) were treated with Bispecific 3, KEYTRUDA, Avelumab, KEYTRUDA + Avelumab combination, or isotype control antibodies. **Figure 17A** shows individual tumor volume traces for each group. Metastases were identified in multiple mice that died prior to the tumor-sizing cutoff. **Figure 17B** shows differences in mean tumor volume across treatment groups, demonstrating that by 15 days after tumor cell inoculation, Bispecific 3 treatment delayed average tumor growth significantly longer than any of the other treatments tested in B16F10-HuPD-L1 mice. ****, $P<0.0001$; **, $P<0.01$, *, $P<0.05$, Two-way ANOVA and Tukey's multiple comparisons test. **Figure 17B** is a graph comparing the effect of different treatments on tumor volume measured at a 15-day cutoff in B16F10-HuPD-L1 mice. Metastases were identified in multiple mice that died prior to the tumor-sizing cutoff. As shown in **Figure 17B**, Bispecific 3 treatment delayed average tumor growth significantly longer than any of the other treatments tested in B16F10-hPD-L1 mice. **Figure 17C** is a survival graph and illustrates that treatment with Bispecific 3 increased the survival of B16F10-hPD-L1 tumor mice as compared to survival with any of the other treatments tested. **Figure 17D** shows that by 21 days after tumor cell inoculation, Bispecific 3 treatment

continued to delay average tumor growth significantly better than treatment with KEYTRUDA in B16F10-hPD-L1 mice. **Figure 17E** is a survival graph and illustrates that treatment with Bispecific 3 increased the survival of B16F10-HuPD-L1 tumor mice as compared to survival with any of the other treatments tested. **Figure 17F** provides a Table showing the number of tumor-free mice for each of the different treatment groups. The group treated with Bispecific 3 had 3 mice that were tumor-free, while the combination of KEYTRUDA and avelumab had 1 mouse that was tumor-free.

Figures 18A-18D demonstrate that Bispecific 3 has drug-like properties (DLP's) similar to a well-behaved monoclonal antibody and maintains parental PD-1 and PD-L1 binding. **Figure 18A** shows that Bispecific 3 shows similar binding to CHO cells expressing human PD-1 as parental clone mAb28 (top) and to CHO cells expressing human PD-L1 as parental clone mAb1 (bottom). **Figure 18B** shows that Bispecific 3 shows similar binding to CHO cells expressing cynomolgus PD-1 as parental clone mAb28 (top) and to CHO cells expressing cynomolgus PD-L1 as parental clone mAb1 (bottom). **Figure 18C** shows that Bispecific 3 shows similar binding to CHO cells expressing mouse PD-1 as parental clone mAb28 (top) and to CHO cells expressing mouse PD-L1 as parental clone mAb1 (bottom). **Figure 18D** shows a size-exclusion chromatography trace of Bispecific 3 after Protein A chromatography (top) demonstrating a single peak with greater than 98% purity and a differential scanning fluorimetry (DSF) trace of Bispecific 3 (bottom) demonstrating that the molecule has high thermal stability.

DETAILED DESCRIPTION

Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this disclosure pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodologies by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* 2nd ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. As appropriate, procedures involving the use of commercially available kits and reagents are

generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

As used herein, the singular forms “a,” “an,” and “the” include the plural referents unless the context clearly indicates otherwise.

5 In the specification and claims, the term “about” is used to modify, for example, the quantity of an ingredient in a composition, concentration, volume, process temperature, process time, yield, flow rate, pressure, and like values, and ranges thereof, employed in describing the embodiments of the disclosure. The term “about” refers to variation in the numerical quantity that can occur, for example, through typical measuring and handling
10 procedures used for making compounds, compositions, concentrates or use formulations; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of starting materials or ingredients used to carry out the methods, and like proximate considerations. The term “about” also encompasses amounts that differ due to aging of a formulation with a particular initial concentration or mixture, and amounts that differ due to
15 mixing or processing a formulation with a particular initial concentration or mixture. Where modified by the term “about” the claims appended hereto include equivalents to these quantities. If there are uses of the term which are not clear to persons of ordinary skill given the context in which it is used, “about” will mean up to plus or minus 10% of the particular value.

20 With regard to the binding of an antigen-binding protein/region/arm to a target molecule, the terms “specific binding,” “specifically binds to,” “specific for,” “selectively binds,” and “selective for” a particular antigen (*e.g.*, a polypeptide target) or an epitope on a particular antigen mean binding that is measurably different from a non-specific or non-selective interaction. Specific binding can be measured, for example, by determining binding
25 of a molecule compared to binding of a control molecule. Specific binding can also be determined by competition with a control molecule that is similar to the target, such as an excess of non-labeled target. In that case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by the excess non-labeled target.

 The term “epitope” means a component of an antigen capable of specific binding to
30 an antigen-binding protein. Epitopes frequently consist of surface-accessible amino acid residues and/or sugar side chains and can have specific three-dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. An epitope can comprise amino acid residues that

are directly involved in the binding, and other amino acid residues, which are not directly involved in the binding. The epitope to which an antigen-binding protein binds can be determined using known techniques for epitope determination such as, for example, testing for antigen-binding protein binding to antigen variants with different point-mutations.

5 Percent “identity” between a polypeptide sequence and a reference sequence, is defined as the percentage of amino acid residues in the polypeptide sequence that are identical to the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved
10 in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, MEGALIGN (DNASTAR), CLUSTALW, or CLUSTAL OMEGA software. In some embodiments, alignment is performed using the CLUSTAL OMEGA software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve
15 maximal alignment over the full length of the sequences being compared.

 The term “capable of” as used herein means that an agent or method (*e.g.*, any of the multispecific antigen-binding constructs or methods disclosed herein) has the ability to achieve a specified property in the appropriate context (as would be understood by the skilled worker), but is not required to be associated with that property at any particular moment in
20 time. For example, any of the multispecific antigen-binding constructs disclosed herein may be capable of binding PD-1 and/or PD-L1 when administered to cells expressing PD-1 and/or PD-L1, but the constructs would not be expected to bind PD-1 and/or PD-L1 when the constructs are in a composition devoid of PD-1 or PD-L1 protein.

 A “conservative substitution” or a “conservative amino acid substitution,” refers to
25 the substitution of one or more amino acids with one or more chemically or functionally similar amino acids. Conservative substitution tables providing similar amino acids are well known in the art. Polypeptide sequences having such substitutions are known as “conservatively modified variants,” or “variants.” Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles.
30 Some examples of conservative substitutions can be found, for example, in Creighton, *Proteins: Structures and Molecular Properties* 2nd ed. (1993) W. H. Freeman & Co., New York, NY.

 A polypeptide disclosed herein can comprise an amino acid sequence which is not naturally occurring. Such variants necessarily have less than 100% sequence identity or

similarity with the starting molecule. In certain embodiments, the variant will have an amino acid sequence from about 75% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of the starting (*e.g.*, naturally-occurring or wild-type) polypeptide, more preferably from about 80% to less than 100%, more preferably from about 85% to less than 100%, more preferably from about 90% to less than 100% (*e.g.*, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) and most preferably from about 95% to less than 100%, *e.g.*, over the length of the variant molecule.

An “antibody,” as used herein, can refer to an intact antibody (*e.g.*, an intact immunoglobulin) and antibody portion, for example, an antigen-binding portion. Antigen-binding portions comprise at least one antigen-binding domain. One example of an antigen-binding domain is an antigen-binding domain formed by a V_H-V_L dimer. Antibodies and antigen-binding portions can be described by the antigen to which they specifically bind. For example, a PD-L1 antibody, or anti-PD-L1 antibody, is an antibody that specifically binds to PD-L1.

The V_H and V_L regions can be further subdivided into regions of hypervariability (hypervariable regions (HVRs), also called complementarity determining regions (CDRs)) interspersed with regions that are more conserved. The more conserved regions are called framework regions (FRs). Each V_H and V_L generally comprises three CDRs and four FRs, arranged in the following order (from N-terminus to C-terminus): FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. The CDRs are involved in antigen-binding, and confer antigen specificity and binding affinity to the antibody. (*See* Kabat et al. (1991) *Sequences of Proteins of Immunological Interest* 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD.)

CDRs are involved in antigen binding and confer antigen specificity and binding affinity to the antibody. There are three CDRs in each of the variable domains of the heavy chain and the light chain, which are designated CDR1, CDR2 and CDR3, for each of the variable domains. The term “CDR set” as used herein refers to a group of three CDRs that occur in a single heavy or light chain variable domain capable of binding a target antigen. The exact boundaries of these CDRs have been defined differently according to different systems. The three heavy chain CDRs can be referred to as CDRH1, CDRH2, and CDRH3, and the three light chain CDRs can be referred to as CDRL1, CDRL2, and CDRL3.

The system described by Kabat, also referred to as “numbered according to Kabat,” “Kabat numbering,” “Kabat definitions,” and “Kabat labeling,” provides an unambiguous residue numbering system applicable to any variable domain of an antibody, and provides

precise residue boundaries defining the three CDRs of each chain. (Kabat et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, Md. (1987) and (1991), the contents of which are incorporated by reference in their entirety. These CDRs are referred to as Kabat CDRs and comprise about residues 24-34 (CDR1), 50-56 (CDR2) and
 5 89-97 (CDR3) in the light chain variable domain, and 31-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3) in the heavy chain variable domain. When the CDRs are defined according to Kabat, the light chain FR residues are positioned at about residues 1-23 (LCFR1), 35-49 (LCFR2), 57-88 (LCFR3), and 98-107 (LCFR4) and the heavy chain FR residues are positioned about at residues 1-30 (HCFR1), 36-49 (HCFR2), 66-94 (HCFR3), and 103-113
 10 (HCFR4) in the heavy chain residues. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

Other CDR numbering systems are also used in the art (see, for example, **Table A**). Chothia and coworkers found that certain sub-portions within Kabat CDRs adopt nearly identical peptide backbone conformations, despite having great diversity at the level of amino
 15 acid sequence. (Chothia et al. (1987) J. Mol. Biol. 196: 901-917; and Chothia et al. (1989) Nature 342: 877-883). These sub-portions were designated as L1, L2, and L3 or H1, H2, and H3 where the "L" and the "H" designates the light chain and the heavy chains regions, respectively. These CDRs can be referred to as "Chothia CDRs," "Chothia numbering," or "numbered according to Chothia," and comprise about residues 24-34 (CDR1), 50-56
 20 (CDR2) and 89-97 (CDR3) in the light chain variable domain, and 26-32 (CDR1), 52-56 (CDR2) and 95-102 (CDR3) in the heavy chain variable domain. Mol. Biol. 196:901-917 (1987).

The system described by MacCallum, also referred to as "numbered according to MacCallum," or "MacCallum numbering" comprises about residues 30-36 (CDR1), 46-55
 25 (CDR2) and 89-96 (CDR3) in the light chain variable domain, and 30-35 (CDR1), 47-58 (CDR2) and 93-101 (CDR3) in the heavy chain variable domain. MacCallum et al. ((1996) J. Mol. Biol. 262(5):732-745).

The system described by AbM, also referred to as "numbering according to AbM," or "AbM numbering" comprises about residues 24-34 (CDR1), 50-56 (CDR2) and 89-97
 30 (CDR3) in the light chain variable domain, and 26-35 (CDR1), 50-58 (CDR2) and 95-102 (CDR3) in the heavy chain variable domain.

The IMGT (INTERNATIONAL IMMUNOGENETICS INFORMATION SYSTEM) numbering of variable regions can also be used, which is the numbering of the residues in an immunoglobulin variable heavy or light chain according to the methods of the IMGT, as

described in Lefranc, M.-P., "The IMGT unique numbering for immunoglobulins, T cell Receptors and Ig-like domains", *The Immunologist*, 7, 132-136 (1999), and is expressly incorporated herein in its entirety by reference. As used herein, "IMGT sequence numbering" or "numbered according to IMTG," refers to numbering of the sequence encoding a variable region according to the IMGT. For the heavy chain variable domain, when numbered according to IMGT, the hypervariable region ranges from amino acid positions 27 to 38 for CDR1, amino acid positions 56 to 65 for CDR2, and amino acid positions 105 to 117 for CDR3. For the light chain variable domain, when numbered according to IMGT, the hypervariable region ranges from amino acid positions 27 to 38 for CDR1, amino acid positions 56 to 65 for CDR2, and amino acid positions 105 to 117 for CDR3.

In some embodiments of the constructs and antigen-binding arms described herein, the CDRs recited herein comprise about residues 24-34 (CDR1), 49-56 (CDR2) and 89-97 (CDR3) in the light chain variable domain, and 27-35 (CDR1), 49-60 (CDR2) and 93-102 (CDR3) in the heavy chain variable domain, when numbered according to Chothia numbering. In some embodiments, CDR2 in the light chain variable domain can comprise amino acids 49-56, when numbered according to Chothia numbering.

Table A: CDR Definitions

	CDRH1	CDRH2	CDRH3	CDRL1	CDRL2	CDRL3
Kabat	31-35	50-65	95-102	24-34	50-56	89-97
Alternative CDRs numbered according to Chothia	27-35	49-60	93-102	24-34	50-56	89-97
Chothia	26-32	52-56 or 50-56	95-102	24-34	50-56	89-97
MacCallum	30-35	47-58	93-101	30-36	46-55	89-96
AbM	26-35	50-58	95-102	24-34	50-56	89-97
IMGT	27-38	56-65	105-117	27-38	56-65	105-117

Preferred methods and materials are described herein, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the presently disclosed methods and compositions. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

Various aspects of the invention are described in further detail below. Additional definitions are set out throughout the specification.

The immune system has the capability of recognizing and eliminating tumor cells; however, tumors can use multiple strategies to evade immunity. Recent studies have shown
5 that inhibitory immune checkpoint molecules promote cancer progression through various antitumor inhibitory mechanisms. Blockade of immune checkpoints is one of the approaches to activating or reactivating therapeutic antitumor immunity. Various ligands have been described for a number of cognate inhibitory immune checkpoint receptors. Reviewed in, e.g., Nair & Elkord, *Immunology & Cell Biology* (2018), 96:21-33; and Jenkins et al., *British*
10 *J. of Cancer* (2017), 118:9-16.

The Programmed Death 1 (PD-1) protein is an inhibitory member of the extended CD28/CTLA-4 family of T cell regulators (Okazaki et al. (2002) *Curr Opin Immunol* 14: 391779-82; Bennett et al. (2003) *J. Immunol.* 170:711-8). Other members of the CD28 family include CD28, CTLA-4, ICOS and BTLA. PD-1 is suggested to exist as a monomer, lacking
15 the unpaired cysteine residue characteristic of other CD28 family members. PD-1 is expressed on activated B cells, T cells, and monocytes.

The PD-1 gene encodes a 55 kDa type I transmembrane protein (Agata et al. (1996) *Int Immunol.* 8:765-72). Although structurally similar to CTLA-4, PD-1 lacks the MYPPY motif that is important for B7-1 and B7-2 binding. Two ligands for PD-1 have been
20 identified, PD-L1 (B7-H1) and PD-L2 (B7-DC), that have been shown to downregulate T cell activation upon binding to PD-1 (Freeman et al. (2000) *J. Exp. Med.* 192:1027-34; Carter et al. (2002) *Eur. J. Immunol.* 32:634-43). Both PD-L1 and PD-L2 are B7 homologs that bind to PD-1, but do not bind to other CD28 family members. PD-L1 is abundant in a variety of human cancers (Dong et al. (2002) *Nat. Med.* 8:787-9).

PD-1 is known as an immunoinhibitory protein that negatively regulates TCR signals (Ishida, Y. et al. (1992) *EMBO J.* 11:3887-3895; Blank, C. et al. (Epub 2006 Dec. 29) *Immunol. Immunother.* 56(5):739-745). The interaction between PD-1 and PD-L1 can act as an immune checkpoint, which can lead to, e.g., a decrease in tumor infiltrating lymphocytes, a decrease in T-cell receptor mediated proliferation, and/or immune evasion by cancerous
30 cells (Dong et al. (2003) *J. Mol. Med.* 81:281-7; Blank et al. (2005) *Cancer Immunol. Immunother.* 54:307-314; Konishi et al. (2004) *Clin. Cancer Res.* 10:5094-100). Immune suppression can be reversed by inhibiting the local interaction of PD-1 with PD-L1 or PD-L2; the effect is additive when the interaction of PD-1 with PD-L2 is blocked as well (Iwai et al.

(2002) *Proc. Nat'l. Acad. Sci. USA* 99:12293-7; Brown et al. (2003) *J. Immunol.* 170:1257-66).

PD-L1, also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1), is a 40kDa type 1 transmembrane protein that plays a role in suppressing the immune system during particular events such as pregnancy, tissue allografts, autoimmune disease and other disease states such as hepatitis. By way of example, human PD-L1 comprises the amino acid sequence of SEQ ID NO: 115 (UniProt Q9NZQ7). Normally the immune system reacts to foreign antigens that are associated with exogenous or endogenous danger signals, which triggers a proliferation of antigen-specific CD8⁺ T cells and/or CD4⁺ helper cells. In cancer, PD-L1 expressed on cancer cells binds to its ligand PD-1 on immune effector cells, for example, T cells. The binding of PD-L1 to PD-1 transmits an inhibitory signal that reduces the proliferation of antigen-specific T-cells in lymph nodes, while simultaneously reducing apoptosis in regulatory T cells (anti-inflammatory, suppressive T cells). The PD-1/PD-L1 interaction also induces apoptosis of tumor-specific T cells, promotes the differentiation of CD4⁺ T cells into Foxp3⁺ regulatory T cells, and promotes resistance of tumor cells to cytotoxic T lymphocyte (CTL) attack, thus allowing tumors to evade the host immune system.

The present disclosure relates to compositions and methods for inhibiting tumor evasion by reducing immune checkpoint suppression that results from the interaction between PD-1 and its ligand (*e.g.*, PD-L1 and/or PD-L2). In particular, provided herein are compositions comprising novel multispecific and multivalent constructs, such as a bispecific and tetravalent construct, that block the interaction between PD-1 and its ligand (*e.g.*, PD-1 and/or PD-L2) while promoting the interaction of (bridging) the cells on which PD-1 and its ligand are expressed. Such compositions of the present disclosure with the capacity to “block and bridge” have increased potency *in vitro* and *in vivo* and strongly enhance, for example, T cell proliferation, IFN γ production and/or secretion, the cytolytic activity of T cells, and/or rescue T cells from functional exhaustion, to provide superior anti-tumor efficacy (a biological effect which can be manifested by various means, including, but not limited to, *e.g.*, a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in tumor cell proliferation, and/or a decrease in tumor cell survival), as compared to combinations of individual antibodies, as well as compared to clinical checkpoint blockade agents. Also provided herein are novel monoclonal anti-PD-1 antibodies and antigen-binding fragments thereof, and novel monoclonal anti-PD-L1 antibodies and antigen-binding fragments thereof, for use in such multispecific and multivalent constructs. Some of these novel monoclonal

anti-PD-1 antibodies and novel monoclonal anti-PD-L1 antibodies share a common light chain, thereby allowing for the generation of multispecific and multivalent constructs having excellent drug-like properties and manufacturability, as well as affinities similar to their parental antibodies.

5 Accordingly, the disclosure provides a multispecific antigen-binding construct comprising at least two antigen-binding arms or units of antigen-binding, wherein a first arm or unit of antigen-binding binds PD-1 expressed by an immune cell, and a second arm or unit of antigen-binding binds one or more PD-1 ligands (*e.g.*, PD-L1 and/or PD-L2) which is expressed by a second cell. In some embodiments, the multispecific antigen-binding
10 construct blocks the interaction of PD-1 and its ligand. In some embodiments, the multispecific antigen-binding construct bridges the cells on which PD-1 and its ligand are expressed to promote the interaction and/or efficacy of the immune cell that expresses PD-1. In some embodiments, at least one of the antigen-binding arms is bivalent for PD-1. In some
15 embodiments, at least one of the antigen-binding arms is bivalent for PD-L1. In some embodiments, at least one of the antigen-binding arms is bivalent for PD-1, and at least one of the antigen-binding arms is bivalent for PD-L1. In some embodiments, the multispecific antigen-binding construct comprises at least two units of antigen-binding that bind PD-1. In
20 some embodiments, the multispecific antigen-binding construct comprises two units of antigen-binding that bind PD-1. In some embodiments, the multispecific antigen-binding construct comprises at least two units of antigen-binding that bind a PD-1 ligand, such as PD-L1 or PD-L2. In some
25 embodiments, the multispecific antigen-binding construct comprises two units of antigen-binding that bind a PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments, the multispecific antigen-binding construct comprises at least four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind a PD-1 ligand, such as PD-L1 or PD-L2. In some
30 embodiments, the multispecific antigen-binding construct comprises four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind a PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments of any of the aspects described herein, the construct is a bispecific antibody. In some
 embodiments, the bispecific antibody is an antagonist of both PD-1 and PD-1 ligand. In some embodiments, the construct comprises a common light chain. In some embodiments, one or both of the antigen-binding arms is an aptamer. In some
 embodiments, one or both of the antigen-binding arms is a protein other than an antibody. In some embodiments, the construct comprises at least two antibodies. In
 some embodiments, at least one of the antigen-binding arms is a bivalent antibody specific

for PD-1. In some embodiments, at least one of the antigen-binding arms is a bivalent antibody specific for PD-L1. In some embodiments, at least one of the antigen-binding arms is a bivalent antibody specific for PD-1, and at least one of the antigen-binding arms is a bivalent antibody specific for PD-L1, such that the construct is tetravalent. In some
5 embodiments, the bispecific antibody binds two different epitopes on PD-1. In some
embodiments, the bispecific antibody binds two different epitopes on the PD-1 ligand. Also
provided herein, in some aspects, are novel isolated antibodies and antigen-binding portions
thereof that specifically bind PD-L1 or PD-1. In some embodiments, these novel isolated
antibodies and antigen-binding portions thereof, such as CDRs, variable heavy chains, and/or
10 variable light chains, that specifically bind PD-L1 or PD-1 can be used in one or more arms or
units of antigen-binding of the multispecific antigen-binding constructs described herein.

Accordingly, as described herein, the disclosed multispecific antigen-binding
constructs include bispecific, trispecific, tetraspecific, or multispecific antibodies or antigen-
binding portions thereof. The described multispecific constructs are preferably bivalent for at
15 least one, preferably both, antigen-binding arm(s), *i.e.*, are bispecific and trivalent, or
bispecific and tetravalent molecules. The multispecific constructs described herein can, in
various aspects and embodiments, comprise one or more antibodies and/or antigen-binding
portions thereof. For example, an antigen-binding arm can comprise a variable heavy and/or
variable light chain, or complementarity determining regions (CDRs) thereof, of a given
20 antibody to PD-1 and/or a given antibody to PD-L1. Accordingly, in some embodiments of
any of the aspects described herein, the first antigen-binding arm, second antigen-binding
arm, first unit of antigen-binding, second unit of binding, or any combination thereof, can
comprise an antibody or an antigen-binding portion thereof. In some embodiments of any of
the aspects described herein, the first antigen-binding arm, second antigen-binding arm, first
25 unit of antigen-binding, second unit of binding, or any combination thereof, is an antibody or
an antigen-binding portion thereof.

A. PD-L1 Antagonists

In some aspects and embodiments, the disclosure provides for anti-PD-L1 antagonists.
In some embodiments, the anti-PD-L1 antagonist is any of the anti-PD-L1 antibodies or
30 antigen-binding molecules disclosed herein. In some embodiments, the anti-PD-L1 antibody
or antigen-binding molecule is not a part of a multispecific antigen-binding construct, *i.e.*, the
anti-PD-L1 antibody or antigen-binding molecule is not a part of a protein construct that
binds to multiple epitopes. In some embodiments, the anti-PD-L1 antibody or antigen-
binding portion can be combined with a different antibody or antigen-binding portion to form

a multispecific antigen-binding construct. In some embodiments, the multispecific antigen-binding construct is capable of binding an epitope on PD-L1 and an epitope on another protein. In some embodiments, the epitope on the other protein is on PD-1.

Accordingly, in some aspects, provided herein are antibodies or antigen-binding
 5 portions thereof that specifically bind PD-L1. In some aspects, the antibody or antigen-binding portion thereof that specifically binds PD-L1 comprises (a) a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN); (ii) a CDRH2 comprising SEQ ID NO: 2 (GGIIPX₁X₂GX₃ATYA, wherein X₁ is V or I; X₂ is F, L, or V; and X₃ is T or A); and (iii) a CDRH3 comprising SEQ ID NO: 3 (ARLKX₁ELKDAFDI,
 10 wherein X₁ is G, F, or N); and (b) a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 4 (RASQX₁ISSYLN, wherein X₁ is S, W, or Q); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 6 (X₁QSYSTPLT, wherein X₁ is Q or F).

In some embodiments of these aspects and all such aspects described herein, CDRH1
 15 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 7 (GGIIPILGAATYA) and CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 7 (GGIIPILGAATYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 comprises SEQ ID NO: 9
 20 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb1.

In some embodiments of these aspects and all such aspects described herein, CDRH1
 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11
 25 (GGIIPVFGTATYA) and CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 comprises SEQ ID NO: 12 (RASQWISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3
 30 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb2. In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 comprises SEQ ID NO: 13 (RASQQISSYLN); CDRL2

comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb3.

In some embodiments of these aspects and all such aspects described herein, CDRH1
 5 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb4. Another
 10 representative antibody having such heavy and light chain variable CDR regions is mAb24.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 14 (GTFSSYAFS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA) and CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some
 15 embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 14 (GTFSSYAFS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb5.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 15 (GGIIPIFGIANYA) and CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some
 20 embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 15 (GGIIPIFGIANYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb6.

In some embodiments of these aspects and all such aspects described herein, CDRH1
 30 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 16 (GGIIPNFGTATYA) and CDRH3 comprises SEQ ID NO: 17 (ARLKGELKGAGDI). In some embodiments of these aspects and all such aspects described herein SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 16 (GGIIPNFGTATYA), CDRH3 comprises SEQ ID NO: 17 (ARLKGELKGAGDI); CDRL1 comprises SEQ ID NO: 9

(RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb7.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 comprises SEQ ID NO: 18 (ARLKFELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 18 (ARLKFELKDAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb8.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 comprises SEQ ID NO: 19 (ARLKGELKDAFDE). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 19 (ARLKGELKDAFDE), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb9.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 comprises SEQ ID NO: 20 (ARLKNELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 20 (ARLKNELKDAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb10.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 21 (GGVIPFLGTANYA), and CDRH3 comprises SEQ ID NO: 22 (ARLKGILKDALDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises

SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 21 (GGVIPFLGTANYA), CDRH3 comprises SEQ ID NO: 22 (ARLKGILKDALDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb11.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSYAIS), CDRH2 comprises SEQ ID NO: 24 (GGIPIVGIANYA), and CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSYAIS), CDRH2 comprises SEQ ID NO: 24 (GGIPIVGIANYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb12.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSYAIS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 comprises SEQ ID NO: 25 (ARLKGEFKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSYAIS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 25 (ARLKGEFKDAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb13.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSYAIS), CDRH2 comprises SEQ ID NO: 26 (GRIIPLFGTAHYA), and CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSYAIS), CDRH2 comprises SEQ ID NO: 26 (GRIIPLFGTAHYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb14.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSYAIS), CDRH2 comprises SEQ ID NO: 27

(GRINPILGTANYA), and CDRH3 comprises SEQ ID NO: 28 (ARLKGELKDAFSI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSY AIS), CDRH2 comprises SEQ ID NO: 27 (GRINPILGTANYA), CDRH3 comprises SEQ ID NO: 28 (ARLKGELKDAFSI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb15.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 29 (GRIIPFGTADYA), and CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 29 (GRIIPFGTADYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb16.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSY AIS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 comprises SEQ ID NO: 30 (ARLKGELKCAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 23 (GTFSSY AIS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 30 (ARLKGELKCAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT).

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 122 (GTKSSY AIS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 comprises SEQ ID NO: 30 (ARLKGELKCAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 122 (GTKSSY AIS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 30 (ARLKGELKCAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb17.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 31 (GGIIPILGTATYA), and CDRH3 comprises SEQ ID NO: 32 (ARRKGELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises
 5 SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 31 (GGIIPILGTATYA), CDRH3 comprises SEQ ID NO: 32 (ARRKGELKDAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb18.

10 In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 33 (GGIPIVATANYA), and CDRH3 comprises SEQ ID NO: 32 (ARRKGELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 33 (GGIPIVATANYA),
 15 CDRH3 comprises SEQ ID NO: 32 (ARRKGELKDAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb19.

In some embodiments of these aspects and all such aspects described herein, CDRH1
 20 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 34 (GGIPIFGKATYA), and CDRH3 comprises SEQ ID NO: 32 (ARRKGELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 34 (GGIPIFGKATYA), CDRH3 comprises SEQ ID NO: 32 (ARRKGELKDAFDI), CDRL1 comprises SEQ ID NO:
 25 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb20.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 36 (GPFRSHAVS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), and CDRH3 comprises SEQ ID NO: 37 (ARLKSELKDAFDI). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 36 (GPFRSHAVS), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 37 (ARLKSELKDAFDI), CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises

SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb21.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 38 (FQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb22.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 39 (QQSYSTILT). A representative antibody having such heavy and light chain variable CDR regions is mAb23.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 1 (GTFSSYAIN), CDRH2 comprises SEQ ID NO: 11 (GGIIPVFGTATYA), CDRH3 comprises SEQ ID NO: 8 (ARLKGELKDAFDI); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb24.

In each case, where specific sequences are recited, embodiments comprising a sequence having at least 90% (*e.g.*, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) identity to the recited sequence (*e.g.*, SEQ ID NO: 1-34 and 36-39) are also provided.

The disclosure also provides, in some aspects, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises a heavy chain variable region comprising an amino acid sequence that is at least 90% identical (*e.g.*, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58, and a light chain variable region comprising an amino acid sequence that is at least 90% identical (*e.g.*, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 59, 60, 61, 62, or 63.

The disclosure also provides, in some aspects, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises a heavy chain variable region comprising an amino acid sequence that is at least 90% identical (*e.g.*, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58 and a light chain variable region comprising an amino acid sequence that is at least 90% identical (*e.g.*, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 59.

The disclosure also provides, in some aspects, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises a heavy chain variable region comprising an amino acid sequence that is at least 90% identical (*e.g.*, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 35 and a light chain variable region comprising an amino acid sequence that is at least 90% identical (*e.g.*, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 59, 60, 61, 62, or 63.

Antibodies mAb1-mAb23 are affinity matured antibodies derived from parent antibody mAb24, as described in the Examples. An affinity matured antibody or antigen-binding portion thereof is an antibody or antigen-binding fragment with one or more alterations (*e.g.*, in one or more CDRs or FRs) that result in an improvement in the affinity of an antibody for its antigen, compared to a parent antibody lacking the alteration(s). In some embodiments, an affinity matured antibody has nanomolar or picomolar affinity for PD-L1. In some embodiments, the PD-L1 antibody or antigen-binding portion thereof has a K_D of at least 1×10^{-7} M, at least 1×10^{-8} M, at least 1×10^{-9} M, at least 1×10^{-10} M, at least 1×10^{-11} M, at least 1×10^{-12} M, or at least 1×10^{-13} M.

Table 1 shows the binding affinities (K_D) of mAb1, mAb2, mAb3, mAb4, mAb5, mAb6, mAb7, mAb8, mAb9, mAb10, mAb11, mAb12, mAb13, mAb14, mAb15, mAb16, mAb17, mAb18, mAb19, mAb20, mAb21, mAb22, and mAb23 (*i.e.*, affinity matured variants of mAb24) to human PD-L1. The term K_D , as used herein, refers to the dissociation equilibrium constant of a particular antibody-antigen interaction. $K_D = k_d/k_a$. The term k_d (sec^{-1}), as used herein, refers to the dissociation rate constant of a particular antibody-antigen interaction. The value is also referred to as the k_{off} value. The term k_a ($\text{M}^{-1} \times \text{sec}^{-1}$), as used

herein, refers to the association rate constant of a particular antibody-antigen interaction. The value is also referred to as the k_{on} value.

Table 1. Binding affinities of mAb1 – mAb23 to human PD-L1

mAb	K_D (nM)	Fold Improvement
1	4.16	1.1
2	2.2	2.0
3	0.9	4.8
4	4.9	0.9
5	27.1	0.2
6	35.9	0.1
7	--	--
8	3.7	1.2
9	5.4	0.8
10	6.3	0.7
11	156	--
12	358	--
13	52.3	0.1
14	--	--
15	--	--
16	--	--
17	--	--
18	527	--
19	817	--
20	53.9	0.1
21	91.5	0.1
22	2.5	1.8
23	369	--

5

Table 2 provides cell binding data for mAb1, mAb2, mAb3, mAb4, mAb5, mAb6, mAb7, mAb8, mAb9, mAb10, mAb11, mAb12, mAb13, mAb14, mAb15, mAb16, mAb17, mAb18, mAb19, mAb20, mAb21, mAb22, and mAb23 (*i.e.*, affinity matured variants of mAb24) to human PD-L1 ("huPDL1"), cyno PD-L1, or murine PD-L1 ("muPDL1"). Human

10 or cyno PD-L1 was expressed on HEK cells; murine PD-L1 was expressed on A20 cells. Binding is expressed as an EC_{50} value, which can be estimated from titrating different concentrations of mAb on cells that exogenously express the antigen of interest. Fluorescent tagged secondaries can be used to detect and quantify the mAb binding. The data shown in Table 2 was fit to a 1:1 binding model using built-in functions in GRAPHPAD, which
 15 yielded the EC_{50} value.

Table 2. Cell binding of mAb1 – mAb23 to human, cyno, and murine PD-L1 expressed on cells

mAb	huPDL1 EC ₅₀ (nM)	Fold Improvement	cynoPDL1 EC ₅₀ (nM)	Fold Improvement	muPDL1 EC ₅₀ (nM)	Fold Improvement
1	0.04	3.3	0.09	3.21	0.74	5.26
2	0.06	2.2	0.08	3.76	2.00	1.96
3	0.11	1.3	ND	--	1.27	3.08
4	0.04	3.3	0.07	4.10	NB	--
5	0.09	1.5	0.39	0.74	NB	--
6	0.13	1.1	0.47	0.61	NB	--
7	ND	--	NB	--	NB	--
8	ND	--	0.08	3.42	NB	--
9	0.06	2.5	0.07	4.15	NB	--
10	0.04	3.2	0.09	3.05	NB	--
11	1.37	0.1	NB	--	NB	--
12	1.23	0.1	NB	--	NB	--
13	1.43	1.0	ND	--	NB	--
14	1.54	0.1	NB	--	NB	--
15	ND	--	NB	--	NB	--
16	NB	--	NB	--	NB	--
17	0.76	0.2	NB	--	NB	--
18	0.38	0.4	0.06	4.96	0.84	4.67
19	1.20	0.1	0.26	1.08	2.84	1.38
20	0.13	1.0	0.12	2.31	2.66	1.47
21	0.14	1.0	NB	--	NB	--
22	0.18	0.8	0.32	0.89	NB	--
23	0.83	0.2	NB	--	NB	--

5 *NB = no binding; ND = not determined; human and cyno PDL1 expressed on HEK cells; murine PDL1 expressed on A20 cells

The disclosure also provides an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises

10 a heavy chain variable region comprising an amino acid sequence that is at least 90% identical (for example, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical) to any one of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58,

and a light chain variable region comprising an amino acid sequence that is at least 90%

15 identical (for example, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical) to any one of

SEQ ID NO: 59, 60, 61, 62, or 63. In some embodiments, the heavy chain variable region comprises an amino acid sequence that differs by 15 amino acids or less, 14 amino acids or less, 13 amino acids or less, 12 amino acids or less, 11 amino acids or less, 10 amino acids or less, 9 amino acids or less, 8 amino acids or less, 7 amino acids or less, 6 amino acids or less, 5 amino acids or less, 4 amino acids or less, 3 amino acids or less, 2 amino acids or less, or 1 amino acid from any one of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58. In some embodiments, the light chain variable region comprises an amino acid sequence that differs by 15 amino acids or less, 14 amino acids or less, 13 amino acids or less, 12 amino acids or less, 11 amino acids or less, 10 amino acids or less, 9 amino acids or less, 8 amino acids or less, 7 amino acids or less, 6 amino acids or less, 5 amino acids or less, 4 amino acids or less, 3 amino acids or less, 2 amino acids or less, or 1 amino acid from any one of SEQ ID NO: 59, 60, 61, 62, or 63. Tables 3 and 4 provide the sequences for heavy chain variable sequences SEQ ID Nos: 35 and 40-58, and light chain variable sequences SEQ ID Nos: 59-63, respectively.

Table 3. Heavy chain anti-PD-L1 variable sequences

SEQ ID NO	Sequence
35	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
40	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAFSWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
41	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
42	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
43	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
44	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
45	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
46	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
47	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
48	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
49	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS
50	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGGGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARLKGELKDAFDIWGQGTMLTVSS

SEQ ID NO	Sequence
51	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAISWVRQAPGQGLEWMGRINPILGTANY AQKFQGRVTITADESTSTAYMELSSLRSED TAVYYCARLKGELKDAF DIWGQGTILVTVSS
52	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGQGLEWMGRIIPVFGTADY AQKFQGRVTITADESTSTAYMELSSLRSED TAVYYCARLKGELKDAF DIWGQGTILVTVSS
53	QVQLVQSGAEVKKPGSSVKVSKASGGKTFSSYAISWVRQAPGQGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSED TAVYYCARLKGELKDAF DIWGQGTILVTVSS
54	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGQGLEWMGGIIPILGTATY AQKFQGRVTITADESTSTAYMELSSLRSED TAVYYCARRKGELKDAF DIWGQGTILVTVSS
55	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGQGLEWMGGIIPILGAATY AQKFQGRVTITADESTSTAYMELSSLRSED TAVYYCARLKGELKDAF DIWGQGTILVTVSS
56	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGQGLEWMGGIIPIVATANY AQKFQGRVTITADESTSTAYMELSSLRSED TAVYYCARRKGELKDAF DIWGQGTILVTVSS
57	QVQLVQSGAEVKKPGSSVKVSKASGGTFSSYAINWVRQAPGQGLEWMGGIIPVFGKATY AQKFQGRVTITADESTSTAYMELSSLRSED TAVYYCARRKGELKDAF DIWGQGTILVTVSS
58	QVQLVQSGAEVKKPGSSVKVSKASGGPFSSHAVSWVRQAPGQGLEWMGGIIPVFGTATY AQKFQGRVTITADESTSTAYMELSSLRSED TAVYYCARLSELKDAF DIWGQGTILVTVSS

Table 4. Light chain variable sequences

SEQ ID NO	Sequence
59	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSL QSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGKVEIK
60	DIQMTQSPSSLSASVGDRVTITCRASQWISSYLNWYQQKPGKAPKLLIYAASSL QSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGKVEIK
61	DIQMTQSPSSLSASVGDRVTITCRASQQISSYLNWYQQKPGKAPKLLIYAASSL QSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGKVEIK
62	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSL QSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCFQSYSTPLTFGGGKVEIK
63	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSL QSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTILTFGGGKVEIK

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58, and light chain CDRs of any of the light chain variable regions of SEQ ID NOs: 59, 60, 61, 62, or 63.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58,

and light chain CDRs of any of the light chain variable regions of SEQ ID NOs: 59, 60, 61, 62, or 63, wherein the heavy and light chain CDR residues are numbered according to Kabat.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58, and light chain CDRs of any of the light chain variable regions of SEQ ID NOs: 59, 60, 61, 62, or 63, wherein the heavy and light chain CDR residues are numbered according to Chothia.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58, and light chain CDRs of any of the light chain variable regions of SEQ ID NOs: 59, 60, 61, 62, or 63, wherein the heavy and light chain CDR residues are numbered according to MacCallum.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58, and light chain CDRs of any of the light chain variable regions of SEQ ID NOs: 59, 60, 61, 62, or 63, wherein the heavy and light chain CDR residues are numbered according to AbM.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58, and light chain CDRs of any of the light chain variable regions of SEQ ID NOs: 59, 60, 61, 62, or 63, wherein the heavy and light chain CDR residues are numbered according to IMGT.

B. PD-1 Antagonists

In some aspects and embodiments, the disclosure provides for anti-PD-1 antagonists. In some embodiments, the anti-PD-1 antagonist is any of the anti-PD-1 antibodies or antigen-binding molecules disclosed herein. In some embodiments, the anti-PD-1 antibody or antigen-binding molecule is not a part of a multispecific antigen-binding construct, *i.e.*, the anti-PD-1 antibody or antigen-binding molecule is not a part of a protein construct that binds

to multiple epitopes. In some embodiments, the anti-PD-1 antibody or antigen-binding portion can be combined with a different antibody or antigen-binding portion to form a multispecific antigen-binding construct. In some embodiments, the multispecific antigen-binding construct is capable of binding an epitope on PD-1 and an epitope on another protein.

5 In some embodiments, the epitope on the other protein is on PD-L1.

In some embodiments, any of the multispecific antigen-binding constructs disclosed herein comprises a PD-1 antagonist. In some embodiments, the PD-1 antagonist is an “inhibitory receptor.” As used herein, an “inhibitory receptor” refers generally to an immune checkpoint molecule that, when bound by a cognate ligand, causes suppression or inhibition of an immune response, such as those known to enhance tumor evasion. However, in some instances as used herein, “inhibitory receptor” refers specifically to PD-1.

PD-1 is an immune checkpoint inhibitory receptor that contains an “Immunoreceptor Tyrosine-based Inhibition Motif” or “ITIM”, comprising a conserved sequence of amino acids (S/I/V/L)xYxx(I/V/L) where x is any amino acid. Methods for assaying whether PD-1 activity has been inhibited are known in the art and can be readily designed by those of skill in the art. Such assays include, for example, testing the effects of any downstream signaling pathway(s) of PD-1 *in vitro* or *in vivo*. After PD-1 interacts with its ligand, the ITIM motif becomes phosphorylated by enzymes of, *e.g.*, the Src kinase family, allowing them to recruit other enzymes, *e.g.*, the phosphotyrosine phosphatases SHP-1 and SHP-2, or the inositol-phosphatase called SHIP. These phosphatases have been shown to decrease the activation of molecules involved in cell signaling. See, *e.g.*, Barrow & Trowsdale (2006) Eur J Immunol. 36 (7): 1646–53. Thus, the phosphorylation state of the ITIM motif within PD-1 can be assessed using known methods in the art. Also, the presence of downstream factors, such as phosphotyrosine phosphatases can also be examined. Moreover, various cell-based assays and kits that detect the presence of downstream factors (*e.g.*, nuclear factor of activated T-cells – NFAT – as a measure of PD-1 inhibition) as a proxy for PD-1 activity state are known in the art. In other examples, simple binding assays can be used to determine whether the construct of the present disclosure can block binding PD-1 and its ligand, as discussed above.

Accordingly, in some aspects, provided herein are antibodies or antigen-binding portions thereof that specifically bind PD-1. In some aspects, the antibody or antigen-binding portion thereof that specifically binds PD-1 comprises (a) a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 70 (FTFX₁X₂YAX₃X₄, wherein X₁=S, R, G, or N; X₂=D, S, N, A, R, or G; X₃= M or L; X₄= S, L, or N); (ii) a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA); and (iii) a CDRH3 comprising SEQ ID NO: 72

(ARGLDFIVGX₅TGNDY, wherein X₅=A, Y, or R); and (b) a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

5 In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 73 (FTFSDYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 74 (ARGLDFIVGATGNDY). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 73 (FTFSDYAMS); CDRH2 comprises SEQ ID NO: 71
10 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 74 (ARGLDFIVGATGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb25.

In some embodiments of these aspects and all such aspects described herein, CDRH1
15 comprises SEQ ID NO: 73 (FTFSDYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 73 (FTFSDYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
20 CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb26.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 76 (FTFSSYAMS), CDRH2 comprises SEQ ID NO: 71
25 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 76 (FTFSSYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
30 CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb27.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 77 (FTFSSYAML), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY).

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 77 (FTFSSYAML), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb28.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 78 (FTFSNYALS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY).

10 In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 78 (FTFSNYALS); CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb29.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 79 (FTFSAYAMN), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY).

20 In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 79 (FTFSAYAMN); CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb30.

25 In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 80 (FTFRSYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY).

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 80 (FTFRSYAMS); CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb31.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 81 (FTFGRYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY).

In some embodiments of these aspects and all such aspects described herein, CDRH1
 5 comprises SEQ ID NO: 81 (FTFGRYAMS); CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb32.

10 In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 82 (FTFNSYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY).

In some embodiments of these aspects and all such aspects described herein, CDRH1
 15 comprises SEQ ID NO: 82 (FTFNSYAMS); CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 75 (ARGLDFIVGYTGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb33.

In some embodiments of these aspects and all such aspects described herein, CDRH1
 20 comprises SEQ ID NO: 83 (FTFSNYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 74 (ARGLDFIVGATGNDY).

In some embodiments of these aspects and all such aspects described herein, CDRH1
 comprises SEQ ID NO: 83 (FTFSNYAMS); CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 74 (ARGLDFIVGATGNDY);
 25 CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb34.

In some embodiments of these aspects and all such aspects described herein, CDRH1
 comprises SEQ ID NO: 84 (FTFSGYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 85 (ARGLDFIVGRTGNDY). In
 30 some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 84 (FTFSGYAMS); CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 85 (ARGLDFIVGRTGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3

comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb35.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 86 (FTFSSYAMN), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 85 (ARGLDFIVGRTGNDY). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 86 (FTFSSYAMN); CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 85 (ARGLDFIVGRTGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb36.

In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 80 (FTFRSYAMS), CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA) and CDRH3 comprises SEQ ID NO: 85 (ARGLDFIVGRTGNDY). In some embodiments of these aspects and all such aspects described herein, CDRH1 comprises SEQ ID NO: 80 (FTFRSYAMS); CDRH2 comprises SEQ ID NO: 71 (SAISNSGTYTYYA); CDRH3 comprises SEQ ID NO: 85 (ARGLDFIVGRTGNDY); CDRL1 comprises SEQ ID NO: 9 (RASQSISSYLN); CDRL2 comprises SEQ ID NO: 5 (AASSLQS); and CDRL3 comprises SEQ ID NO: 10 (QQSYSTPLT). A representative antibody having such heavy and light chain variable CDR regions is mAb37.

In each case, where specific sequences are recited, embodiments comprising a sequence having at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) identity to the recited sequence (*e.g.*, SEQ ID NO: 5, 9, 10, or 71-86) are also provided.

The disclosure also provides, in some aspects, an antibody or antigen-binding portion thereof that specifically binds PD-1, wherein the antibody or antigen-binding portion thereof comprises a heavy chain variable region comprising an amino acid sequence that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to any one of SEQ ID NO: 87-99, and a light chain variable region comprising an amino acid sequence that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 59. In some embodiments, the heavy chain variable region comprises an amino

acid sequence that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 90, and a light chain variable region comprising an amino acid sequence that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 59. Table 7 provides the sequences for heavy chain variable sequences SEQ ID Nos: 87-99, and Table 4 provides light chain variable sequence SEQ ID NO: 59, respectively. In some embodiments, the heavy chain variable region comprises an amino acid sequence that differs by 15 amino acids or less, 14 amino acids or less, 13 amino acids or less, 12 amino acids or less, 11 amino acids or less, 10 amino acids or less, 9 amino acids or less, 8 amino acids or less, 7 amino acids or less, 6 amino acids or less, 5 amino acids or less, 4 amino acids or less, 3 amino acids or less, 2 amino acids or less, or 1 amino acid from any one of SEQ ID NOs: 87-99. In some embodiments, the light chain variable region comprises an amino acid sequence that differs by 15 amino acids or less, 14 amino acids or less, 13 amino acids or less, 12 amino acids or less, 11 amino acids or less, 10 amino acids or less, 9 amino acids or less, 8 amino acids or less, 7 amino acids or less, 6 amino acids or less, 5 amino acids or less, 4 amino acids or less, 3 amino acids or less, 2 amino acids or less, or 1 amino acid from SEQ ID NO: 59.

Antibodies mAb26-mAb37 are affinity matured antibodies derived from parent antibody mAb25, as described in the Examples. An affinity matured antibody or antigen-binding portion thereof is an antibody or antigen-binding fragment with one or more alterations (*e.g.*, in one or more CDRs or FRs) that result in an improvement in the affinity of an antibody for its antigen, compared to a parent antibody lacking the alteration(s). In some embodiments, an affinity matured antibody has nanomolar or picomolar affinity for PD-1. In some embodiments, the PD-1 antibody or antigen-binding portion thereof has a K_D of at least 1×10^{-7} M, at least 1×10^{-8} M, at least 1×10^{-9} M, at least 1×10^{-10} M, at least 1×10^{-11} M, at least 1×10^{-12} M, or at least 1×10^{-13} M.

Tables 5 and 6 provide cell binding data for mAb25, mAb26, mAb27, mAb28, mAb29, mAb30, mAb31, mAb32, mAb33, mAb34, mAb35, mAb36, and mAb37 (*i.e.*, affinity matured variants of mAb25) to human PD-1 ("huPD-1"), cyno PD-1 ("cyPD-1"), or murine PD-1 ("muPD-1"). Human, cyno, or murine PD-1 was expressed on CHO cells. Binding is expressed as an EC_{50} value, which can be estimated from titrating different concentrations of mAb on cells that exogenously express the antigen of interest. Fluorescent tagged secondaries can be used to detect and quantify the mAb binding. The data shown in

Table 5 was fit to a 1:1 binding model using built-in functions in GRAPHPAD, which yielded the EC₅₀ value.

Table 5: Binding affinities of mAb25 – mAb37 to human PD-1

mAb	K _D (nM)	Fold improvement over Parent
mAb25	240	
mAb26	10	24
mAb27	2.3	104
mAb28	5.6	43
mAb29	8.6	28
mAb30	3.1	77
mAb31	5.5	44
mAb32	2.1	114
mAb33	7	34
mAb34	7.2	33
mAb35	9	27
mAb36	7.7	31
mAb37	14	17

5

Table 6: Cell binding of mAb25 – mAb37 to human, cyno, and murine PD-1 expressed on cells

mAb	huPD-1 EC ₅₀ (nM)	Fold improvement over Parent	cynoPD-1 EC ₅₀ (nM)	Fold improvement over Parent	muPD-1 EC ₅₀ (nM)
mAb25	0.915		8.59		NB
mAb26	3.74	0.2	1.54	5.6	17.9
mAb27	1.75	0.5	1.53	5.6	6.14
mAb28	3.94	0.2	1.48	5.8	0.814
mAb29	1.27	0.7	1.73	5.0	2.14
mAb30	2.77	0.3	2.05	4.2	4.05
mAb31	1.71	0.5	1.9	4.5	1.87

mAb32	1.51	0.6	1.29	6.7	2.8
mAb33	1.04	0.9	1.33	6.5	10.4
mAb34	3.87	0.2	1.33	6.5	20.5
mAb35	1.64	0.6	2.66	3.2	PF
mAb36	1.01	0.9	1.19	7.2	PF
mAb37	0.75	1.2	1.09	7.9	0.942

*NB = no binding; PF = poor fit; human, cyno, murine PD-1 expressed on CHO cells

Table 7. Heavy chain anti-PD-1 variable sequences

SEQ ID NO	Sequence
87	EVQLLESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGATGNDYWGQGTLLTVSS
88	EVQLLESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTLLTVSS
89	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTLLTVSS
90	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMLWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTLLTVSS
91	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSNYALSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTLLTVSS
92	EVQLLESGGGLVQPGGSLRLSCAASGFTFSAYAMNVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTLLTVSS
93	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSYAMSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTLLTVSS
94	EVQLLESGGGLVQPGGSLRLSCAASGFTFGRYAMSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTLLTVSS
95	EVQLLESGGGLVQPGGSLRLSCAASGFTFNSYAMSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTLLTVSS
96	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSNYAMSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGATGNDYWGQGTLLTVSS
97	EVQLLESGGGLVQPGGSLRLSCAASGFTFSGYAMSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGRTGNDYWGQGTLLTVSS
98	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMNVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGRTGNDYWGQGTLLTVSS
99	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSYAMSWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGRTGNDYWGQGTLLTVSS

5 The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99, and light chain CDRs of the light chain variable region of SEQ ID NO: 59.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99, and light chain CDRs of the light chain variable region of SEQ ID NO: 59, wherein the heavy and light chain CDR residues are numbered according to Kabat.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99, and light chain CDRs of the light chain variable region of SEQ ID NO: 59, wherein the heavy and light chain CDR residues are numbered according to Chothia.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99, and light chain CDRs of the light chain variable region of SEQ ID NO: 59, wherein the heavy and light chain CDR residues are numbered according to MacCallum.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99, and light chain CDRs of the light chain variable region of SEQ ID NO: 59, wherein the heavy and light chain CDR residues are numbered according to AbM.

The disclosure also provides, in some embodiments, an antibody or antigen-binding portion thereof that specifically binds PD-L1, wherein the antibody or antigen-binding portion thereof comprises heavy chain CDRs of any of the heavy chain variable regions of SEQ ID NOs: 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99, and light chain CDRs of the light chain variable region of SEQ ID NO: 59, wherein the heavy and light chain CDR residues are numbered according to IMGT.

C. Multispecific Antigen-Binding Constructs

The present disclosure provides, in some aspects, compositions and methods for enhancing an immune response to tumor cells by inhibiting the interaction between PD-L1 and PD-1, for example, the interaction between PD-L1 expressed on a tumor cell and PD-1

expressed on a T cell. Antibodies or antigen-binding portions thereof that specifically or selectively bind PD-L1 or PD-1 are provided. As used herein, the terms “specifically binds to,” “specific for,” “selectively binds” and “selective for” PD-L1 or PD-1, or an epitope on PD-L1 or PD-1, mean binding that is measurably different from a non-specific or non-selective interaction. In some embodiments, the antibody or antigen-binding portion thereof specifically binds to human PD-L1 or PD-1 and/or mouse PD-L1 or PD-1. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule. Specific binding can also be determined by competition with a control molecule that is similar to the target, such as an excess of non-labeled target. In that case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by the excess non-labeled target.

In some embodiments, any of the multispecific antigen-binding constructs disclosed herein binds to at least two different receptors or epitopes (*e.g.*, PD-1 and PD-L1), wherein the two different receptors or epitopes bound by the multispecific antigen-binding construct are expressed on the surface of the same cell. For example, in some embodiments, the multispecific antigen-binding construct simultaneously binds to PD-1 and PD-L1, wherein the PD-1 and PD-L1 are expressed on the surface of the same cell. In some embodiments, any of the multispecific antigen-binding constructs disclosed herein binds to at least two different receptors or epitopes (*e.g.*, PD-1 and PD-L1), wherein the two different receptors or epitopes bound by the multispecific antigen-binding construct are expressed on the surface of two different cells. For example, in some embodiments, the multispecific antigen-binding construct simultaneously binds to PD-1 expressed on the surface of a first cell and to PD-L1 expressed on the surface of a second cell.

In some embodiments, the multispecific antigen-binding construct is capable of binding human PD-1. In some embodiments, the multispecific antigen-binding construct is capable of binding murine PD-1. In some embodiments, the multispecific antigen-binding construct is capable of binding cynomolgus monkey PD-1. In some embodiments, the multispecific antigen-binding construct is capable of binding human, murine, and cynomolgus monkey PD-1 with similar affinity.

In some aspects and embodiments, the disclosure provides for a multispecific antigen-binding construct comprising at least two units of antigen-binding, wherein a first unit of antigen-binding binds PD-1, and a second unit of antigen-binding binds a PD-1 ligand. In some embodiments, the first unit of antigen-binding binds PD-1 expressed by an immune cell. In some embodiments, the second unit of antigen-binding binds PD-1 expressed by a

second cell. In some embodiments, the multispecific antigen-binding construct blocks the interaction of PD-1 and a PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments, the multispecific antigen-binding construct blocks the interaction of PD-1 and a PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments, the multispecific antigen-binding construct comprises at least two units of antigen-binding that bind PD-1. In some embodiments, the multispecific antigen-binding construct comprises two units of antigen-binding that bind PD-1. In some embodiments, the multispecific antigen-binding construct comprises at least two units of antigen-binding that bind a PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments, the multispecific antigen-binding construct comprises two units of antigen-binding that bind a PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments, the multispecific antigen-binding construct comprises at least four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind a PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments, the multispecific antigen-binding construct comprises four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind a PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments, each unit of antigen-binding is capable of binding independently to its cognate antigen, *i.e.*, PD-1 or a PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments, the multispecific antigen-binding construct promotes loss of PD-1 expression from a cell. In some embodiments, the loss of PD-1 expression is due to PD-1 shedding. In some embodiments, the multispecific antigen-binding construct blocks interaction of PD-1 and the PD-1 ligand, such as PD-L1 or PD-L2. In some embodiments, the multispecific antigen-binding construct comprises a common light chain. For example, at least two units of antigen-binding comprise a common light chain.

In some embodiments, the first unit of antigen-binding binds PD-1 and comprises:

(a) a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 70 (FTFX₁X₂YAX₃X₄, wherein X₁=S, R, G, or N; X₂=D, S, N, A, R, or G; X₃=M or L; X₄=S, L, or N); (ii) a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA); and (iii) a CDRH3 comprising SEQ ID NO: 72 (ARGLDFIVGX₅TGNDY, wherein X₅=A, Y, or R); and

(b) a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some such embodiments, the first unit of antigen-binding binds PD-1 and comprises:

- (a) a CDRH1 comprising SEQ ID NO: 73 (FTFSDYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 74 (ARGLDFIVGATGNDY);
- 5 (b) a CDRH1 comprising SEQ ID NO: 73 (FTFSDYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- (c) a CDRH1 comprising SEQ ID NO: 76 (FTFSSYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- 10 (d) a CDRH1 comprising SEQ ID NO: 77 (FTFSSYAML), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- (e) a CDRH1 comprising SEQ ID NO: 78 (FTFSNYALS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- 15 (f) a CDRH1 comprising SEQ ID NO: 79 (FTFSAYAMN), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- (g) a CDRH1 comprising SEQ ID NO: 80 (FTFRSYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- 20 (h) a CDRH1 comprising SEQ ID NO: 81 (FTFGRYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- 25 (i) a CDRH1 comprising SEQ ID NO: 82 (FTFN SYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 75 (ARGLDFIVGYTGNDY);
- (j) a CDRH1 comprising SEQ ID NO: 83 (FTFSNYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 74 (ARGLDFIVGATGNDY);
- 30 (k) a CDRH1 comprising SEQ ID NO: 84 (FTFSGYAMS), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 85 (ARGLDFIVGRTGNDY);

(l) a CDRH1 comprising SEQ ID NO: 86 (FTFSSYAMN), a CDRH2 comprising SEQ ID NO: 71 (SAISNSGTYTYYA) and a CDRH3 comprising SEQ ID NO: 85 (ARGLDFIVGRTGNDY); or

5 (m) a CDRH1 comprising SEQ ID NO: 80 (FTFRSYAMS), a comprising SEQ ID NO: 71 (SAISNSGTYTYYA), and a CDRH3 comprising SEQ ID NO: 85 (ARGLDFIVGRTGNDY).

In some embodiments, the first unit of antigen-binding binds PD-1 and comprises:

- (a) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 87;
- 10 (b) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 88;
- (c) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 89;
- (d) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 90;
- 15 (e) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 91;
- (f) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 92;
- 20 (g) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 93;
- (h) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 94;
- (i) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 95;
- 25 (j) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 96;
- (k) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 97;
- 30 (l) a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 98; or
- (m) a heavy chain variable region comprising amino acid sequence that is at least 90% identical to SEQ ID NO: 99.

In some embodiments, the first unit of antigen-binding binds PD-1 and comprises a light chain variable region comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 59.

In some embodiments, the second unit of antigen-binding binds PD-L2. In some
5 embodiments, the second unit of antigen-binding binds PD-L1. In some embodiments, the second unit of antigen-binding binds PD-L1 and comprises:

- a. a heavy chain variable region comprising (i) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN); (ii) a CDRH2 comprising SEQ ID NO: 2 (GGIIPX₁X₂GX₃ATYA, wherein X₁ is V or I; X₂ is F, L, or V; and X₃ is T or A); and (iii) a CDRH3
10 comprising SEQ ID NO: 3 (ARLKX₁ELKDAFDI, wherein X₁ is G, F, or N); and
- b. a light chain variable region comprising: (i) a CDRL1 comprising SEQ ID NO: 4 (RASQX₁ISSYLN, wherein X₁ is S, W, or Q); (ii) a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and (iii) a CDRL3 comprising SEQ ID NO: 6 (X₁QSYSTPLT, wherein X₁ is Q or F).

15 In some such embodiments, the second unit of antigen-binding binds PD-L1 and comprises:

- (a) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 7 (GGIIPILGAATYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);
- 20 (b) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 7 (GGIIPILGAATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- 25 (c) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- 30 (d) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA) and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);
- (e) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8

- (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 12 (RASQWISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- (f) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 13 (RASQISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- (g) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- (h) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 15 (GGIPIFGIANYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);
- (i) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 15 (GGIPIFGIANYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- (j) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 16 (GGIIPNFGTATYA), and a CDRH3 comprising SEQ ID NO: 17 (ARLKGELKGAGDI);
- (k) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 16 (GGIIPNFGTATYA), a CDRH3 comprising SEQ ID NO: 17 (ARLKGELKGAGDI); a CDRL1 comprising SEQ ID NO: 9 (RASQISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- (l) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 18 (ARLKFELKDAFDI);
- (m) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 18

- (ARLKFELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 5 (n) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 19 (ARLKGELKDAFDE);
- (o) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 19 (ARLKGELKDAFDE), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN);
- 10 a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- (p) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 20 (ARLKNELKDAFDI);
- 15 (q) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 20 (ARLKNELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- 20 (r) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 21 (GGVIPFLGTANYA), and a CDRH3 comprising SEQ ID NO: 22 (ARLKGILKDALDI);
- (s) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 21 (GGVIPFLGTANYA), a CDRH3 comprising SEQ ID NO: 22 (ARLKGILKDALDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a
- 25 CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- (t) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising EQ ID NO: 29 (GRIIPIFGTADYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);
- 30 (u) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 29 (GRIIPIFGTADYA), a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a

- CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- (v) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 31 (GGIIPILGTATYA), and a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI);
- (w) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 31 (GGIIPILGTATYA), a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- (x) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 33 (GGIPIVATANYA), and a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI);
- (y) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 33 (GGIPIVATANYA), a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- (z) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 34 (GGIPIFGKATYA), and a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI);
- (aa) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 34 (GGIPIFGKATYA), a CDRH3 comprising SEQ ID NO: 32 (ARRKGELKDAFDI), a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);
- (bb) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARRKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 38 (FQSYSTPLT);
- (cc) a CDRH1 comprising SEQ ID NO: 1 (GTFSSYAIN), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), a CDRH3 comprising SEQ ID NO: 8 (ARRKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a

CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 39 (QSYSTILT);

(dd) a CDRH1 comprising SEQ ID NO: 14 (GTFSSYAFS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA) and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);

(ee) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), a CDRH2 comprising SEQ ID NO: 24 (GGIPIVGIANYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);

(ff) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), a CDRH2 comprising SEQ ID NO: 24 (GGIPIVGIANYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);

(gg) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 25 (ARLKGELKDAFDI);

(hh) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 25 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);

(ii) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), a CDRH2 comprising SEQ ID NO: 26 (GRIIPLFGTAHYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI);

(jj) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), a CDRH2 comprising SEQ ID NO: 26 (GRIIPLFGTAHYA), and a CDRH3 comprising SEQ ID NO: 8 (ARLKGELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QSYSTPLT);

(kk) a CDRH1 comprising SEQ ID NO: 23 (GTFSSYAIS), a CDRH2 comprising SEQ ID NO: 27 (GRINPILGTANYA), and a CDRH3 comprising SEQ ID NO: 28 (ARLKGELKDAFSI);

- (ll) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 27 (GRINPILGTANYA), and a CDRH3 comprising SEQ ID NO: 28 (ARLKGELKDAFSI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- (mm) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 30 (ARLKGELKCAFDI);
- (nn) a CDRH1 comprising SEQ ID NO: 23 (GTFSSY AIS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a comprising SEQ ID NO: 30 (ARLKGELKCAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT);
- (oo) a CDRH1 comprising SEQ ID NO: 36 (GPFRSHAVS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 37 (ARLKSELKDAFDI); or
- (pp) a CDRH1 comprising SEQ ID NO: 36 (GPFRSHAVS), a CDRH2 comprising SEQ ID NO: 11 (GGIIPVFGTATYA), and a CDRH3 comprising SEQ ID NO: 37 (ARLKSELKDAFDI); a CDRL1 comprising SEQ ID NO: 9 (RASQSISSYLN); a CDRL2 comprising SEQ ID NO: 5 (AASSLQS); and a CDRL3 comprising SEQ ID NO: 10 (QQSYSTPLT).

In some such embodiments, the second unit of antigen-binding binds PD-L1 and comprises a heavy chain variable region comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, or 58, and a light chain variable region comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 59, 60, 61, 62, or 63.

In some embodiments, the second unit of antigen-binding binds PD-L1 and comprises a heavy chain variable region comprising:

- (a) an amino acid sequence that is at least 90% identical to SEQ ID NO: 35;
- (b) an amino acid sequence that is at least 90% identical to SEQ ID NO: 40;
- (c) an amino acid sequence that is at least 90% identical to SEQ ID NO: 41;
- (d) an amino acid sequence that is at least 90% identical to SEQ ID NO: 42;
- (e) an amino acid sequence that is at least 90% identical to SEQ ID NO: 43;
- (f) an amino acid sequence that is at least 90% identical to SEQ ID NO: 44;

- (g) an amino acid sequence that is at least 90% identical to SEQ ID NO: 45;
- (h) an amino acid sequence that is at least 90% identical to SEQ ID NO: 46;
- (i) an amino acid sequence that is at least 90% identical to SEQ ID NO: 47;
- (j) an amino acid sequence that is at least 90% identical to SEQ ID NO: 48;
- 5 (k) an amino acid sequence that is at least 90% identical to SEQ ID NO: 49;
- (l) an amino acid sequence that is at least 90% identical to SEQ ID NO: 50;
- (m) an amino acid sequence that is at least 90% identical to SEQ ID NO: 51;
- (n) an amino acid sequence that is at least 90% identical to SEQ ID NO: 52;
- (o) an amino acid sequence that is at least 90% identical to SEQ ID NO: 53;
- 10 (p) an amino acid sequence that is at least 90% identical to SEQ ID NO: 54;
- (q) an amino acid sequence that is at least 90% identical to SEQ ID NO: 55;
- (r) an amino acid sequence that is at least 90% identical to SEQ ID NO: 56;
- (s) an amino acid sequence that is at least 90% identical to SEQ ID NO: 57; or
- (t) an amino acid sequence that is at least 90% identical to SEQ ID NO: 58;
- 15 In some embodiments, the second unit of antigen-binding binds PD-L1 and comprises a light chain variable region comprising:
 - (a) an amino acid sequence that is at least 90% identical to SEQ ID NO: 59;
 - (b) an amino acid sequence that is at least 90% identical to SEQ ID NO: 60;
 - (c) an amino acid sequence that is at least 90% identical to SEQ ID NO: 61;
 - 20 (d) an amino acid sequence that is at least 90% identical to SEQ ID NO: 62; or
 - (e) an amino acid sequence that is at least 90% identical to SEQ ID NO: 63.
- In some embodiments, the disclosure provides for a multispecific antigen-binding construct comprising any of the PD-1 antagonists disclosed herein and any of the antagonists of a PD-1 ligand, such as PD-L1, disclosed herein. For example, Bispecific 3 is a
 - 25 multispecific, tetravalent antigen-binding construct that specifically binds human PD-1 and human PD-L1. The construct comprises an anti-PD-1 IgG1 antibody (mAb28) in which the heavy chain of the antibody is a fusion protein further comprising at its C-terminus the heavy chain variable region of an anti-PD-L1 antibody (mAb1), which is connected to the Fc region of the anti-BCMA antibody by way of a poly-GGGS (SEQ ID NO: 120) linker. The light
 - 30 chains for the anti-PD-1 portion and the anti-PD-L1 portions of the construct are identical (SEQ ID NO: 101). Bispecific 3, the structure for which is represented by the illustration in FIG. 13A, comprises the heavy chain sequence recited in SEQ ID NO: 100 and the light chain sequence recited in SEQ ID NO: 101.

In some embodiments, the disclosure provides for a multispecific antibody or antigen-binding portion thereof that specifically binds PD-1 and PD-L1, wherein the antibody or antigen-binding portion thereof comprises a heavy chain region comprising an amino acid sequence that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 100 or 102 and a light chain region comprising an amino acid sequence that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 101 or 103.

In some embodiments, the disclosure provides for a multispecific antibody or antigen-binding portion thereof that specifically binds PD-1 and PD-L1, wherein the antibody or antigen-binding portion thereof comprises a heavy chain region comprising an amino acid sequence that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 100, and a light chain region comprising an amino acid sequence that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 101. In some embodiments, the heavy chain region comprises an amino acid sequence that differs by 15 amino acids or less, 14 amino acids or less, 13 amino acids or less, 12 amino acids or less, 11 amino acids or less, 10 amino acids or less, 9 amino acids or less, 8 amino acids or less, 7 amino acids or less, 6 amino acids or less, 5 amino acids or less, 4 amino acids or less, 3 amino acids or less, 2 amino acids or less, or 1 amino acid from SEQ ID NO: 100. In some embodiments, the light chain region comprises an amino acid sequence that differs by 15 amino acids or less, 14 amino acids or less, 13 amino acids or less, 12 amino acids or less, 11 amino acids or less, 10 amino acids or less, 9 amino acids or less, 8 amino acids or less, 7 amino acids or less, 6 amino acids or less, 5 amino acids or less, 4 amino acids or less, 3 amino acids or less, 2 amino acids or less, or 1 amino acid from SEQ ID NO: 101.

In some embodiments, the disclosure provides for a multispecific antibody or antigen-binding portion thereof that specifically binds PD-1 and PD-L1, wherein the antibody or antigen-binding portion thereof comprises a heavy chain region comprising an amino acid sequence that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 102, and a light chain region comprising an amino acid sequence

that is at least 85%, (*e.g.*, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical) to SEQ ID NO: 103. In some embodiments, the heavy chain region comprises an amino acid sequence that differs by 15 amino acids or less, 14 amino acids or less, 13 amino acids or less, 12 amino acids or less, 11 amino acids or less, 10 amino acids or less, 9 amino acids or less, 8 amino acids or less, 7 amino acids or less, 6 amino acids or less, 5 amino acids or less, 4 amino acids or less, 3 amino acids or less, 2 amino acids or less, or 1 amino acid from SEQ ID NO: 102. In some embodiments, the light chain region comprises an amino acid sequence that differs by 15 amino acids or less, 14 amino acids or less, 13 amino acids or less, 12 amino acids or less, 11 amino acids or less, 10 amino acids or less, 9 amino acids or less, 8 amino acids or less, 7 amino acids or less, 6 amino acids or less, 5 amino acids or less, 4 amino acids or less, 3 amino acids or less, 2 amino acids or less, or 1 amino acid from SEQ ID NO: 103.

Also provided herein, in some aspects and embodiments, is a multispecific antigen-binding construct comprising four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind PD-L1, and wherein the construct comprises a heavy chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 100 or 102, and a light chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 101 or 103.

Also provided herein, in some aspects and embodiments, is a multispecific antigen-binding construct comprising four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind PD-L1, and wherein the construct comprises a heavy chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 100 and a light chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 101.

Also provided herein, in some aspects and embodiments, is a multispecific antigen-binding construct comprising four units of antigen-binding, wherein two units of antigen-binding bind PD-1 and two units of antigen-binding bind PD-L1, and wherein the construct comprises a heavy chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 102 and a light chain amino acid sequence that is at least 85%, identical to the amino acid sequence of SEQ ID NO: 103.

Table 8. Multispecific Heavy Chain Sequences

SEQ ID NO	Sequence
100	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMLWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTILVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPS VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCVMHEALHNHYTQKSLSLSPGGGGSGGGGSGGGGSGGGGSGVQLVQSGAEVKKPGSSV KVSCASGGTFSSYAINWVRQAPGQGLEWMGGIIPILGAATYAQKFQGRVTITADESTSTA YME LSSLRSED TAVYYCARLKGELKDAFDIWGQGTILVTVSSASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTY ICNVNHKPSNTKVDKKVEPKSCDKTHT
102	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMLWVRQAPGKGLEWVSAISNSGTYTYYA DSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGLDFIVGYTGNDYWGQGTILVTVSS AKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLES DL YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDDKIVPRDCGCKPCICTVPEVSSVFIFPP KPKDVLTTITLTPKVTCTVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFASTFRSVSEL PIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPTPPKEQMAKDKVSLTC MITDFFPEDITVEWQWNGQPAENYKNTQFIMNTNGSYFVYSKLNQKSNWEAGNTFTCSVL HEGLHNHTEKSLSHSPGGGGSGGGGSGGGGSGGGGSGVQLVQSGAEVKKPGSSVKVSCA SGGTFSSYAINWVRQAPGQGLEWMGGIIPILGAATYAQKFQGRVTITADESTSTAYME LSS LRSED TAVYYCARLKGELKDAFDIWGQGTILVTVSSAKTTPPSVYPLAPGSAAQTNSMVTLG CLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLES DLYTLSSSVTVPSSPRPSETVTCNVAHP ASSTKVDDKIVPRDCG

Table 9. Multispecific Light Chain Sequences

SEQ ID NO	Sequence
101	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPKAPKLLIYAASSLQSGVPSR FSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGKVEIKRTVAAPSVFIFPPSD EQLKSGTASVVCLLNFFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
103	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPKAPKLLIYAASSLQSGVPSR FSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGKVEIKRADAAPT VSI FPPSS EQLTSGGASVVCFLNFFYPKDINVKKIDGSE RQNGVLNSWTDQDSKDSSTYSMSSTLT LTK DEYERHNSYTCEATHKTSTSPIVKSFNRNEC

The term "multispecific antigen-binding construct", as used herein refers to bispecific, tri-specific, or multispecific antigen-binding constructs, and antigen-binding portions or fragments thereof. A multispecific antigen-binding construct can be a single multifunctional polypeptide, or it can be a multimeric complex of two or more molecules (*e.g.*, polypeptides and/or aptamers) that are covalently or non-covalently associated with one another. The term "multispecific antigen-binding constructs" includes antibodies (or antigen-binding fragments

thereof) that may be linked to or co-expressed with another functional molecule, *e.g.*, another peptide, protein, and/or aptamer. For example, an antibody or fragment thereof can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, non-covalent association or otherwise) to one or more other molecular entities, such as a protein or fragment thereof to produce a bispecific or a multispecific antigen-binding molecule with a second binding specificity. As used herein, the term "multispecific antigen-binding constructs" also includes bispecific, trispecific or multispecific antibodies or antigen-binding fragments thereof. In certain embodiments, an antibody is functionally linked to another antibody or antigen-binding fragment thereof to produce a bispecific antibody with a second binding specificity. Bispecific and multispecific antibodies of the present invention are described elsewhere herein.

As used herein, an antigen-binding "arm" refers to a unit, domain, region, or the like, of the multispecific antigen-binding construct that forms an area of the construct that binds to an antigen. Thus, a "first arm" forms a separate binding area of the multispecific antigen-binding construct from a "second arm" of the construct, each arm forming a unit of antigen binding. Generally, one "arm" (first arm) is distinct from the other "arm" (second arm) in its antigen binding or antigen specificity. Thus, in the example of a bispecific, bivalent antibody, one arm of the antibody binds to antigen A, while the other arm of the antibody binds to antigen B. In some embodiments, in the example of a bispecific, bivalent antibody, one arm of the antibody binds to antigen A, while the other arm of the antibody binds to antigen B or C (cross-reacts with two antigens such as PD-L1 and PD-L2 due to, *e.g.*, similarity in structure). *See, e.g.*, US Patent 9,845,356. Similarly, in the example of a tetravalent bispecific antibody (formed, *e.g.*, by joining two different antibodies), one "arm" refers to the area of the antibody that binds to antigen A (even if two binding sites – of a bivalent antibody – bind to antigen A) and the "other arm" refers to the area of the antibody that binds to antigen B (even if two binding sites – of a bivalent antibody – bind to antigen B). In some embodiments, in the example of a tetravalent bispecific antibody (formed, *e.g.*, by joining two different antibodies), one "arm" refers to the area of the antibody that binds to antigen A (even if two binding sites – of a bivalent antibody – bind to antigen A) and the "other arm" refers to the area of the antibody that binds to antigen B or C (even if two binding sites – of a bivalent antibody – can bind to antigen B or C). *See, e.g.*, US Patent 9845356. As would be apparent to those of skill in the art, "first" or "second" can be used interchangeably.

The term “valency,” when used to describe an antigen-binding construct or protein or antigen-binding arm, refers to the number of recognition (binding) sites in the antigen-binding construct or protein, regardless of whether those different recognition or binding sites bind to the same epitope. Each recognition site specifically recognizes, and is therefore
5 capable of binding, one epitope (binding site) on an antigen. When an antigen-binding protein comprises more than one recognition site (*e.g.*, when an antigen-binding protein is an IgG, which has two recognition sites in its variable regions), each recognition site can specifically recognize the same epitope on the same antigen, or different epitopes, whether on the same or different antigens. Multivalency can increase the avidity, *i.e.*, the strength of
10 binding between an antigen-binding arm or construct and the pertinent antigen or target receptor. Avidity is related to both the affinity between an epitope or antigenic determinant and its binding site on the antigen-binding unit, and the actual number of pertinent binding sites present on the antigen-binding unit.

In some embodiments, any of the multispecific antigen-binding constructs disclosed
15 herein comprises a multivalent (*e.g.*, bivalent) antibody or antigen-binding fragment, wherein at least two of the valencies specifically bind PD-1. In some embodiments, any of the multispecific antigen-binding constructs disclosed herein comprises a multivalent (*e.g.*, bivalent) antibody or antigen-binding fragment, wherein at least two of the valencies specifically bind a PD-1 ligand (*e.g.*, PD-L1 or PD-L2). In some embodiments, any of the
20 multispecific antigen-binding constructs disclosed herein comprises a multivalent (*e.g.*, bivalent) antibody or antigen-binding fragment, wherein at least two of the valencies specifically bind PD-L1. In some embodiments, any of the multispecific antigen-binding constructs disclosed herein comprises a first multivalent (*e.g.*, bivalent) antibody or antigen-binding fragment and a second multivalent (*e.g.*, bivalent) antibody or antigen-binding
25 fragment, wherein at least two of the valencies of the first multivalent antibody or antigen-binding fragment specifically bind PD-1, and wherein at least two of the valencies of the second multivalent antibody or antigen-binding fragment specifically bind PD-L1. In some embodiments, any of the multispecific antigen-binding constructs disclosed herein is a tetravalent construct, wherein the tetravalent construct comprises a first bivalent antibody or
30 antigen-binding fragment and a second bivalent antibody or antigen-binding fragment, wherein both valencies of the first bivalent antibody or antigen-binding fragment are specific for the same epitope on PD-1, and wherein both valencies of the second bivalent antibody or antigen-binding fragment are specific for the same epitope on PD-L1. In some embodiments of such tetravalent constructs, the first and second bivalent antibody or antigen-binding

fragment or portion thereof use a light chain having the same amino acid sequence. In other words, the tetravalent construct comprises a common light chain. For example, a light chain having the sequence of SEQ ID NO: 101 or SEQ ID NO: 103.

In some embodiments, the first arm is an antagonist of PD-1. In some embodiments, the second arm is an antagonist of a PD-1 ligand – *e.g.*, PD-L1 and/or PD-L2. In some
5 embodiments, the first arm is an antagonist of PD-1, and the second arm is an antagonist of cognate PD-1 ligand – *e.g.*, PD-L1 and/or PD-L2.

The terms “antagonist,” “antagonize,” and “inhibit” when used to refer to the biological activity of the antigen-binding arm indicate that the antigen-binding arm binds its target (*e.g.*,
10 PD-1) on the respective cell and partially or fully blocks, inhibits, and/or reduces the biological response through PD-1. In some embodiments, inhibition in the presence of the antagonist is observed in a dose-dependent manner. In some embodiments, the measured signal (*e.g.*, biological activity) is at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about
15 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100% lower than the signal measured with a negative control under comparable conditions. Also disclosed herein, are methods of identifying antagonists suitable for use in the methods of the disclosure. For example, these
20 methods include, but are not limited to, binding assays such as enzyme-linked immunoabsorbent assay (ELISA), FORTE BIO© systems, and radioimmunoassay (RIA). These assays determine the ability of an antagonist to bind the polypeptide of interest (*e.g.*, PD-1 or its ligand) and therefore indicate the ability of the antagonist to inhibit, neutralize or block the activity of the polypeptide of interest. Efficacy of an antagonist can also be determined using
25 functional assays, such as the ability of an antagonist to inhibit the function of the polypeptide. For example, a functional assay may comprise contacting a polypeptide with a candidate antagonist molecule and measuring a detectable change in one or more biological activities normally associated with the polypeptide. The potency of an antagonist is usually defined by its IC₅₀ value (concentration required to inhibit 50% of the agonist response). The
30 lower the IC₅₀ value the greater the potency of the antagonist and the lower the concentration that is required to inhibit the maximum biological response.

In some embodiments, at least one antigen-binding arm has a K_D of at least 1 × 10⁻⁷ M, at least 1 × 10⁻⁸ M, at least 1 × 10⁻⁹ M, at least 1 × 10⁻¹⁰ M, at least 1 × 10⁻¹¹ M, at least 1 ×

10⁻¹² M, or at least 1 x 10⁻¹³ M. In some embodiments, both antigen-binding arms have the same or similar K_D. The term “K_D” (M), as used herein, refers to the dissociation equilibrium constant of a particular antigen-binding arm/antigen interaction. $K_D = k_d/k_a$. The term “k_d” (sec⁻¹), as used herein, refers to the dissociation rate constant of a particular antigen-binding arm/antigen interaction. This value is also referred to as the k_{off} value. The term “k_a” (M⁻¹×sec⁻¹), as used herein, refers to the association rate constant of a particular antigen-binding arm/antigen interaction. This value is also referred to as the k_{on} value.

In some embodiments, the binding of one arm (*e.g.*, the first arm) of the multispecific antigen-binding construct to its target does not block the binding of the other arm (*e.g.*, the second arm) to its target. In some embodiments, the binding of one arm does not sterically hinder the second arm from binding its target. For example, upon the binding of a first arm to PD-1, the second arm is free to bind a ligand of PD-1 (*e.g.*, PD-L1 and/or PD-L2). Thus, in some embodiments, the first arm and second arm bind to their respective targets and both arms remain bound concurrently.

In some embodiments, binding of the first arm and the second arm to their respective targets bridges the immune cell and the second cell together, bringing the two cells in close proximity. As used herein, “bridge” refers to the joining of two cell types (*e.g.*, one immune cell that expresses PD-1, and a second cell that expresses its ligand – PD-L1), or bringing of the two cells together in close proximity; the two cells need not be in physical contact. Thus, the multispecific antigen-binding construct acts as a connector (*e.g.*, a bridge) to the two cells, each one expressing PD-1 or either of its ligands.

Methods for determining whether two cells are bridged or connected together by a construct of the present invention are known in the art. For example, in some embodiments, the bridging of the immune cell and the second cell is determined by, *e.g.*, flow cytometry, FRET, immunoprecipitation, microscopy, or fluorescence plate reader.

In some embodiments, binding of the first arm and the second arm of a multispecific construct to their respective targets results in down-regulation and/or shedding of the ectodomain and/or degradation of a target, *e.g.*, PD-1. As used herein, “down-regulation” refers to the process by which a cell decreases the quantity of a cellular component, such as RNA or protein. In the case of cell-surface protein receptors, down-regulation can occur through internalization of the receptor as a consequence of binding to a ligand or any of the constructs described herein. Shedding or ectodomain shedding refers to a process by which cell surface proteins are proteolytically cleaved resulting in the release of their ectodomains into the extracellular milieu. Non-limiting examples of sheddases that regulate ectodomain

shedding include members of the disintegrin and metalloproteinase (ADAM) family, such as ADAM8, ADAM9, ADAM 10, ADAM12, ADAM15, ADAM 17, and ADAM 28, and matrix metalloproteinases (MMPs), such as MMP2, MMP3, MMP7, MMP9, and MMP14. It is believed that the distance from the plasma membrane and structure of the cleavage site region are more important than the specific sequence in ectodomain shedding. Protein degradation or proteolysis refers to a set of processes that result in the hydrolysis of one or more of the peptide bonds in a protein, either through catalysis by proteolytic enzymes called proteases, or nonenzymatically, for example at very low or very high pH. In eukaryotic cells, two major pathways—the ubiquitin-proteasome pathway and lysosomal proteolysis—mediate protein degradation. Methods for determining whether a target receptor is down-regulated and/or shedded and/or degraded by a multispecific construct disclosed herein are known in the art, and are described in the Examples, *see*, for example, Figures 12A-12C; *e.g.*, flow cytometry, Western blotting, immunoprecipitation, microscopy, or fluorescence plate reader.

As described herein, the constructs of the present invention can bridge an immune cell that expresses PD-1, and a second cell that expresses its ligand, such as a second immune cell, and/or a cancer or tumor cell. As those of skill in the art would recognize, the type of immune cell depends on the context of the disease to be treated; the particular type of immune cell can be readily determined depending on the disorder under consideration. In some embodiments, the immune cell is a T cell, *e.g.*, regulatory T cells (a.k.a. suppressor T cells), including CD8⁺ T cells and CD4⁺ T cells, and subtypes, such as CD4⁺ FOXP3⁺ T_{reg} cells, CD4⁺FOXP3⁻ T_{reg} cells, Tr1 cells, Th3 cells, and T_{reg}17 cells. In some embodiments, the immune cell is a natural killer (NK) cell. In some embodiments, the immune cell is a B cell. In some embodiments, the immune cell is a macrophage.

Similarly, the type of second cell depends on the disorder under consideration. In some embodiments, the second cell (the cell that expresses a PD-1 ligand) is a second immune cell, *e.g.*, a regulatory immune cell. In some embodiments, the regulatory immune cell is any one or more of a regulatory T cell, a B cell, a macrophage, a myeloid-derived suppressor cell, a dendritic cell, or a mesenchymal stromal cell. In some embodiments, the regulatory immune cell is a regulatory T cell, *e.g.*, CD8⁺ T cell or CD4⁺ T cell.

In some embodiments, the second cell is a tumor cell. As used herein, “tumor cell” is sometimes used interchangeably with “cancer cell”, but also encompasses non-malignant (non-cancerous) cells exhibiting increased proliferation as compared to a normal cell. In some embodiments, the tumor cell is a cancer that can be treated by blocking the interaction between PD-1 expressed by an immune cell and its ligand (*e.g.*, PD-L1 or PD-L2) expressed

on a second cell, while bridging the immune cell and the tumor cell. In some embodiments, the tumor cell is selected from the group consisting of a hematological cancer, a lymphoma, a myeloma, a leukemia, a neurological cancer, melanoma, breast cancer, a prostate cancer, a colorectal cancer, lung cancer, head and neck cancer, a gastrointestinal cancer, liver cancer, pancreatic cancer, a genitourinary cancer, a bone cancer, renal cancer, and a vascular cancer.

In some embodiments, the tumor cell is selected from the group consisting of Kaposi's sarcoma, leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, myeloblasts promyelocyte myelomonocytic monocytic erythroleukemia, chronic leukemia, chronic myelocytic (granulocytic) leukemia, chronic lymphocytic leukemia, mantle cell lymphoma, primary central nervous system lymphoma, Burkitt's lymphoma and marginal zone B cell lymphoma, Polycythemia vera Lymphoma, Hodgkin's disease, non-Hodgkin's disease, multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, solid tumors, sarcomas, and carcinomas, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, osteosarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon sarcoma, colorectal carcinoma, 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, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, uterine cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, retinoblastoma, nasopharyngeal carcinoma, esophageal carcinoma, basal cell carcinoma, biliary tract cancer, bladder cancer, bone cancer, brain and central nervous system (CNS) cancer, cervical cancer, choriocarcinoma, colorectal cancers, connective tissue cancer, cancer of the digestive system, endometrial cancer, esophageal cancer, eye cancer, head and neck cancer, gastric cancer, intraepithelial neoplasm, kidney cancer, larynx cancer, liver cancer, lung cancer (small cell, large cell), melanoma, neuroblastoma; oral cavity cancer (for example lip, tongue, mouth and pharynx), ovarian cancer, pancreatic cancer, retinoblastoma, rhabdomyosarcoma, rectal cancer; cancer of the respiratory system, sarcoma, skin cancer, stomach cancer, testicular cancer, thyroid cancer, uterine cancer, and cancer of the urinary system.

As described herein, multispecific antigen-binding constructs of the present invention include bispecific, trispecific, tetraspecific, or multispecific antibodies (immunoglobulins) or antigen-binding portions or fragments thereof.

5 The term “immunoglobulin” refers to a class of structurally related proteins generally comprising two pairs of polypeptide chains: one pair of light (L) chains and one pair of heavy (H) chains. In an “intact immunoglobulin,” all four of these chains are interconnected by disulfide bonds. The structure of immunoglobulins has been well characterized. *See, e.g.,* Paul, *Fundamental Immunology* 7th ed., Ch. 5 (2013) Lippincott Williams & Wilkins, Philadelphia, PA. Briefly, each heavy chain typically comprises a heavy chain variable region (V_H) and a heavy chain constant region (C_H). The heavy chain constant region
10 typically comprises three domains, C_{H1}, C_{H2}, and C_{H3}. Each light chain typically comprises a light chain variable region (V_L) and a light chain constant region. The light chain constant region typically comprises one domain, abbreviated C_L. The term “immunoglobulin” (Ig) is sometimes used interchangeably with the term “antibody” herein.

15 The term “antibody” describes a type of immunoglobulin molecule and is used herein in its broadest sense. An antibody specifically includes intact antibodies (*e.g.*, intact immunoglobulins), and antibody fragments such as antigen-binding fragments of an antibody, as described herein. Thus, “antibody” can refer to an intact antibody as well as an antigen-binding fragment thereof. Antibodies comprise at least one antigen-binding domain. One
20 example of an antigen-binding domain is an antigen binding domain formed by a V_H-V_L dimer. Antibodies can be described by the antigen to which they specifically bind. For example, a PD-1 antibody, alternatively referred to as an anti-PD-1 antibody, is an antibody that specifically binds to the inhibitory receptor PD-1.

The V_H and V_L regions can be further subdivided into regions of hypervariability
25 (“hypervariable regions (HVRs),” also called “complementarity determining regions” (CDRs)) interspersed with regions that are more conserved. The more conserved regions are called framework regions (FRs). Each V_H and V_L generally comprises three CDRs and four FRs, arranged in the following order (from N-terminus to C-terminus): FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. The CDRs are involved in antigen binding, and confer antigen
30 specificity and binding affinity to the antibody. *See* Kabat et al., *Sequences of Proteins of Immunological Interest* 5th ed. (1991) Public Health Service, National Institutes of Health, Bethesda, MD, incorporated by reference in its entirety.

The light chain from vertebrate species can be assigned to one of two types, called kappa and lambda, based on the sequence of the constant domain.

The heavy chain from vertebrate species can be assigned to one of five different classes (or isotypes): IgA, IgD, IgE, IgG, and IgM. These classes are also designated α , δ , ϵ , γ , and μ , respectively. The IgG and IgA classes are further divided into subclasses on the basis of differences in sequence and function. Humans express the following subclasses:

5 IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2.

Methods of generating and screening for an antibody against a desired target is well-known in the art. Methods of further modifying antibodies for enhanced properties (*e.g.*, enhanced affinity, chimerization, humanization) as well as generating antigen-binding fragments, as described herein, are also well-known in the art.

10 The term “chimeric antibody” refers to an antibody in which a component of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

“Humanized” forms of non-human antibodies are chimeric antibodies that contain minimal sequence derived from the non-human antibody. A humanized antibody is generally
15 a human immunoglobulin (recipient antibody) in which residues from one or more CDRs are replaced by residues from one or more CDRs of a non-human antibody (donor antibody).

The donor antibody can be any suitable non-human antibody, such as a mouse, rat, rabbit, chicken, or non-human primate antibody having a desired specificity, affinity, or biological effect. In some instances, selected framework region residues of the recipient antibody are
20 replaced by the corresponding framework region residues from the donor antibody.

Humanized antibodies can also comprise residues that are not found in either the recipient antibody or the donor antibody. Such modifications can be made to further refine antibody function. For further details, *see* Jones et al., (1986) *Nature*, 321:522-525; Riechmann et al., (1988) *Nature*, 332:323-329; and Presta, (1992) *Curr. Op. Struct. Biol.*, 2:593-596, each of
25 which is incorporated by reference in its entirety.

A “human antibody” is one which possesses an amino acid sequence corresponding to that of an antibody produced by a human or a human cell, or derived from a non-human source that utilizes a human antibody repertoire or human antibody-encoding sequences (*e.g.*, obtained from human sources or designed *de novo*). Human antibodies specifically exclude
30 humanized antibodies.

In some embodiments, an antibody molecule comprises a diabody, and a single-chain molecule, as well as an antigen-binding fragment of an antibody (*e.g.*, Fab, F(ab')₂, and Fv). For example, an antibody molecule can include a heavy (H) chain variable domain sequence (abbreviated herein as VH), and a light (L) chain variable domain sequence (abbreviated

herein as VL). In some embodiments, an antibody molecule comprises or consists of a heavy chain and a light chain (referred to as a half antibody). In another example, an antibody molecule includes two heavy (H) chain variable domain sequences and two light (L) chain variable domain sequence, thereby forming two antigen binding sites, such as Fab, Fab', F(ab')₂, Fc, Fd, Fd', Fv, single chain antibodies (scFv, for example), single variable domain antibodies, diabodies (Dab) (bivalent and bispecific), and chimeric (*e.g.*, humanized) antibodies, which may be produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA technologies. These functional antibody fragments retain the ability to selectively bind with their respective antigen. Antibodies and antibody fragments can be from any class of antibodies including, but not limited to, IgG, IgA, IgM, IgD, and IgE, and from any subclass (*e.g.*, IgA1, IgA2, IgG1, IgG2, IgG3, and IgG4) of antibodies. The preparation of antibody molecules can be monoclonal or polyclonal. An antibody molecule can also be a human, humanized, CDR-grafted, or *in vitro* generated antibody. The antibody can have a heavy chain constant region chosen from, *e.g.*, IgG1, IgG2, IgG3, or IgG4. The antibody can also have a light chain chosen from, *e.g.*, kappa or lambda. In some embodiments, the antibody comprises an IgG1 heavy chain constant region having an amino acid sequence that is at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 64

(ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAP ELLGGSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSD GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK). In some

embodiments, the antibody comprises an IgG4 heavy chain constant region having an amino acid sequence that is at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 68

(ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGKTYTCNVNHDHKPSNTKVDKRVESKYGPPCPSCPAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVDVVSQEDPEVQFNWYVDGVEVHNAKTKP REEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQV YTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSPGK). In some

embodiments, the antibody comprises an IgG4 heavy chain constant region having an amino

acid sequence that is at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 69

(ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYTCNVDPKPSNTKVDKRVESKYGPPCPPCPAPEFL
5 GGPSVFLFPPKPKDTLMISRTPEVTCVVDVDSQEDPEVQFNWYVDGVEVHNAKTKP
REEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQV
YTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFF
LYSRLTVDKSRWQEGNVFSCSV MHEALHNHYTQKSLSL SLG)

Antigen-binding portions or fragments of an antibody molecule are well known in the art, and include, for example: (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 diabody (dAb) fragment, which consists of a VH domain; (vi) 15 a camelid or camelized variable domain; (vii) a 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; (viii) a single domain antibody. These antibody fragments are obtained using conventional techniques known to those skilled in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

20 Antibody molecules can also be single domain antibodies. Single domain antibodies can include antibodies whose complementary determining regions are part of a single domain polypeptide. Examples include, but are not limited to, heavy chain antibodies, antibodies naturally devoid of light chains, single domain antibodies derived from conventional 4-chain antibodies, engineered antibodies and single domain scaffolds other than those derived from 25 antibodies. Single domain antibodies may be any of the art, or any future single domain antibodies. Single domain antibodies may be derived from any species including, but not limited to mouse, human, camel, llama, fish, shark, goat, rabbit, and bovine. According to another aspect of the invention, a single domain antibody is a naturally-occurring single-domain antibody known as heavy chain antibody devoid of light chains. Such single domain 30 antibodies are disclosed in WO 9404678, for example. For clarity reasons, this variable domain derived from a heavy chain antibody naturally devoid of light chain is known herein as a VHH or nanobody to distinguish it from the conventional VH of four chain immunoglobulins. Such a VHH molecule can be derived from antibodies raised in *Camelidae* species, for example in camel, llama, dromedary, alpaca and guanaco. Other species besides

Camelidae may produce heavy chain antibodies naturally devoid of light chain; such VHHs are within the scope of the invention.

In some embodiments, the multispecific antigen-binding construct comprises a bispecific antibody. A bispecific antibody has specificity for no more than two antigens, but
5 can have more than two binding sites, as described herein. A bispecific antibody molecule is characterized by a first immunoglobulin variable domain sequence which has binding specificity for a first antigen (*e.g.*, PD-1) and a second immunoglobulin variable domain sequence that has binding specificity for a second antigen (*e.g.*, a PD-1 ligand such as PD-L1 ligand). In some embodiments a bispecific antibody molecule comprises a scFv, or fragment
10 thereof, have binding specificity for a first antigen and a scFv, or fragment thereof, have binding specificity for a second antigen. *See, e.g.*, Kontermann & Brinkmann, (2015), *Drug Discovery Today*, 20(7):838-47, incorporated by reference in its entirety.

Various formats and methods are known in the art that can be used to generate the multivalent and/or multispecific constructs described herein, such as multivalent and/or
15 multispecific antibody formats of both asymmetric and symmetric architectures. Non-limiting examples of such formats include (i) Fc-less bispecific antibody formats, such as tandem single-chain variable fragments (scFv2, taFv) and triplebodies, including bi-specific T cell engager (BiTE) and bispecific killer cell engagers (BiKE) molecules; bispecific single-domain antibody fusion proteins comprising single-domain antibodies, such as VH or VL
20 domains, VHH, VNAR and Nanobodies; diabodies and diabody derivatives, including tandem diabody and dual-affinity retargeting (DART) proteins; Fab fusion proteins; and other Fc-less fusion proteins, through the use of heterodimerizing peptides or miniantibodies from various proteins, *e.g.*, leucine zippers with a coiled coil structure; (ii) bispecific IgGs with asymmetric architecture, such as asymmetric IgGs with heavy and light chains from two
25 different antibodies; bispecific IgGs with an asymmetric Fc region using knobs-into-holes approaches, electrostatic interactions (steering) to avoid homodimerization of CH3 domains, preferential heavy chain heterodimerization by introducing charge pairs into the hinge region of IgG1 and IgG2, strand-exchange engineered domain (SEED) heterodimers, and bispecific engagement by antibodies based on the T cell receptor (BEAT) technologies; asymmetric Fc and CH3 fusion proteins; (iii) bispecific antibodies with a symmetric architecture, such as
30 appended IgGs by fusion of scFvs, fusion of domain antibodies and scaffold proteins, fusion of Fab arms, and fusion of additional variable heavy and light chain domains; modified IgG molecules; symmetric Fc- and CH3-based bispecific antibodies; and bispecific antibodies using immunoglobulin-derived homodimerization domains. *See, for example*, “The making

of bispecific antibodies,” Brinkmann and Kontermann, MABS 2017, Vol. 9:2, pp. 182-212, the contents of which are herein incorporated by reference in its entirety. *See also*, the “knob in a hole” approach described in, *e.g.*, U.S. Pat. No. 5,731,168; the electrostatic steering Fc pairing as described in, *e.g.*, WO 09/089004, WO 06/106905 and WO 2010/129304; Strand Exchange Engineered Domains (SEED) heterodimer formation as described in, *e.g.*, WO 07/110205; Fab arm exchange as described in, *e.g.*, WO 08/119353, WO 2011/131746, and WO 2013/060867; double antibody conjugate, *e.g.*, by antibody cross-linking to generate a bi-specific structure using a heterobifunctional reagent having an amine-reactive group and a sulfhydryl reactive group as described in, *e.g.*, U.S. Pat. No. 4,433,059; bispecific antibody determinants generated by recombining half antibodies (heavy-light chain pairs or Fabs) from different antibodies through cycle of reduction and oxidation of disulfide bonds between the two heavy chains, as described in, *e.g.*, U.S. Pat. No. 4,444,878; trifunctional antibodies, *e.g.*, three Fab’ fragments cross-linked through sulfhydryl reactive groups, as described in, *e.g.*, U.S. Pat. No. 5,273,743; biosynthetic binding proteins, *e.g.*, pair of scFvs cross-linked through C-terminal tails preferably through disulfide or amine-reactive chemical cross-linking, as described in, *e.g.*, U.S. Pat. No. 5,534,254; bifunctional antibodies, *e.g.*, Fab fragments with different binding specificities dimerized through leucine zippers (*e.g.*, c-fos and c-jun) that have replaced the constant domain, as described in, *e.g.*, U.S. Pat. No. 5,582,996; bispecific and oligospecific mono- and oligovalent receptors, *e.g.*, VH-CH1 regions of two antibodies (two Fab fragments) linked through a polypeptide spacer between the CH1 region of one antibody and the VH region of the other antibody typically with associated light chains, as described in, *e.g.*, U.S. Pat. No. 5,591,828; bispecific DNA-antibody conjugates, *e.g.*, crosslinking of antibodies or Fab fragments through a double stranded piece of DNA, as described in, *e.g.*, U.S. Pat. No. 5,635,602; bispecific fusion proteins, *e.g.*, an expression construct containing two scFvs with a hydrophilic helical peptide linker between them and a full constant region, as described in, *e.g.*, U.S. Pat. No. 5,637,481; multivalent and multispecific binding proteins, *e.g.*, dimer of polypeptides having first domain with binding region of Ig heavy chain variable region, and second domain with binding region of Ig light chain variable region, generally termed diabodies (higher order structures are also encompassed creating for bispecific, trispecific, or tetraspecific molecules, as described in, *e.g.*, U.S. Pat. No. 5,837,242; minibody constructs with linked VL and VH chains further connected with peptide spacers to an antibody hinge region and CH3 region, which can be dimerized to form bispecific/multivalent molecules, as described in, *e.g.*, U.S.

Pat. No. 5,837,821; VH and VL domains linked with a short peptide linker (*e.g.*, 5 or 10 amino acids) or no linker at all in either orientation, which can form dimers to form bispecific diabodies; trimers and tetramers, as described in, *e.g.*, U.S. Pat. No. 5,844,094; String of VH domains (or VL domains in family members) connected by peptide linkages with

5 crosslinkable groups at the C-terminus further associated with VL domains to form a series of FVs (or scFVs), as described in, *e.g.*, U.S. Pat. No. 5,864,019; and single chain binding polypeptides with both a VH and a VL domain linked through a peptide linker are combined into multivalent structures through non-covalent or chemical crosslinking to form, *e.g.*, homobivalent, heterobivalent, trivalent, and tetravalent structures using both scFV or diabody

10 type format, as described in, *e.g.*, U.S. Pat. No. 5,869,620. Additional exemplary multispecific and bispecific molecules and methods of making the same are found, for example, in U.S. Pat. No. 5,910,573, U.S. Pat. No. 5,932,448, U.S. Pat. No. 5,959,083, U.S. Pat. No. 5,989,830, U.S. Pat. No. 6,005,079, U.S. Pat. No. 6,239,259, U.S. Pat. No. 6,294,353, U.S. Pat. No. 6,333,396, U.S. Pat. No. 6,476,198, U.S. Pat. No. 6,511,663, U.S.

15 Pat. No. 6,670,453, U.S. Pat. No. 6,743,896, U.S. Pat. No. 6,809,185, U.S. Pat. No. 6,833,441, U.S. Pat. No. 7,129,330, U.S. Pat. No. 7,183,076, U.S. Pat. No. 7,521,056, U.S. Pat. No. 7,527,787, U.S. Pat. No. 7,534,866, U.S. Pat. No. 7,612,181, US2002004587A1, US2002076406A1, US2002103345A1, US2003207346A1, US2003211078A1, US2004219643A1, US2004220388A1, US2004242847A1, US2005003403A1,

20 US2005004352A1, US2005069552A1, US2005079170A1, US2005100543A1, US2005136049A1, US2005136051A1, US2005163782A1, US2005266425A1, US2006083747A1, US2006120960A1, US2006204493A1, US2006263367A1, US2007004909A1, US2007087381A1, US2007128150A1, US2007141049A1, US2007154901A1, US2007274985A1, US2008050370A1, US2008069820A1,

25 US2008152645A1, US2008171855A1, US2008241884A1, US2008254512A1, US2008260738A1, US2009130106A1, US2009148905A1, US2009155275A1, US2009162359A1, US2009162360A1, US2009175851A1, US2009175867A1, US2009232811A1, US2009234105A1, US2009263392A1, US2009274649A1, EP346087A2, WO0006605A2, WO02072635A2, WO04081051A1, WO06020258A2,

30 WO2007044887A2, WO2007095338A2, WO2007137760A2, WO2008119353A1, WO2009021754A2, WO2009068630A1, WO9103493A1, WO9323537A1, WO9409131A1, WO9412625A2, WO9509917A1, WO9637621A2, WO9964460A1. The contents of the above-referenced applications are incorporated herein by reference in their entireties.

In some embodiments, the multispecific antigen-binding construct of the present invention is a bispecific antibody. Bispecific antibodies according to the present disclosure can be generated against PD-1 and PD-L1, or against PD-1 and PD-L2. The antibody arms of the bispecific antibody can be generated by standard techniques, as disclosed herein. In some
5 embodiments, any known antibodies against PD-1 and its ligand can be used to generate a bispecific antibody according to the present disclosure. For example, such bispecific constructs have been exemplified herein (*see*, for example, Pembrolizumab (PD-1 antibody) joined with Atezolizumab (PD-L1 antibody) in Figure 3; Nivolumab (PD-1 antibody) joined with Atezolizumab (PD-L1 antibody) in Figure 4). As exemplified herein, multispecific
10 antigen-binding constructs (*e.g.*, bispecific antibodies) described herein can be generated using known and/or available antibodies in the art.

In some embodiments, the bispecific antibody is bivalent – *e.g.*, one arm is monovalent for PD-1 while the other arm is monovalent for either PD-L1 or PD-L2, or both – *e.g.*, cross-reacts with both ligands). In some embodiments, the bispecific antibody is
15 tetravalent, such as the novel Bispecific 3 and Bispecific 4 antibodies described herein. For example, as illustrated in Figure 3, the Pembrolizumab binding arm is bivalent for PD-1, each binding the same epitope on PD-1, while the Atezolizumab binding arm is bivalent for PD-L1, each binding the same epitope on PD-L1. This can also be seen in, for example, the bispecific format in Figure 8 (the exemplary common light chain bispecific illustrated in the
20 left panel of step 2 of the workflow presented in Figure 8). The exemplary common light chain bispecific format in Figure 8 (the format illustrated in the right panel of step 2 of the workflow) represents another example of a tetravalent bispecific format. In contrast to the tetravalent bispecific format in which the first antigen binding arms are joined to the second antigen binding arms on opposite ends of the Fc region, here each Fab of the first antigen
25 binding arms is joined to each Fab of the second antigen binding arm. For example, one a Fab of the first antigen binding arm is linked to a Fab of the second antigen binding arm using a linker, where each antigen binding arm shares a common light chain. *See*, Figure 8, the format illustrated in the right panel of step 2 of the workflow.

In some embodiments, the bispecific antibody is tetravalent, wherein one arm is
30 bivalent for PD-1, each binding two different epitopes on PD-1. In some embodiments, the bispecific antibody is tetravalent, wherein one arm is bivalent for a ligand of PD-1 (PD-L1 and/or PD-L2), each binding two different epitopes on a ligand of PD-1. In some embodiments, the bispecific antibody is tetravalent, wherein one arm is bivalent for PD-1, each binding two different but overlapping epitopes on PD-1. In some embodiments, the

bispecific antibody is tetravalent, wherein one arm is bivalent for PD-1 ligand (PD-L1 and/or PD-L2), each binding two different but overlapping epitopes on the PD-1 ligand (PD-L1 and/or PD-L2). In some embodiments, the bispecific antibody is tetravalent, wherein one arm is bivalent for PD-1 and each binds the same epitope on PD-1. In some embodiments, the bispecific antibody is tetravalent, wherein one arm is bivalent for a PD-1 ligand (PD-L1 and/or PD-L2), and each binds the same epitope on the PD-1 ligand (PD-L1 and/or PD-L2). In some embodiments, the bispecific antibody is tetravalent, where one arm is bivalent for a same epitope on PD-1; and the other arm is bivalent for a same epitope on a PD-1 ligand (PD-L1 and/or PD-L2).

In some embodiments, the bispecific antibody is an antagonist of both PD-1 and PD-L1. In some embodiments, the bispecific antibody is an antagonist of both PD-1 and PD-L2. In some embodiments, the bispecific antibody is an antagonist of PD-1 and both ligands PD/L1 and PD-L2 (*e.g.*, cross reacts with both ligands).

In certain embodiments, the first antigen-binding arm and the second antigen-binding arm are linked by at least one amino linker amino acid sequence. Optionally, the linker amino acid sequence comprises GGGGS_x (SEQ ID NO: 121), wherein x is an integer between and including 1 to 6.

In some embodiments, the multispecific antigen-binding construct does not comprise an immunoglobulin Fc domain.

In some embodiments, the construct comprises an immunoglobulin Fc domain. In some embodiments, the first arm or second arm, or both, of the construct, comprises a heavy chain comprising one or more immunoglobulin Fc modifications. In some embodiments, the immunoglobulin Fc domain of the heavy chain comprises one or more amino acid mutations that, *e.g.*, promote heterodimerization of the first and second arms, promote serum half-life, and/or modify effector function. In some embodiments, the mutation is present in a CH3 domain of the heavy chain. *See, e.g.*, Xu et al., mAbs 7(1): 231-42, 2015.

While traditional Fc fusion proteins and antibodies are examples of unguided interaction pairs, a variety of engineered Fc domains have been designed as asymmetric interaction pairs (Spiess et al., (2015) Molecular Immunology 67(2A): 95-106) to promote heterodimerization, *e.g.*, of a first antigen-binding arm and a second antigen-bind arm. Various methods are known in the art that increase desired pairing of Fc-containing polypeptide chains in a single cell line to produce a preferred asymmetric fusion protein at acceptable yields [*see, for example*, Klein et al. (2012) mAbs 4:653-663; and Spiess et al. (2015) Molecular Immunology 67(2PartA): 95-106. Methods to obtain desired pairing of Fc-

containing polypeptides include, but are not limited to, charge-based pairing (electrostatic steering), "knobs-into-holes" steric pairing, SEEDbody pairing, and leucine zipper-based pairing. *See*, for example, Ridgway et al. (1996) *Protein Eng* 9:617-621; Merchant et al. (1998) *Nat Biotech* 16:677-681; Davis et al. (2010) *Protein Eng Des Sel* 23:195-202; 5 Gunasekaran et al. (2010); 285:19637-19646; Wranik et al. (2012) *J Biol Chem* 287:43331-43339; US5932448; WO 1993/011162; WO 2009/089004, and WO 2011/034605.

For example, one means by which interaction between specific polypeptides may be promoted is by engineering protuberance-into-cavity (knob-into-holes) complementary regions such as described in Arathoon et al., U.S.7,183,076; Carter et al., U.S.5,731,168; and 10 Kumar et al., WO 2016/164089, incorporated herein by reference in their entireties. "Protuberances" are constructed by replacing small amino acid side chains from the interface of the first polypeptide (*e.g.*, a first interaction pair) with larger side chains (*e.g.*, tyrosine or tryptophan). Complementary "cavities" of identical or similar size to the protuberances are optionally created on the interface of the second polypeptide (*e.g.*, a second interaction pair) 15 by replacing large amino acid side chains with smaller ones (*e.g.*, alanine or threonine). Where a suitably positioned and dimensioned protuberance or cavity exists at the interface of either the first or second polypeptide, it is only necessary to engineer a corresponding cavity or protuberance, respectively, at the adjacent interface.

At neutral pH (7.0), aspartic acid and glutamic acid are negatively charged and lysine, 20 arginine, and histidine are positively charged. These charged residues can be used to promote heterodimer formation and at the same time hinder homodimer formation. Attractive interactions take place between opposite charges and repulsive interactions occur between like charges. In part, protein complexes disclosed herein make use of the attractive interactions for promoting heteromultimer formation (*e.g.*, heterodimer formation), and 25 optionally repulsive interactions for hindering homodimer formation (*e.g.*, homodimer formation) by carrying out site directed mutagenesis of charged interface residues.

For example, the IgG1 CH3 domain interface comprises four unique charge residue pairs involved in domain-domain interactions: Asp356-Lys439', Glu357-Lys370', Lys392-Asp399', and Asp399-Lys409' [residue numbering in the second chain is indicated by (')]. It 30 should be noted that the numbering scheme used here to designate residues in the IgG1 CH3 domain conforms to the EU numbering scheme of Kabat. Due to the 2-fold symmetry present in the CH3-CH3 domain interactions, each unique interaction will be represented twice in the structure (*e.g.*, Asp-399-Lys409' and Lys409-Asp399'). In the wild-type sequence, K409-D399' favors both heterodimer and homodimer formation. A single

mutation switching the charge polarity (*e.g.*, K409E; positive to negative charge) in the first chain leads to unfavorable interactions for the formation of the first chain homodimer. The unfavorable interactions arise due to the repulsive interactions occurring between the same charges (negative-negative; K409E-D399' and D399-K409E'). A similar mutation switching the charge polarity (D399K'; negative to positive) in the second chain leads to unfavorable interactions (K409'-D399K' and D399K-K409') for the second chain homodimer formation. But, at the same time, these two mutations (K409E and D399K') lead to favorable interactions (K409E-D399K' and D399-K409') for the heterodimer formation. The electrostatic steering effect on heterodimer formation and homodimer discouragement can be further enhanced by mutation of additional charge residues which may or may not be paired with an oppositely charged residue in the second chain including, for example, Arg355 and Lys360. *See, e.g.*, WO 2016/164089.

Thus, in some embodiments, the multispecific antigen-binding constructs (*e.g.*, bispecific constructs) described herein can comprise a constant domain of an immunoglobulin, including, for example, the Fc portion of an immunoglobulin. For example, a first arm may comprise an amino acid sequence that is derived from an Fc domain of an IgG (IgG1, IgG2, IgG3, or IgG4), IgA (IgA1 or IgA2), IgE, or IgM immunoglobulin. Optionally, a second arm may comprise an amino acid sequence that is derived from an Fc domain of an IgG (IgG1, IgG2, IgG3, or IgG4), IgA (IgA1 or IgA2), IgE, or IgM. Such immunoglobulin domains may comprise one or more amino acid modifications (*e.g.*, deletions, additions, and/or substitutions) that promote heterodimer formation. In some embodiments, a multispecific antigen-binding construct is of the IgG1 isotype. In some embodiments, a multispecific antigen-binding construct is of the IgG1 isotype and comprises a substitution. In some embodiments, a multispecific antigen-binding construct is of the IgG2 isotype. In some embodiments, a multispecific antigen-binding construct is of the IgG3 isotype. In some embodiments, a multispecific antigen-binding construct is of the IgG4 isotype. In some embodiments, a multispecific antigen-binding construct is of the IgG4 isotype and comprises a substitution. In some embodiments, the substitution is at Ser228 when numbered according to EU numbering. In some embodiments, the substitution at Ser228 is S228P. In some embodiments, a first arm and a second arm comprise Fc domains derived from the same immunoglobulin class and subtype. In some embodiments, a first arm and a second arm comprise Fc domains derived from different immunoglobulin classes or subtypes. Similarly, a first arm and/or a second arm (*e.g.*, an asymmetric pair or an unguided interaction pair) comprise a modified constant domain of an immunoglobulin, including, for

example, one or more amino acid modifications (*e.g.*, deletions, additions, and/or substitutions) that promote heterodimer formation. Methods of generating Fc modifications having the desired heterodimer formation are known in the art.

In some embodiments, the Fc domain can be modified to enhance serum half-life of the multispecific antigen-binding construct disclosed herein. Fc domains comprising one or more mutations which enhance or diminish antibody binding to the Fc receptor, *e.g.*, at acidic pH as compared to neutral pH, are known in the art. For example, the constructs disclosed herein may comprise a mutation in the CH2 or a CH3 region of the Fc domain, wherein the mutation(s) increases the affinity of the Fc domain to FcRn in an acidic environment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0). Such mutations may result in an increase in serum half-life of the construct when administered to an animal. Methods of modifying the Fc domain for desired characteristics, such as enhanced serum half-life are known in the art.

In some embodiments, the constructs described herein comprise an altered heavy chain constant region that has reduced (or no) effector function relative to its corresponding unaltered constant region. Effector functions involving the constant region of the constructs described herein may be modulated by altering properties of the constant or Fc region. Altered effector functions include, for example, a modulation in one or more of the following activities: antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), apoptosis, binding to one or more Fc-receptors, and pro-inflammatory responses. Modulation refers to an increase, decrease, or elimination of an effector function activity exhibited by a subject antibody containing an altered constant region as compared to the activity of the unaltered form of the constant region. In particular embodiments, modulation includes situations in which an activity is abolished or completely absent.

An altered constant region with altered FcR binding affinity and/or ADCC activity and/or altered CDC activity is a polypeptide which has either an enhanced or diminished FcR binding activity and/or ADCC activity and/or CDC activity compared to the unaltered form of the constant region. An altered constant region which displays increased binding to an FcR binds at least one FcR with greater affinity than the unaltered polypeptide. An altered constant region which displays decreased binding to an FcR binds at least one FcR with lower affinity than the unaltered form of the constant region. Such variants which display decreased binding to an FcR may possess little or no appreciable binding to an FcR, *e.g.*, 0 to 50% (*e.g.*, less than 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6,

5, 4, 3, 2, or 1%) of the binding to the FcR as compared to the level of binding of a native sequence immunoglobulin constant or Fc region to the FcR. Similarly, an altered constant region that displays modulated ADCC and/or CDC activity may exhibit either increased or reduced ADCC and/or CDC activity compared to the unaltered constant region. For example, in some embodiments, any one or more of the antibodies described herein comprising an altered constant region can exhibit approximately 0 to 50% (*e.g.*, less than 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1%) of the ADCC and/or CDC activity of the unaltered form of the constant region. A multispecific antigen-binding construct described herein comprising an altered constant region displaying reduced ADCC and/or CDC can exhibit reduced or no ADCC and/or CDC activity.

In some embodiments, the multispecific antigen-binding constructs described herein exhibit reduced or no effector function. In some embodiments, the multispecific antigen-binding constructs comprise a hybrid constant region, or a portion thereof, such as a G2/G4 hybrid constant region (see *e.g.*, Burton et al. (1992) *Adv Immun* 51:1-18; Canfield et al. (1991) *J Exp Med* 173:1483-1491; and Mueller et al. (1997) *Mol Immunol* 34(6):441-452).

In some embodiments, the multispecific antigen-binding constructs described herein can contain an altered constant region exhibiting enhanced or reduced complement dependent cytotoxicity (CDC). Modulated CDC activity can be achieved by introducing one or more amino acid substitutions, insertions, or deletions in an Fc region of the antibody. See, *e.g.*, U.S. patent no. 6,194,551.

The constructs and antigen-binding arms described herein can comprise, in part, scaffold domains, proteins, or portions, *e.g.*, molecules which do not provide target receptor-binding activity, but which can provide a portion or domain of the construct which provides spatial organization, structural support, a means of linking of multiple receptor-binding units, or other desired characteristics, *e.g.*, improved half-life. Various scaffold technologies and compositions are known in the art and can be readily linked or conjugated to the antigen-binding units described herein. The scaffold domain, protein, or portion can be derived from an antibody or not derived from an antibody. Such scaffold proteins, and domains thereof, are, generally, obtained through combinatorial chemistry-based adaptation of preexisting antigen-binding proteins.

Non-antibody protein scaffolds can be considered to fall into two structural categories, domain-sized constructs (in the range of 6 to 20 kDa), and constrained peptides (in the 2-4 kDa range). Domain-sized non-antibody scaffolds include, but are not limited to,

affibodies, affilins, anticalins, atrimers, DARPins, FN3 scaffolds (such as adnectins and centyrins), fynomers, Kunitz domains, pronectins and OBodies. Peptide-sized non-antibody scaffolds include, for example, avimers, bicyclic peptides and cysteine knots. These non-antibody scaffolds and the underlying proteins or peptides on which they are based or from which they have been derived are reviewed by, *e.g.*, Simeon and Chen, *Protein Cell* 9(1): 3-14 (2018); Vazquez-Lombardi *et al.*, *Drug Discovery Today* 20: 1271-1283 (2015), and by Binz *et al.*, *Nature Biotechnol.* 23: 1257-1268 (2005), the contents of each of which are herein incorporated by reference in their entireties. Advantages of using non-antibody scaffolds include increased affinity, target neutralization, and stability. Various non-antibody scaffolds also can overcome some of the limitations of antibody scaffolds, *e.g.*, in terms of tissue penetration, smaller size, and thermostability. Some non-antibody scaffolds can also permit easier construction, not being hindered, for example, by the light chain association issue when bispecific constructs are desired. Methods of constructing constructs on a non-antibody scaffold are known to those of ordinary skill in the art. While not formally on an antibody scaffold, such constructs often include antibody binding domains, whether in the form of single-domain antibodies, scFvs or other antibody binding-domain variants that provide specific target-binding capabilities.

Accordingly, in some embodiments of any of the aspects described herein, a construct can comprise a non-antibody scaffold protein. In some embodiments of any of the aspects described herein, at least one of the receptor-binding units can comprise a non-antibody scaffold protein. One of skill in the art would appreciate that the scaffold portion of a non-antibody scaffold protein can include, in some embodiments, *e.g.*, an adnectin scaffold or a portion derived from human tenth fibronectin type III domain (10Fn3); an anticalin scaffold derived from human lipocalin (*e.g.*, such as those described in, *e.g.*, WO2015/104406); an avimer scaffold or a protein fragment derived from the A-domain of low density-related protein (LRP) and/or very low density lipoprotein receptor (VLDLR); a fynomer scaffold or portion of the SH3 domain of FYN tyrosine kinase; a kunitz domain scaffold or portion of Kunitz-type protease inhibitors, such as a human trypsin inhibitor, aprotinin (bovine pancreatic trypsin inhibitor), Alzheimer's amyloid precursor protein, and tissue factor pathway inhibitor; a knottin scaffold (cysteine knot miniproteins), such as one based on a trypsin inhibitor from *E. elaterium*; an affibody scaffold or all or part of the Z domain of *S. aureus* protein A; a β -Hairpin mimetic scaffold; a Designed ankyrin repeat protein (DARPin) scaffold or artificial protein scaffolds based on ankyrin repeat (AR) proteins; or any scaffold derived or based on human transferrin, human CTLA-4, human crystallin, and human

ubiquitin. For example, the binding site of human transferrin for human transferrin receptor can be diversified to create a diverse library of transferrin variants, some of which have acquired affinity for different antigens. See, *e.g.*, Ali *et al.* (1999) *J. Biol. Chem.* 274:24066-24073. The portion of human transferrin not involved with binding the receptor remains
5 unchanged and serves as a scaffold, like framework regions of antibodies, to present the variant binding sites. The libraries are then screened, as an antibody library is, and in accordance with the methods described herein, against a target antigen of interest to identify those variants having optimal selectivity and affinity for the target antigen. See, *e.g.*, Hey *et al.* (2005) *TRENDS Biotechnol.* 23(10):514-522.

10 D. Methods for Producing the Multispecific Antigen-Binding Constructs

The disclosure also features methods for producing any of the multispecific antigen-binding constructs described herein. In some embodiments, methods for producing the construct of the present invention includes methods for preparing an antibody, and/or fragments thereof as described herein. Such methods are well-known in the art, and can
15 include, *e.g.*, immunizing a subject (*e.g.*, a non-human mammal) with an appropriate immunogen. For example, to generate an antibody that binds to PD-1, a skilled artisan can immunize a suitable subject (*e.g.*, a non-human mammal such as a rat, a mouse, a gerbil, a hamster, a dog, a cat, a pig, a goat, a horse, or a non-human primate) with a full-length PD-1 polypeptide such as a full-length human PD-1 polypeptide comprising the amino acid
20 sequence depicted in SEQ ID NO.: 114 (GenBank accession number NP_005009.2; UniProt Q15116), an antigenic fragment thereof, and/or variant thereof. Similarly, to generate an antibody that binds to a ligand of PD-1 (*e.g.*, PD-L1), a skilled artisan can immunize a suitable subject with a full-length PD-L1 polypeptide such as a full-length human PD-L1 polypeptide comprising the amino acid sequence depicted in SEQ ID NO.: 115 (GenBank
25 accession number NP_054862.1, UniProt Q9NZQ7), an antigenic fragment thereof, and/or variant thereof. Similarly, to generate an antibody that binds to PD-L2, a skilled artisan can immunize a suitable subject with a full-length PD-L2 polypeptide such as a full-length human PD-L2 polypeptide comprising the amino acid sequence depicted in SEQ ID NO.: 116 (GenBank accession number NP_079515.2, UniProt Q9BQ51), an antigenic fragment
30 thereof, and/or variant thereof.

As those of skill in the art would recognize, a full-length polypeptide (PD-1, PD-L1, or PD-L2) can be used as an antigen and antibodies can be screened for desired binding properties (*e.g.*, blocks PD-1/ligand interaction; capacity to bridge cells on which PD-1 and its ligand are expressed). As those of skill in the art would also recognize, antigenic

fragments of a polypeptide (PD-1, PD-L1, or PD-L2) can be selected based on known structural features of the polypeptide. For example, the PD-1/PD-L1 and PD-1/PD-L2 interactions have been structurally well-characterized (*see, e.g.,* Zak, K., et al. (2015) Structure 23(12):2341-48; Ghiotto, M., et al. (2010) Int'l Immuno. 22(8):651-60; Freeman, G. (2008) PNAS 105(30):10275-76; Lazar-Molnar, E. et al. (2008) PNAS 105:10483-88, incorporated by reference in their entireties). Thus, regions within, *e.g.,* PD-1, PD-L1, and/or PD-L2, based on receptor/ligand interface information available in the art, can be used to design a suitable antigenic fragment having desired binding properties. For example, the PD-1 ectodomain contains a single IgV domain typical of the CD28 family, wherein PD-L1 and PD-L2 are composed of IgV and IgC domains typical of the B7 family. The structures of PD-1, PD-L1 and/or PD-L2 show a 1:1 stoichiometry, with interaction primarily between the faces of the IgV domains. An IgV domain comprises about 120 amino acids organized into nine parallel beta strands (ABCC'C'D'EF'G) with loops connecting the strands. It has been shown that PD-1 uses the front beta-face (GFCC' strands and CC', CC'', and FG loops) to bind to the beta-face of PD-L1 (GFCC') or PD-L2 (AGFC strands and FG loop). Further, six amino acids of the C, F, and G strands of PD-1 form a concave, hydrophobic core that interacts with the F and G strands as well as the FG loop of PD-L2. Eight if 14 residues involved in binding to PD-1 are identical or highly conserved between PD-L1 and PD-L2. Using such information, those of skill in the art can determine suitable antigenic regions to generate antibodies having desirable properties. For example, those of skill in the art can generate an antibody that cross reacts with both ligands PD-L1 and PD-L2 (*see, e.g.,* US Patent 9845356).

A suitable subject (*e.g.,* a non-human mammal) can be immunized with the appropriate antigen along with subsequent booster immunizations a number of times sufficient to elicit the production of an antibody by the mammal. The immunogen can be administered to a subject (*e.g.,* a non-human mammal) with an adjuvant. Adjuvants useful in producing an antibody in a subject include, but are not limited to, protein adjuvants; bacterial adjuvants, *e.g.,* whole bacteria (BCG, *Corynebacterium parvum* or *Salmonella minnesota*) and bacterial components including cell wall skeleton, trehalose dimycolate, monophosphoryl lipid A, methanol extractable residue (MER) of tubercle *bacillus*, complete or incomplete Freund's adjuvant; viral adjuvants; chemical adjuvants, *e.g.,* aluminum hydroxide, and iodoacetate and cholesteryl hemisuccinate. Other adjuvants that can be used in the methods for inducing an immune response include, *e.g.,* cholera toxin and parapoxvirus proteins. See also Bieg et al. (1999) Autoimmunity 31(1):15-24. See also, *e.g.,* Lodmell et al. (2000)

Vaccine 18:1059-1066; Johnson et al. (1999) *J Med Chem* 42:4640-4649; Baldrige et al. (1999) *Methods* 19:103-107; and Gupta et al. (1995) *Vaccine* 13(14): 1263-1276.

In some embodiments, the methods include preparing a hybridoma cell line that secretes a monoclonal antibody that binds to the immunogen. For example, a suitable
 5 mammal such as a laboratory mouse is immunized with a polypeptide (*e.g.*, PD-1, PD-L1, PD-L2) or antigenic fragment as described above. Antibody-producing cells (*e.g.*, B cells of the spleen) of the immunized mammal can be isolated two to four days after at least one booster immunization of the immunogen and then grown briefly in culture before fusion with
 10 cells of a suitable myeloma cell line. The cells can be fused in the presence of a fusion promoter such as, *e.g.*, vaccinia virus or polyethylene glycol. The hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected. For example, spleen cells of Balb/c mice immunized with a suitable immunogen can be fused with cells of the myeloma cell line PA1 or the myeloma cell line Sp2/0-Ag 14. After the fusion, the cells are expanded in suitable culture medium, which is supplemented with a selection medium,
 15 for example HAT medium, at regular intervals in order to prevent normal myeloma cells from overgrowing the desired hybridoma cells. The obtained hybridoma cells are then screened for secretion of the desired antibodies, *e.g.*, an antibody that binds to PD-1.

In some embodiments, a skilled artisan can identify an anti-PD-1 antibody from a non-immune biased library as described in, *e.g.*, U.S. patent no. 6,300,064 (to Knappik et al.;
 20 Morphosys AG) and Schoonbroodt et al. (2005) *Nucleic Acids Res* 33(9):e81.

In some embodiments, the methods described herein can involve, or be used in conjunction with, *e.g.*, phage display technologies, bacterial display, yeast surface display, eukaryotic viral display, mammalian cell display, and cell-free (*e.g.*, ribosomal display) antibody screening techniques (*see, e.g.*, Etz et al. (2001) *J Bacteriol* 183:6924-6935;
 25 Cornelis (2000) *Curr Opin Biotechnol* 11:450-454; Klemm et al. (2000) *Microbiology* 146:3025-3032; Kieke et al. (1997) *Protein Eng* 10:1303-1310; Yeung et al. (2002) *Biotechnol Prog* 18:212-220; Boder et al. (2000) *Methods Enzymology* 328:430-444; Grabherr et al. (2001) *Comb Chem High Throughput Screen* 4:185-192; Michael et al. (1995) *Gene Ther* 2:660-668; Pereboev et al. (2001) *J Virol* 75:7107-7113; Schaffitzel et al. (1999) *J*
 30 *Immunol Methods* 231:119-135; and Hanes et al. (2000) *Nat Biotechnol* 18:1287-1292).

Methods for identifying antibodies using various phage display methods are known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. Such phage can be utilized to display antigen-binding domains of antibodies, such as Fab, Fv, or disulfide-

bond stabilized Fv antibody fragments, expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Phage used in these methods are typically filamentous phage such as fd and M13. The antigen binding domains are expressed as a recombinantly-fused protein to any of the phage coat proteins pIII, pVIII, or pIX. *See, e.g.*, Shi et al. (2010) *JMB* 5 397:385-396. Examples of phage display methods that can be used to make the immunoglobulins, or fragments thereof, described herein include those disclosed in Brinkman et al. (1995) *J Immunol Methods* 182:41-50; Ames et al. (1995) *J Immunol Methods* 184:177-186; Kettleborough et al. (1994) *Eur J Immunol* 24:952-958; Persic et al. (1997) *Gene* 187:9-18; Burton et al. (1994) *Advances in Immunology* 57:191-280; and PCT publication nos. WO 10 90/02809, WO 91/10737, WO 92/01047, WO 92/18619, WO 93/11236, WO 95/15982, and WO 95/20401. Suitable methods are also described in, *e.g.*, U.S. patent nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108.

In some embodiments, the phage display antibody libraries can be generated using 15 mRNA collected from B cells from the immunized mammals. For example, a splenic cell sample comprising B cells can be isolated from mice immunized with a PD-1 polypeptide as described above. mRNA can be isolated from the cells and converted to cDNA using standard molecular biology techniques. *See, e.g.*, Sambrook et al. (1989) "Molecular Cloning: A Laboratory Manual, 2nd Edition," Cold Spring Harbor Laboratory Press, Cold 20 Spring Harbor, N.Y.; Harlow and Lane (1988), *supra*; Benny K. C. Lo (2004), *supra*; and Borreback (1995), *supra*. The cDNA coding for the variable regions of the heavy chain and light chain polypeptides of immunoglobulins are used to construct the phage display library. Methods for generating such a library are described in, *e.g.*, Merz et al. (1995) *J Neurosci Methods* 62(1-2):213-9; Di Niro et al. (2005) *Biochem J* 388(Pt 3):889-894; and Engberg et 25 al. (1995) *Methods Mol Biol* 51:355-376.

In some embodiments, a combination of selection and screening can be employed to identify an antibody of interest from, *e.g.*, a population of hybridoma-derived antibodies or a phage display antibody library. Suitable methods are known in the art and are described in, *e.g.*, Hoogenboom (1997) *Trends in Biotechnology* 15:62-70; Brinkman et al. (1995), *supra*; 30 Ames et al. (1995), *supra*; Kettleborough et al. (1994), *supra*; Persic et al. (1997), *supra*; and Burton et al. (1994), *supra*. For example, a plurality of phagemid vectors, each encoding a fusion protein of a bacteriophage coat protein (*e.g.*, pIII, pVIII, or pIX of M13 phage) and a different antigen-combining region are produced using standard molecular biology techniques and then introduced into a population of bacteria (*e.g.*, *E. coli*). Expression of the

bacteriophage in bacteria can, in some embodiments, require use of a helper phage. In some embodiments, no helper phage is required (*see, e.g.*, Chasteen et al., (2006) *Nucleic Acids Res* 34(21):e145). Phage produced from the bacteria are recovered and then contacted to, *e.g.*, a target antigen bound to a solid support (immobilized). Phage may also be contacted to
5 antigen in solution, and the complex is subsequently bound to a solid support.

A subpopulation of antibodies screened using the above methods can be characterized for their specificity and binding affinity for a particular antigen (*e.g.*, human PD-1) using any immunological or biochemical based method known in the art. For example, specific binding of an antibody to PD-1, may be determined for example using immunological or biochemical
10 based methods such as, but not limited to, an ELISA assay, SPR assays, immunoprecipitation assay, affinity chromatography, and equilibrium dialysis as described above. Immunoassays which can be used to analyze immunospecific binding and cross-reactivity of the antibodies include, but are not limited to, competitive and non-competitive assay systems using techniques such as Western blots, RIA, ELISA (enzyme linked immunosorbent assay),
15 “sandwich” immunoassays, immunoprecipitation assays, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein A immunoassays. Such assays are routine and well known in the art.

It is understood that the above methods can also be used to determine if, *e.g.*, an anti-
20 PD-1 antibody does not bind to full-length, human PD-1 and/or PD-1 proteins.

In embodiments where the selected CDR amino acid sequences are short sequences (*e.g.*, fewer than 10-15 amino acids in length), nucleic acids encoding the CDRs can be chemically synthesized as described in, *e.g.*, Shiraishi et al. (2007) *Nucleic Acids Symposium Series* 51(1):129-130 and U.S. Patent No. 6,995,259. For a given nucleic acid sequence
25 encoding an acceptor antibody, the region of the nucleic acid sequence encoding the CDRs can be replaced with the chemically synthesized nucleic acids using standard molecular biology techniques. The 5' and 3' ends of the chemically synthesized nucleic acids can be synthesized to comprise sticky end restriction enzyme sites for use in cloning the nucleic acids into the nucleic acid encoding the variable region of the donor antibody. Alternatively,
30 fragments of chemically synthesized nucleic acids, together capable of encoding an antibody, can be joined together using DNA assembly techniques known in the art (*e.g.* Gibson Assembly).

Any antibody of choice can be further modified to generate an antigen-binding fragment, as described herein, and/or manipulated using known techniques in the art to

generate the multispecific antigen-binding constructs as described herein. For example, cross-linking methods can be used to generate a bispecific structure using a heterobifunctional reagent having an amine-reactive group and a sulfhydryl reactive group as described in, e.g., U.S. Pat. No. 4,433,059; bispecific antibody determinants can be generated by recombining half antibodies (heavy-light chain pairs or Fabs) from different antibodies through cycle of reduction and oxidation of disulfide bonds between the two heavy chains, as described in, e.g., U.S. Pat. No. 4,444,878; trifunctional antibodies, e.g., three Fab' fragments can be cross-linked through sulfhydryl reactive groups, as described in, e.g., U.S. Pat. No. 5,273,743. Other methods of generating bispecific constructs, e.g., methods of generating bispecific constructs having common light chains, are described herein. Non-limiting examples of amino acid sequences of common light chains used in the constructs described herein include SEQ ID NOs: 59-63.

E. Expression and Purification of Multispecific Antigen-Binding Constructs

The multispecific antigen-binding constructs thereof described herein can be produced using a variety of techniques known in the art of molecular biology and protein chemistry. For example, a nucleic acid encoding the multispecific antigen-binding construct (as a single multifunctional polypeptide, or as separate molecules of a multimeric complex – e.g., one antigen-binding arm separately from the other antigen-binding arm) can be inserted into an expression vector that contains transcriptional and translational regulatory sequences, which include, e.g., promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, transcription terminator signals, polyadenylation signals, and enhancer or activator sequences. The regulatory sequences include a promoter and transcriptional start and stop sequences. In addition, the expression vector can include more than one replication system such that it can be maintained in two different organisms, for example in mammalian or insect cells for expression and in a prokaryotic host for cloning and amplification.

Several possible vector systems are available for the expression of cloned heavy chain and light chain polypeptides from nucleic acids in mammalian cells. One class of vectors relies upon the integration of the desired gene sequences into the host cell genome. Cells which have stably integrated DNA can be selected by simultaneously introducing drug resistance genes such as *E. coli* gpt (Mulligan and Berg (1981) *Proc Natl Acad Sci USA* 78:2072) or *Tn5* neo (Southern and Berg (1982) *Mol Appl Genet* 1:327). The selectable marker gene can be either linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection (Wigler et al. (1979) *Cell* 16:77). A second class of

vectors utilizes DNA elements which confer autonomously replicating capabilities to an extrachromosomal plasmid. These vectors can be derived from animal viruses, such as bovine papillomavirus (Sarver et al. (1982) *Proc Natl Acad Sci USA*, 79:7147), cytomegalovirus, polyoma virus (Deans et al. (1984) *Proc Natl Acad Sci USA* 81:1292), or
5 SV40 virus (Lusky and Botchan (1981) *Nature* 293:79).

The expression vectors can be introduced into cells in a manner suitable for subsequent expression of the nucleic acid. The method of introduction is largely dictated by the targeted cell type, discussed below. Exemplary methods include CaPO₄ precipitation, liposome fusion, cationic liposomes, electroporation, viral infection, dextran-mediated
10 transfection, polybrene-mediated transfection, protoplast fusion, and direct microinjection.

Appropriate host cells for the expression of antibodies or antigen-binding fragments thereof include yeast, bacteria, insect, plant, and mammalian cells. Of particular interest are bacteria such as *E. coli*, fungi such as *Saccharomyces cerevisiae* and *Pichia pastoris*, insect cells such as SF9, mammalian cell lines (e.g., human cell lines), as well as primary cell lines.

15 In some embodiments, an antibody or fragment thereof can be expressed in, and purified from, transgenic animals (e.g., transgenic mammals). For example, an antibody can be produced in transgenic non-human mammals (e.g., rodents) and isolated from milk as described in, e.g., Houdebine (2002) *Curr Opin Biotechnol* 13(6):625-629; van Kuik-Romeijn et al. (2000) *Transgenic Res* 9(2):155-159; and Pollock et al. (1999) *J Immunol*
20 *Methods* 231(1-2):147-157.

The antibodies and fragments thereof can be produced from the cells by culturing a host cell transformed with the expression vector containing nucleic acid encoding the antibodies or fragments, under conditions, and for an amount of time, sufficient to allow expression of the proteins. Such conditions for protein expression will vary with the choice
25 of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation. For example, antibodies expressed in *E. coli* can be refolded from inclusion bodies (see, e.g., Hou et al. (1998) *Cytokine* 10:319-30). Bacterial expression systems and methods for their use are well known in the art (see Current Protocols in Molecular Biology, Wiley & Sons, and Molecular Cloning--A Laboratory Manual --3rd
30 Ed., Cold Spring Harbor Laboratory Press, New York (2001)). The choice of codons, suitable expression vectors and suitable host cells will vary depending on a number of factors, and may be easily optimized as needed. An antibody (or fragment thereof) described herein can be expressed in mammalian cells or in other expression systems including but not limited to yeast, baculovirus, and *in vitro* expression systems (see, e.g., Kaszubska et al.

(2000) *Protein Expression and Purification* 18:213-220).

Following expression, the antibodies and fragments thereof can be isolated. An antibody or fragment thereof can be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard
 5 purification methods include electrophoretic, molecular, immunological, and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography. For example, an antibody can be purified using a standard anti-antibody column (*e.g.*, a protein-A or protein-G column). Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. *See, e.g.*, Scopes
 10 (1994) "Protein Purification, 3rd edition," Springer-Verlag, New York City, New York. The degree of purification necessary will vary depending on the desired use. In some instances, no purification of the expressed antibody or fragments thereof will be necessary.

Methods for determining the yield or purity of a purified antibody or fragment thereof are known in the art and include, *e.g.*, Bradford assay, UV spectroscopy, Biuret protein assay,
 15 Lowry protein assay, amido black protein assay, high pressure liquid chromatography (HPLC), mass spectrometry (MS), and gel electrophoretic methods (*e.g.*, using a protein stain such as Coomassie Blue or colloidal silver stain).

F. Modification of the Multispecific Antigen-Binding Constructs

The multispecific antigen-binding constructs can be modified following their
 20 expression and purification as a single multifunctional polypeptide, or as separate molecules of a multimeric complex – *e.g.*, one antigen-binding arm separately from the other antigen-binding arm. The modifications can be covalent or non-covalent modifications. Such modifications can be introduced into the antibodies or antigen-binding fragments by, *e.g.*, reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent
 25 that is capable of reacting with selected side chains or terminal residues. Suitable sites for modification can be chosen using any of a variety of criteria including, *e.g.*, structural analysis or amino acid sequence analysis of the antibodies or fragments.

The amino acid sequences provided herein are set forth in single-letter amino acid code which can be used interchangeably with three-letter amino acid code. An amino acid
 30 refers to any monomer unit that can be incorporated into a peptide, polypeptide, or protein. The twenty natural or genetically encoded alpha-amino acids are as follows: alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartic acid (Asp or D), cysteine (Cys or C), glutamine (Gln or Q), glutamic acid (Glu or E), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), lysine (Lys or K), methionine (Met or M),

phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), and valine (Val or V). The structures of these twenty natural amino acids are shown in, *e.g.*, Stryer et al., Biochemistry, 5th ed., Freeman and Company (2002). The term amino acid also includes unnatural amino acids, modified amino acids (*e.g.*, having modified side chains and/or backbones), and amino acid analogs.

The terms identical or percent identity, in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same (*e.g.*, 90%, or 95% or greater identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection.

Identity or similarity with respect to a sequence is defined as the percentage of amino acid residues in the candidate sequence that are identical (*i.e.*, same residue) with the starting amino acid residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith and Waterman (*Adv. Appl. Math.* 2:482, 1970), by the homology alignment algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), by the search for similarity method of Pearson and Lipman (*Proc. Natl. Acad. Sci. USA* 85:2444, 1988), by computerized implementations of these algorithms (*e.g.*, GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection (*see, e.g.*, Ausubel *et al.*, *Current Protocols in Molecular Biology* (1995 supplement)).

As with all peptides, polypeptides, and proteins, including fragments thereof, it is understood that additional modifications in the amino acid sequence of the constructs, antibodies or antigen-binding portions thereof described herein, for example, in the heavy chain variable region and/or light chain variable region, can occur that do not alter the nature or function of the antibodies or antigen-binding fragments thereof. Such modifications include conservative amino acids substitutions, such that each recited sequence optionally contains one or more conservative amino acid substitutions. The following groups each contain amino acids that are conservative substitutions for one another. These groups are exemplary as other conservative substitutions are known to those of skill in the art.

- 1) Alanine (A), Glycine (G);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 5 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
- 7) Serine (S), Threonine (T); and
- 8) Cysteine (C), Methionine (M)

10

By way of example, when an aspartic acid at a specific residue is mentioned, also contemplated is a conservative substitution at the residue, for example, glutamic acid. Nonconservative substitutions, for example, substituting a proline with glycine, are also contemplated.

15

In some embodiments, the constructs, antibodies or antigen-binding portions thereof can be conjugated to a heterologous moiety. The heterologous moiety can be, *e.g.*, a heterologous polypeptide, a therapeutic agent (*e.g.*, a toxin or a drug), or a detectable label such as, but not limited to, a radioactive label, an enzymatic label, a fluorescent label, a heavy metal label, a luminescent label, or an affinity tag such as biotin or streptavidin. Suitable

20 heterologous polypeptides include, *e.g.*, an antigenic tag (*e.g.*, FLAG (DYKDDDDK) (SEQ ID NO: 117), polyhistidine (6-His; HHHHHH (SEQ ID NO: 118)), hemagglutinin (HA; YPYDVPDYA (SEQ ID NO: 119)), glutathione-S-transferase (GST), or maltose-binding protein (MBP)) for use in purifying the antibodies or fragments. Heterologous polypeptides also include polypeptides (*e.g.*, enzymes) that are useful as diagnostic or detectable markers,

25 for example, luciferase, a fluorescent protein (*e.g.*, green fluorescent protein (GFP)), or chloramphenicol acetyl transferase (CAT). Suitable radioactive labels include, *e.g.*, ³²P, ³³P, ¹⁴C, ¹²⁵I, ¹³¹I, ³⁵S, and ³H. Suitable fluorescent labels include, without limitation, fluorescein, fluorescein isothiocyanate (FITC), green fluorescent protein (GFP), DYLIGHT™ 488, phycoerythrin (PE), propidium iodide (PI), PerCP, PE-ALEXA FLUOR® 700, Cy5,

30 allophycocyanin, and Cy7. Luminescent labels include, *e.g.*, any of a variety of luminescent lanthanide (*e.g.*, europium or terbium) chelates. For example, suitable europium chelates include the europium chelate of diethylene triamine pentaacetic acid (DTPA) or tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). Enzymatic labels include, *e.g.*, alkaline phosphatase, CAT, luciferase, and horseradish peroxidase.

Two proteins (*e.g.*, an antibody and a heterologous moiety) can be cross-linked using any of a number of known chemical cross linkers. Examples of such cross linkers are those which link two amino acid residues via a linkage that includes a “hindered” disulfide bond. In these linkages, a disulfide bond within the cross-linking unit is protected (by hindering groups on either side of the disulfide bond) from reduction by the action, for example, of reduced glutathione or the enzyme disulfide reductase. One suitable reagent, 4-succinimidylloxycarbonyl- α -methyl- α -(2-pyridyldithio) toluene (SMPT), forms such a linkage between two proteins utilizing a terminal lysine on one of the proteins and a terminal cysteine on the other. Heterobifunctional reagents that cross-link by a different coupling moiety on each protein can also be used. Other useful cross-linkers include, without limitation, reagents which link two amino groups (*e.g.*, N-5-azido-2-nitrobenzoyloxysuccinimide), two sulfhydryl groups (*e.g.*, 1,4-bis-maleimidobutane), an amino group and a sulfhydryl group (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester), an amino group and a carboxyl group (*e.g.*, 4-[p-azidosalicylamido]butylamine), and an amino group and a guanidinium group that is present in the side chain of arginine (*e.g.*, p-azidophenyl glyoxal monohydrate).

In some embodiments, a radioactive label can be directly conjugated to the amino acid backbone of the antibody. Alternatively, the radioactive label can be included as part of a larger molecule (*e.g.*, ^{125}I in meta-[^{125}I]iodophenyl-N-hydroxysuccinimide ([^{125}I]mIPNHS) which binds to free amino groups to form meta-iodophenyl (mIP) derivatives of relevant proteins (*see, e.g.*, Rogers et al. (1997) *J Nucl Med* 38:1221-1229) or chelate (*e.g.*, to DOTA or DTPA) which is in turn bound to the protein backbone. Methods of conjugating the radioactive labels or larger molecules/chelates containing them to the antibodies or antigen-binding fragments described herein are known in the art. Such methods involve incubating the proteins with the radioactive label under conditions (*e.g.*, pH, salt concentration, and/or temperature) that facilitate binding of the radioactive label or chelate to the protein (*see, e.g.*, U.S. Patent No. 6,001,329).

Methods for conjugating a fluorescent label (sometimes referred to as a “fluorophore”) to a protein (*e.g.*, an antibody) are known in the art of protein chemistry. For example, fluorophores can be conjugated to free amino groups (*e.g.*, of lysines) or sulfhydryl groups (*e.g.*, cysteines) of proteins using succinimidyl (NHS) ester or tetrafluorophenyl (TFP) ester moieties attached to the fluorophores. In some embodiments, the fluorophores can be conjugated to a heterobifunctional cross-linker moiety such as sulfo-SMCC. Suitable conjugation methods involve incubating an antibody protein, or fragment thereof, with the fluorophore under conditions that facilitate binding of the fluorophore to the protein. *See,*

e.g., Welch and Redvanly (2003) "Handbook of Radiopharmaceuticals: Radiochemistry and Applications," John Wiley and Sons (ISBN 0471495603).

In some embodiments, the antibodies or fragments can be modified, e.g., with a moiety that improves the stabilization and/or retention of the antibodies in circulation, e.g., in blood, serum, or other tissues. For example, the antibody or fragment can be PEGylated as described in, e.g., Lee et al. (1999) *Bioconjug Chem* 10(6): 973-8; Kinstler et al. (2002) *Advanced Drug Deliveries Reviews* 54:477-485; and Roberts et al. (2002) *Advanced Drug Delivery Reviews* 54:459-476 or HESylated (Fresenius Kabi, Germany; see, e.g., Pavisić et al. (2010) *Int J Pharm* 387(1-2):110-119). The stabilization moiety can improve the stability, or retention of, the antibody (or fragment) by at least 1.5 (e.g., at least 2, 5, 10, 15, 20, 25, 30, 40, or 50 or more) fold.

In some embodiments, the antibodies or antigen-binding fragments thereof described herein can be glycosylated. In some embodiments, an antibody or antigen-binding fragment thereof described herein can be subjected to enzymatic or chemical treatment, or produced from a cell, such that the antibody or fragment has reduced or absent glycosylation. Methods for producing antibodies with reduced glycosylation are known in the art and described in, e.g., U.S. patent no. 6,933,368; Wright et al. (1991) *EMBO J* 10(10):2717-2723; and Co et al. (1993) *Mol Immunol* 30:1361. In some embodiments, the antibodies or antigen-binding fragments thereof are aglycosylated.

G. Pharmaceutical Compositions and Formulations

The present disclosure also provides for a pharmaceutical composition comprising the multispecific antigen-binding constructs of the present disclosure with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant to be used with the methods disclosed herein. Such pharmaceutical compositions can be used in a subject having e.g., cancer, as disclosed herein.

In certain embodiments, acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed. In certain embodiments, the formulation material(s) are for s.c. and/or I.V. administration. In certain embodiments, the pharmaceutical composition can contain formulation materials for modifying, maintaining or preserving, for example, the pH, osmolality, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. In certain embodiments, suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine or lysine); antimicrobials; antioxidants (such as ascorbic acid, sodium sulfite or sodium hydrogen- sulfite); buffers (such as borate,

bicarbonate, Tris-HCl, citrates, phosphates or other organic acids); bulking agents (such as mannitol or glycine); chelating agents (such as ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta- cyclodextrin); fillers; monosaccharides; disaccharides; and other
5 carbohydrates (such as glucose, mannose or dextrans); proteins (such as serum albumin, gelatin or immunoglobulins); coloring, flavoring and diluting agents; emulsifying agents; hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (such as sodium); preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben,
10 chlorhexidine, sorbic acid or hydrogen peroxide); solvents (such as glycerin, propylene glycol or polyethylene glycol); sugar alcohols (such as mannitol or sorbitol); suspending agents; surfactants or wetting agents (such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20, polysorbate 80, triton, tromethamine, lecithin, cholesterol, tyloxapal); stability enhancing agents (such as sucrose or sorbitol); tonicity enhancing agents (such as
15 alkali metal halides, preferably sodium or potassium chloride, mannitol sorbitol); delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants. (Remington's Pharmaceutical Sciences, 18th Edition, A. R. Gennaro, ed., Mack Publishing Company (1995). In certain embodiments, the formulation comprises PBS; 20 mM NaOAC, pH 5.2, 50 mM NaCl; and/or 10 mM NAOAC, pH 5.2, 9% Sucrose. In certain embodiments, the optimal pharmaceutical
20 composition will be determined by one skilled in the art depending upon, for example, the intended route of administration, delivery format and desired dosage. *See*, for example, Remington's Pharmaceutical Sciences, *supra*. In certain embodiments, such compositions may influence the physical state, stability, rate of *in vivo* release and/or rate of *in vivo* clearance of the multispecific antigen-binding construct.

25 In certain embodiments, the primary vehicle or carrier in a pharmaceutical composition can be either aqueous or non-aqueous in nature. For example, in certain embodiments, a suitable vehicle or carrier can be water for injection, physiological saline solution or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. In certain embodiments, the saline comprises
30 isotonic phosphate-buffered saline. In certain embodiments, neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. In certain embodiments, pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which can further include sorbitol or a suitable substitute therefore. In certain embodiments, a composition comprising the multispecific antigen-binding constructs

disclosed herein can be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents (Remington's Pharmaceutical Sciences, supra) in the form of a lyophilized cake or an aqueous solution. Further, in certain embodiments, a composition comprising the multispecific antigen-binding construct
5 disclosed herein can be formulated as a lyophilizate using appropriate excipients such as sucrose.

In certain embodiments, the pharmaceutical composition can be selected for parenteral delivery. In certain embodiments, the compositions can be selected for inhalation or for delivery through the digestive tract, such as orally. The preparation of such
10 pharmaceutically acceptable compositions is within the ability of one skilled in the art.

In certain embodiments, the formulation components are present in concentrations that are acceptable to the site of administration. In certain embodiments, buffers are used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

15 In certain embodiments, when parenteral administration is contemplated, a therapeutic composition can be in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising a multispecific antigen-binding construct, in a pharmaceutically acceptable vehicle. In certain embodiments, a vehicle for parenteral injection is sterile distilled water in which a multispecific antigen-binding construct is formulated as a sterile, isotonic solution,
20 and properly preserved. In certain embodiments, the preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid or polyglycolic acid), beads or liposomes, that can provide for the controlled or sustained release of the product which can then be delivered via a depot injection. In certain embodiments, hyaluronic acid can also be used, and can have
25 the effect of promoting sustained duration in the circulation. In certain embodiments, implantable drug delivery devices can be used to introduce the desired molecule.

In certain embodiments, a pharmaceutical composition can be formulated for inhalation. In certain embodiments, a multispecific antigen-binding construct can be formulated as a dry powder for inhalation. In certain embodiments, an inhalation solution
30 comprising a multispecific antigen-binding construct can be formulated with a propellant for aerosol delivery. In certain embodiments, solutions can be nebulized. Pulmonary administration is further described in PCT application No. PCT/US94/001875, which describes pulmonary delivery of chemically modified proteins.

In certain embodiments, it is contemplated that formulations can be administered orally. In certain embodiments, a multispecific antigen-binding construct that is administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. In certain embodiments, a capsule can be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. In certain embodiments, at least one additional agent can be included to facilitate absorption of a multispecific antigen-binding construct. In certain embodiments, diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

In certain embodiments, a pharmaceutical composition can involve an effective quantity of a multispecific antigen-binding construct in a mixture with non-toxic excipients which are suitable for the manufacture of tablets. In certain embodiments, by dissolving the tablets in sterile water, or another appropriate vehicle, solutions can be prepared in unit-dose form. In certain embodiments, suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional pharmaceutical compositions will be evident to those skilled in the art, including formulations involving a multispecific antigen-binding construct in sustained- or controlled-delivery formulations. In certain embodiments, techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. See for example, PCT Application No. PCT/US93/00829 which describes the controlled release of porous polymeric microparticles for the delivery of pharmaceutical compositions. In certain embodiments, sustained-release preparations can include semipermeable polymer matrices in the form of shaped articles, *e.g.* films, or microcapsules. Sustained release matrices can include polyesters, hydrogels, polylactides (U.S. Pat. No. 3,773,919 and EP 058,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., *Biopolymers*, 22:547-556 (1983)), poly (2-hydroxyethyl-methacrylate) (Langer et al., *J. Biomed. Mater. Res.*, 15: 167-277 (1981) and Langer, *Chem. Tech.*, 12:98-105 (1982)), ethylene vinyl acetate (Langer et al., *supra*) or poly-D(-)-3-hydroxybutyric acid (EP 133,988). In certain embodiments, sustained release compositions can also include liposomes, which can be prepared by any of several methods known in the art. *See, e.g.,*

Eppstein et al, Proc. Natl. Acad. Sci. USA, 82:3688-3692 (1985); EP 036,676; EP 088,046 and EP 143,949.

The pharmaceutical composition to be used for *in vivo* administration typically is sterile. In certain embodiments, this can be accomplished by filtration through sterile
5 filtration membranes. In certain embodiments, where the composition is lyophilized, sterilization using this method can be conducted either prior to or following lyophilization and reconstitution. In certain embodiments, the composition for parenteral administration can be stored in lyophilized form or in a solution. In certain embodiments, parenteral
10 compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

In certain embodiments, once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. In certain embodiments, such formulations can be stored either in a
15 ready-to-use form or in a form (*e.g.*, lyophilized) that is reconstituted prior to administration.

In certain embodiments, kits are provided for producing a single-dose administration unit. In certain embodiments, the kit can contain both a first container having a dried protein and a second container having an aqueous formulation. In certain embodiments, kits
20 containing single and multi-chambered pre-filled syringes (*e.g.*, liquid syringes and lyosyringes) are included.

In certain embodiments, the effective amount of a pharmaceutical composition comprising a multispecific antigen-binding construct to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment, according to certain embodiments,
25 will thus vary depending, in part, upon the molecule delivered, the indication for which a multispecific antigen-binding construct is being used, the route of administration, and the size (body weight, body surface or organ size) and/or condition (the age and general health) of the patient. In certain embodiments, the clinician can titer the dosage and modify the route of administration to obtain the optimal therapeutic effect.

30 In certain embodiments, the frequency of dosing will take into account the pharmacokinetic parameters of a multispecific antigen-binding construct in the formulation used. In certain embodiments, a clinician will administer the composition until a dosage is reached that achieves the desired effect. In certain embodiments, the composition can therefore be administered as a single dose or as two or more doses (which may or may not

contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. In certain embodiments, appropriate dosages can be ascertained through use of appropriate dose-response data.

In certain embodiments, the route of administration of the pharmaceutical composition is in accord with known methods, *e.g.* orally, through injection by intravenous, intraperitoneal, intracerebral (intra-parenchymal), intracerebroventricular, intramuscular, subcutaneously, intra-ocular, intraarterial, intraportal, or intralesional routes; by sustained release systems or by implantation devices. In certain embodiments, the compositions can be administered by bolus injection or continuously by infusion, or by implantation device. In certain embodiments, individual elements of the combination therapy may be administered by different routes.

In certain embodiments, the composition can be administered locally via implantation of a membrane, sponge or another appropriate material onto which the desired molecule has been absorbed or encapsulated. In certain embodiments, where an implantation device is used, the device can be implanted into any suitable tissue or organ, and delivery of the desired molecule can be via diffusion, timed-release bolus, or continuous administration. In certain embodiments, it can be desirable to use a pharmaceutical composition comprising a multispecific antigen-binding construct in an *ex vivo* manner. In such instances, cells, tissues and/or organs that have been removed from the patient are exposed to a pharmaceutical composition comprising a multispecific antigen-binding construct after which the cells, tissues and/or organs are subsequently implanted back into the patient.

In certain embodiments, a multispecific antigen-binding construct can be delivered by implanting certain cells that have been genetically engineered, using methods such as those described herein, to express and secrete the polypeptides. In certain embodiments, such cells can be animal or human cells, and can be autologous, heterologous, or xenogeneic. In certain embodiments, the cells can be immortalized. In certain embodiments, in order to decrease the chance of an immunological response, the cells can be encapsulated to avoid infiltration of surrounding tissues. In certain embodiments, the encapsulation materials are typically biocompatible, semi-permeable polymeric enclosures or membranes that allow the release of the protein product(s) but prevent the destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissues.

H. Methods of Use

As described herein, the present disclosure provides a method of treating a proliferative disorder in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a multispecific antigen-binding construct of the present disclosure. In some embodiments, the present disclosure provides a method of enhancing an immune response (*e.g.*, enhanced T cell function, such as rescue from T cell functional exhaustion; enhanced T cell-mediated response; increased inflammatory cytokine secretion and/or production, *e.g.*, IFN γ secretion and/or production from T cells; enhanced NK cell function; enhanced macrophage function) in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a multispecific antigen-binding construct of the present disclosure. As exemplified herein, the enhancement of the immune response is greater upon administration of the multispecific antigen-binding construct disclosed herein as compared to an agent (*e.g.*, antibody) that binds either PD-1 or its ligand (*e.g.*, PD-L1 or PD-L2), or a cocktail comprising an agent (*e.g.*, antibody) that binds PD-1 and an agent (*e.g.*, antibody) that binds its ligand. In some embodiments, the enhancement of the immune response (*e.g.*, enhanced T cell function, such as rescue from T cell functional exhaustion; enhanced T cell-mediated response; increased inflammatory cytokine, IFN γ secretion and/or production from T cells; enhanced NK cell function; enhanced macrophage function) is greater by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, or more, as compared to an agent (*e.g.*, antibody) that binds either PD-1 or its ligand, or a cocktail comprising an agent (*e.g.*, antibody) that binds PD-1 and an agent (*e.g.*, antibody) that binds its ligand. Also provided herein are methods for treating or delaying progression of a cancer or reducing or inhibiting tumor growth in a subject by administering to the subject an effective amount of a multispecific antigen-binding construct, an antibody or antigen-binding fragment thereof, a pharmaceutical composition, or a protein conjugate as described herein.

The compositions described herein are useful in, *inter alia*, methods for treating or preventing a variety of cancers in a subject.

The compositions can be administered to a subject, *e.g.*, a human subject, using a variety of methods that depend, in part, on the route of administration. The route can be, *e.g.*, intravenous injection or infusion (IV), subcutaneous injection (SC), intraperitoneal (IP) injection, intramuscular injection (IM), or intrathecal injection (IT). The injection can be in a bolus or a continuous infusion.

As used herein, the term “subject” means a mammalian subject. Exemplary subjects include, but are not limited to humans, monkeys, dogs, cats, mice, rats, cows, horses, camels, goats and sheep. In some embodiments, the subject is a human. In some embodiments, the subject has or is suspected to have a disease or condition that can be treated with a multispecific antigen-binding construct provided herein. In some embodiments, the disease or condition is a cancer. In some embodiments, the subject is a human with a cancer that can be treated with a multispecific antigen-binding construct provided herein. In some embodiments, the subject is a human that is suspected to have cancer that can be treated with a multispecific antigen-binding construct provided herein.

“Treating” or “treatment” of any disease or disorder refers, in some embodiments, to ameliorating a disease or disorder that exists in a subject. In another embodiment, “treating” or “treatment” includes ameliorating at least one physical parameter, which can be indiscernible by the subject. In yet another embodiment, “treating” or “treatment” includes modulating the disease or disorder, either physically (*e.g.*, stabilization of a discernible symptom) or physiologically (*e.g.*, stabilization of a physical parameter) or both. In yet another embodiment, “treating” or “treatment” includes delaying or preventing the onset of the disease or disorder.

As used herein, the term “therapeutically effective amount” or “effective amount” refers to an amount of a multispecific antigen-binding construct that, when administered to a subject, is effective to treat a disease or disorder.

As used herein, “administer” or “administration” refers to the act of injecting or otherwise physically delivering a substance as it exists outside the body (*e.g.*, a multispecific antigen-binding construct provided herein) into a patient, such as by mucosal, intradermal, intravenous, intramuscular delivery and/or any other method of physical delivery described herein or known in the art. When a disease, or a symptom thereof, is being treated, administration of the substance typically occurs after the onset of the disease or symptoms thereof. When a disease, or symptoms thereof, are being prevented, administration of the substance typically occurs before the onset of the disease or symptoms thereof.

Administration can be achieved by, *e.g.*, local infusion, injection, or by means of an implant. The implant can be of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. The implant can be configured for sustained or periodic release of the composition to the subject. *See, e.g.*, U.S. Patent Application Publication No. 20080241223; U.S. Patent Nos. 5,501,856; 4,863,457; and 3,710,795; EP488401; and EP 430539, the disclosures of each of which are incorporated

herein by reference in their entirety. The composition can be delivered to the subject by way of an implantable device based on, *e.g.*, diffusive, erodible, or convective systems, *e.g.*, osmotic pumps, biodegradable implants, electrodiffusion systems, electroosmosis systems, vapor pressure pumps, electrolytic pumps, effervescent pumps, piezoelectric pumps, erosion-based systems, or electromechanical systems.

In some embodiments, a multispecific antigen-binding construct of the present disclosure is therapeutically delivered to a subject by way of local administration.

As used herein, the term “enhanced T cell function” or “activation of T cells” refers to a cellular process in which mature T cells, which express antigen-specific T cell receptors on their surfaces, recognize their cognate antigens and respond by entering the cell cycle, secreting cytokines or lytic enzymes, and initiating or becoming competent to perform cell-based effector functions. T cell activation requires at least two signals to become fully activated. The first occurs after engagement of the T cell antigen-specific receptor (TCR) by the antigen-major histocompatibility complex (MHC), and the second by subsequent engagement of co-stimulatory molecules (*e.g.*, CD28). These signals are transmitted to the nucleus and result in clonal expansion of T cells, upregulation of activation markers on the cell surface, differentiation into effector cells, induction of cytotoxicity or cytokine secretion, induction of apoptosis, or a combination thereof. In some embodiments, “enhanced T cell function” also encompasses enhanced survival and/or enhanced proliferation of the T cell.

Methods for measuring such activities are routine and known in the art. In some embodiments, “enhanced T cell function” also encompasses rescue of a T cell from an exhausted phenotype, so that restoration of or an increase in one or more T cell functions is achieved. As known in the art, the state of T cell exhaustion is characterized by sequential loss of T cell effector functions, such as inflammatory cytokine production, proliferative abilities, metabolic fitness, in addition to sustained upregulation of a wide array of co-inhibitory receptors, and unique transcriptional and epigenetic signatures. T cell exhaustion and alterations thereof can be measured using techniques known in the art, and described herein, for example, the *in vitro* nonspecific T cell + K562-PD-L1 tumor target cell assay.

As used herein, the term T cell-mediated response refers to any response mediated by T cells, including, but not limited to, effector T cells (*e.g.*, CD8⁺ cells, effector $\gamma\delta$ T cells) and helper T cells (*e.g.*, CD4⁺ cells, including subsets thereof, such as T_H1, T_H2, T_H3, T_H17, T_H9, and T_{FH} cells). T cell-mediated responses include, for example, T cell cytotoxicity, T cell cytokine secretion, and proliferation. A suitable dose of an antibody or fragment

thereof described herein, which dose is capable of treating or preventing cancer in a subject, can depend on a variety of factors including, *e.g.*, the age, sex, and weight of a subject to be treated and the particular inhibitor compound used. For example, a different dose of a whole multispecific antigen-binding construct may be required to treat a subject with cancer as
5 compared to the dose of a fragment of the multispecific antigen-binding construct (*e.g.*, Fab' antibody fragment) required to treat the same subject. Other factors affecting the dose administered to the subject include, *e.g.*, the type or severity of the cancer. For example, a subject having metastatic melanoma may require administration of a different dosage of multispecific antigen-binding construct than a subject with glioblastoma. Other factors can
10 include, *e.g.*, other medical disorders concurrently or previously affecting the subject, the general health of the subject, the genetic disposition of the subject, diet, time of administration, rate of excretion, drug combination, and any other additional therapeutics that are administered to the subject. It should also be understood that a specific dosage and treatment regimen for any particular subject will also depend upon the judgment of the
15 treating medical practitioner (*e.g.*, doctor or nurse). Suitable dosages are described herein. In some embodiments, the multispecific antigen-binding construct described herein are effective at both high and low doses.

A pharmaceutical composition can include a therapeutically effective amount multispecific antigen-binding construct described herein. Such effective amounts can be
20 readily determined by one of ordinary skill in the art based, in part, on the effect of the administered antibody, or the combinatorial effect of the antibody and one or more additional active agents, if more than one agent is used. A therapeutically effective amount of an antibody or fragment thereof described herein can also vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody (and one
25 or more additional active agents) to elicit a desired response in the individual, *e.g.*, reduction in tumor growth. For example, a therapeutically effective amount of multispecific antigen-binding construct can inhibit (lessen the severity of or eliminate the occurrence of) and/or prevent a particular disorder, and/or any one of the symptoms of the particular disorder known in the art or described herein. A therapeutically effective amount is also one in which
30 any toxic or detrimental effects of the composition are outweighed by the therapeutically beneficial effects.

Suitable human doses of any of the multispecific antigen-binding construct described herein can further be evaluated in, *e.g.*, Phase I dose escalation studies. *See, e.g.*, van Gorp et al. (2008) *Am J Transplantation* 8(8):1711-1718; Hanouska et al. (2007) *Clin Cancer Res*

13(2, part 1):523-531; and Hetherington et al. (2006) *Antimicrobial Agents and Chemotherapy* 50(10): 3499-3500.

Toxicity and therapeutic efficacy of such compositions can be determined by known pharmaceutical procedures in cell cultures or experimental animals (*e.g.*, animal models of
5 any of the cancers described herein). These procedures can be used, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. An antibody or antigen-binding fragment thereof that exhibits a high therapeutic index is preferred. While
10 compositions that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue and to minimize potential damage to normal cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such antibodies or antigen-binding fragments thereof lies generally within a range of circulating concentrations of the
15 antibodies or fragments that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For multispecific antigen-binding construct described herein, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated
20 in animal models to achieve a circulating plasma concentration range that includes the EC₅₀ (*i.e.*, the concentration of the construct – *e.g.*, antibody – which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography. In some embodiments, *e.g.*, where
25 local administration (*e.g.*, to the eye or a joint) is desired, cell culture or animal modeling can be used to determine a dose required to achieve a therapeutically effective concentration within the local site.

In some embodiments, the methods can be performed in conjunction with other therapies for cancer. For example, the composition can be administered to a subject at the
30 same time, prior to, or after, radiation, surgery, targeted or cytotoxic chemotherapy, chemoradiotherapy, hormone therapy, immunotherapy, gene therapy, cell transplant therapy, precision medicine, genome editing therapy, or other pharmacotherapy.

As described above, the multispecific antigen-binding construct described herein be used to treat a variety of cancers selected from the group consisting of a hematological

cancer, a lymphoma, a myeloma, a leukemia, a neurological cancer, skin cancer, breast cancer, a prostate cancer, a colorectal cancer, lung cancer, head and neck cancer, a gastrointestinal cancer, liver cancer, pancreatic cancer, a genitourinary cancer, a bone cancer, renal cancer, and a vascular cancer. Optionally, the cancer is selected from the group

5 consisting of Kaposi's sarcoma, leukemia, acute lymphocytic leukemia (etv6, aml1, cyclophilin b), acute myelocytic leukemia, myeloblasts promyelocyte myelomonocytic monocytic erythroleukemia, chronic leukemia, chronic myelocytic (granulocytic) leukemia, chronic lymphocytic leukemia (cyclophilin b), mantle cell lymphoma, primary central nervous system lymphoma, Burkitt's lymphoma, marginal zone B cell lymphoma (Ig-

10 idiotype), Polycythemia vera Lymphoma, Hodgkin's disease (Imp-1, EBNA-1), non-Hodgkin's disease, myeloma (MUC family, p21ras), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, solid tumors, sarcoma, carcinoma, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, osteosarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma,

15 synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon sarcoma, colon carcinoma (p21ras, HER2/neu, c-erbB-2, MUC family), pancreatic cancer, breast cancer (MUC family, HER2/neu, c-erbB-2), ovarian cancer, prostate cancer (Prostate Specific Antigen (PSA) and its antigenic epitopes PSA-1, PSA-2, and PSA-3, PSMA, HER2/neu, c-erbB-2, ga733 glycoprotein), squamous cell carcinoma, basal cell carcinoma,

20 adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma (HER2/neu, c-erbB-2), hepatoma, hepatocellular cancer (α -fetoprotein), bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, uterine cancer, testicular tumor (NY-ESO-1), lung carcinoma,

25 small cell lung carcinoma, non-small cell lung carcinoma (HER2/neu, c-erbB-2), bladder carcinoma, epithelial carcinoma, glioma (E-cadherin, α -catenin, β -catenin, γ -catenin, p120ctn), astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma (p5 protein, gp75, oncofetal antigen, GM2 and GD2 gangliosides, Melan-A/MART-1, cdc27,

30 MAGE-3, p21ras, gp100), neuroblastoma, retinoblastoma, nasopharyngeal carcinoma (Imp-1, EBNA-1), esophageal carcinoma, basal cell carcinoma, biliary tract cancer (p21ras), bladder cancer (p21ras), bone cancer, brain and central nervous system (CNS) cancer, cervical carcinoma (p53, p21ras), choriocarcinoma (CEA), colorectal cancers (colorectal associated antigen (CRC)-CO17-1A/GA733, APC), connective tissue cancer, cancer of the digestive

system, endometrial cancer, esophageal cancer, eye cancer, head and neck cancer, gastric cancer (HER2/neu, c-erbB-2, ga733 glycoprotein), epithelial cell cancer (cyclophilin b), intraepithelial neoplasm, kidney cancer, larynx cancer, liver cancer, lung cancer (small cell, large cell) (CEA, MAGE-3, NY-ESO-1), oral cavity cancer (for example lip, tongue, mouth, and pharynx cancers), ovarian cancer (MUC family, HER2/neu, c-erbB-2), pancreatic cancer, rectal cancer, cancer of the respiratory system, skin cancer, thyroid cancer, and cancer of the urinary system.

In some embodiments, a multispecific antigen-binding construct described herein can be administered to a subject as a monotherapy. Alternatively, as described above, the antibody or fragment thereof can be administered to a subject as a combination therapy with another treatment, *e.g.*, another treatment for a cancer. For example, the combination therapy can include administering to the subject (*e.g.*, a human patient) one or more additional agents that provide a therapeutic benefit to a subject who has, or is at risk of developing, cancer. Chemotherapeutic agents suitable for co-administration with compositions of the present invention include, for example: taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxyanthracindione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Further agents include, for example, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.* mechlorethamine, thioTEPA, chlorambucil, melphalan, carmustine (BSNU), lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, cis-dichlordiamine platinum (II)(DDP), procarbazine, altretamine, cisplatin, carboplatin, oxaliplatin, nedaplatin, satraplatin, or triplatin tetranitrate), anthracycline (*e.g.* daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.* dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.* vincristine and vinblastine) and temozolomide. In some embodiments, the multispecific antigen-binding construct and the one or more additional active agents are administered at the same time. In other embodiments, the multispecific antigen-binding construct is administered first in time and the one or more additional active agents are administered second in time. In some embodiments, the one or more additional active agents are administered first in time and the multispecific antigen-binding construct is administered second in time.

A multispecific antigen-binding construct described herein can replace or augment a previously or currently administered therapy. For example, upon treating with a multispecific antigen-binding construct, administration of the one or more additional active agents can cease or diminish, *e.g.*, be administered at lower levels or dosages. In some embodiments, administration of the previous therapy can be maintained. In some embodiments, a previous therapy will be maintained until the level of the multispecific antigen-binding construct reaches a level sufficient to provide a therapeutic effect. The two therapies can be administered in combination.

Monitoring a subject (*e.g.*, a human patient) for an improvement in a cancer, as defined herein, means evaluating the subject for a change in a disease parameter, *e.g.*, a reduction in tumor growth. In some embodiments, the evaluation is performed at least one (1) hour, *e.g.*, at least 2, 4, 6, 8, 12, 24, or 48 hours, or at least 1 day, 2 days, 4 days, 10 days, 13 days, 20 days or more, or at least 1 week, 2 weeks, 4 weeks, 10 weeks, 13 weeks, 20 weeks or more, after an administration. The subject can be evaluated in one or more of the following periods: prior to beginning of treatment; during the treatment; or after one or more elements of the treatment have been administered. Evaluation can include evaluating the need for further treatment, *e.g.*, evaluating whether a dosage, frequency of administration, or duration of treatment should be altered. It can also include evaluating the need to add or drop a selected therapeutic modality, *e.g.*, adding or dropping any of the treatments for a cancer described herein.

In some embodiments, a multispecific antigen-binding construct described herein is administered to modulate a T-cell response in a patient, for example, by increasing T-cell activation and/or proliferation. Blocking the interaction between PD-1 expressed by an immune cell and its ligand strongly enhances T cell proliferation, IFN γ production and secretion, and the cytolytic activity of T cells. Bridging an immune cell that expresses PD-1 with a second cell (*e.g.*, another immune cells, or a tumor cell) that expresses a PD-1 ligand (*e.g.*, PD-L1 or PD-L2) can strongly enhance T cell proliferation, IFN γ production and secretion, and the cytolytic activity of T cells. Accordingly, in some embodiments, the multispecific antigen-binding construct of the present disclosure is administered to a patient in need thereof to induce or increase T-cell activation, enhance T cell proliferation, induce the production and/or secretion of IFN γ , and/or induce a cytolytic T cell response.

EXAMPLES

While the present disclosure has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the disclosure. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present disclosure. All such modifications are intended to be within the scope of the disclosure.

Example 1: Induction of Interferon-gamma (IFN γ) in T cells

To assess the effect of PD1/PDL1 bispecific antibody on T-cell activation, IFN γ production was analyzed in a mixed lymphocyte reaction (MLR). Aglycosylated bispecific antibodies combining binding domains of NivolumabxAtezolizumab, 949xAtezolizumab, J43xAtezolizumab, PidilizumabxAtezolizumab, AtezolizumabxNivolumab, or DurvalumabxNivolumab were tested. The antibody 949 refers to a PD-1 antibody as disclosed in US Patent 9102728. Antibody J43 refers to an anti-murine PD-1 antibody. KEYTRUDA, a humanized antibody that blocks PD-1 (Merck) and is known to induce IFN γ production, was used as a comparator. A schematic and amino acid sequence for NivolumabxAtezolizumab, 949 xAtezolizumab, and AtezolizumabxNivolumab are shown in **Figures 4-6**, respectively. Each of the bispecific formats exemplified and tested herein were generated using known methods in the art. For example, as shown, in Figure 4, the heavy chain portion of the Atezolizumab Fab was crosslinked to the heavy chain Fc portion of Nivolumab using methods known in the art. Suitable methods for crosslinking two proteins, such as the heavy chain of a Fab and the heavy chain of IgG molecule, with or without linker sequences, are described herein. This study demonstrated that various concentrations of bispecific antibodies described above can induce an IFN γ response in T cells.

Peripheral blood mononuclear cells (PBMCs) were isolated from leukopaks (HemaCare, Van Nuys, CA) derived from three independent human donors (D985, D7603, and D5004). Total T cells were enriched from PBMC by negative selection using immunomagnetic cell separation (EASYSEPTM; Stemcell Technologies, Vancouver BC). Monocytes were isolated from PBMCs using immunomagnetic cell separation (EASYSEPTM; Stemcell Technologies, Vancouver BC). T cells were resuspended in complete RPMI at 1×10^6 cells/ml concentration and monocytes were adjusted to 5×10^5 cells/ml respectively. In a 96-well plate, 100 μ l of media containing T cells were plated at 1×10^5 cells/well density

followed by adding 100µl of monocyte cell suspension (E:T ratio 2:1). Next, 50 µl of media containing various dilutions of antibodies was added to reach a final concentration of 0 nM, 0.5 nM, 5 nM, or 50 nM. Plates were incubated at 37°C in a CO₂ incubator for five days. At the end of incubation period, culture supernatants were collected and IFN γ levels were
5 analyzed by MSD assay (Mesoscale Diagnostics, Rockville, MD).

Example 2: Induction of Interferon-gamma (IFN γ) in T cells treated with a Pembrolizumab/Atezolizumab bispecific antibody

To assess the effect of PD1/PDL1 bispecific antibody on T-cell activation, IFN γ
10 production was analyzed in a mixed lymphocyte reaction (MLR). A PembrolizumabxAtzolizumab bispecific, Pembrolizumab mAb, Atezolizumab Fab, a mix of KEYTRUDA and Atezolizumab Fab, and KEYTRUDA, a humanized antibody that blocks PD-1 (Merck) and is known to induce IFN- γ production, were used as comparators. A schematic and amino acid sequence for the PembrolizumabxAtzolizumab bispecific
15 antibody is shown in **Figure 3**. As similarly described for the other bispecific formats exemplified and tested herein, the bispecific format shown in Figure 3 was generated using known methods in the art. As shown in Figure 3, the heavy chain portion of the Atezolizumab Fab was crosslinked to the c-terminus of the heavy chain Fc portion of Pembrolizumab using methods known in the art. Suitable methods for crosslinking two
20 proteins, such as the heavy chain of a Fab and the heavy chain of IgG molecule, with or without linker sequences, are described herein. These results indicate that PembrolizumabxAtzolizumab, a bispecific antibody targeting PD-1 and PD-L1, induces an IFN γ response in T cells comparable to Pembrolizumab, Atezolizumab Fab, or KEYTRUDA treatments alone.

25 T cells were prepared as described above. A volume of 50 µl of media containing various dilutions of antibodies was added to reach a final concentration of 0 nM, 0.5 nM, 5 nM, or 50 nM. Plates were incubated at 37°C in a CO₂ incubator for five days. At the end of incubation period, culture supernatants were collected and IFN- γ levels were analyzed by MSD assay (Mesoscale Diagnostics, Rockville, MD).

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Example 3: Comparison of Interferon-gamma (IFN γ) induction in T cells treated with a bispecific antibody targeting PD-1 and PD-L1 or cocktail of monoclonal antibodies targeting PD-1/PD-L1

To assess the effect of PD-1xPD-L1 or PD-L1xPD-L1 bispecific antibodies on T-cell activation, IFN- γ production was analyzed in a mixed lymphocyte reaction (MLR). A PembrolizumabxAtezolizumab bispecific antibody, NivolumabxAtezolizumab bispecific antibody, a cocktail of KEYTRUDA and Atezolizumab, a cocktail of Nivolumab and Atezolizumab were tested and KEYTRUDA alone, a humanized antibody that blocks PD-1 (Merck) and is known to induce IFN- γ production, was used as a comparator.

T cells were prepared as described above. A volume of 50 μ l of media containing various dilutions of antibodies was added to reach a final concentration of 0 nM, 0.01 nM, 0.001 nM, or 0.0001 nM. Plates were incubated at 37°C in a CO₂ incubator for five days. At the end of incubation period, culture supernatants were collected and IFN γ levels were analyzed by MSD assay (Mesoscale Diagnostics, Rockville, MD).

Figure 1 shows the concentration of IFN- γ as pg/mL at the final concentrations of antibodies tested, as indicated. These results indicate that bispecific PD-1xPD-L1 antibodies, such as PembrolizumabxAtezolizumab or the PDL1/PDL1 bispecific antibody Nivolumab/Atezolizumab, induce a higher IFN γ response in T cells than a cocktail of PD1 and PDL1 specific antibodies, or a PD-1 antibody (KEYTRUDA) alone.

Example 4: Comparison of Interferon-gamma (IFN γ) induction in T cells treated with a bispecific antibody targeting PD-1/PDL-1 or monoclonal antibodies targeting PD-1 or PD-L1

To assess the effect of a PD-1xPD-L1 or PD-L1xPD-L1 bispecific antibody on T-cell activation, IFN γ production was analyzed in a mixed lymphocyte reaction (MLR). PembrolizumabxNivolumab bispecific antibody, AtezolizumabxAtezolizumab tetravalent, monospecific antibody were tested and Nivolumab alone, Atezolizumab alone, and KEYTRUDA (Pembrolizumab), a humanized antibody that blocks PD-1 (Merck) and is known to induce IFN- γ production, were used as comparators.

T cells were prepared as described above. A volume of 50 μ l of media containing various dilutions of antibodies was added to reach a final concentration of 0 nM, 0.01 nM, 0.001 nM, or 0.0001 nM. Plates were incubated at 37°C in a CO₂ incubator for five days. At

the end of incubation period, culture supernatants were collected and IFN- γ levels were analyzed by MSD assay (Mesoscale Diagnostics, Rockville, MD).

Figure 2 shows the concentration of IFN- γ as pg/mL at the final concentrations of antibodies tested, in PBS, as indicated. These results indicate that bispecific PD-1xPD-L1 (PembrolizumabxNivolumab) or PD-L1xPD-L1 antibodies (AtezolizumabxAtezolizumab) induce a similar IFN- γ response in T cells as PD-1 (Atezolizumab or KEYTRUDA) or PD-L1 (Nivolumab) specific antibodies alone.

Figure 7 shows the concentration of IFN- γ as pg/mL at the final concentrations of antibodies tested, in PBS, as indicated. These results indicate that bispecific antibodies PD-1xPD-L1 (PembrolizumabxAtezolizumab) or (NivolumabxAtezolizumab) in a multispecific format induce a greater IFN- γ response at femtomolar concentrations in a mixed lymphocyte reaction (MLR) as compared to a cocktail of Pembrolizumab and Atezolizumab or Nivolumab and Atezolizumab. These results suggest a synergistic effect that results from a multispecific format.

Figure 8 shows an exemplary workflow for identifying multispecific (*e.g.*, bispecific) antibodies that demonstrate synergy. The process includes an unbiased screen of checkpoint blocker combinations in mixed lymphocyte reaction (MLR), which measures IFN γ release, in pg/mL, at various concentrations, as described elsewhere herein (see, for example, the section below entitled *Nonspecific T cell + K562-PD-L1 tumor target cell assay*). In the second step of the illustrated workflow, common light chain bispecifics were generated to further test their efficacy and various exemplary common light chain bispecific formats are depicted. The identified common light chain bispecific formats outperform known PD-1 blockers in a T-cell activation assay.

Example 5: Generation and Characterization of Anti-PD-1 and Anti-PD-L1 Antibody Constructs and Multispecific Antigen-Binding Molecules

Affinity Maturation of anti-PD-L1 Antibodies

Affinity matured anti-PD-L1 antibodies derived from PD-L1 antibody mAb24 were generated via construction of a mutant library, mammalian display sorting, and screening of monoclonal IgG. The library contained mutations in the heavy chain, where synthetic diversity in CDRH1, CDRH2, and CDRH3 was introduced, while the light chain sequence was held constant, except in some cases, to maintain compatibility with single light chain constructs. The library went through 3 rounds of mammalian display sorting aimed at

increasing affinity for human PD-L1 and maintaining mouse cross-reactivity. In each round, an off-rate competition step was employed after initial binding to biotinylated antigens (*i.e.*, 1 hour incubation with excess unlabeled antigen or parental IgG). After the final round of sorting, clones were picked, their sequences analyzed, and unique clones were assayed via
 5 Wasatch SPR binding kinetics and cell-binding equilibrium assays to identify lead candidates.

The resulting anti-PD-L1 antibodies from different selection rounds were plotted on k_d/k_a double log plots. Apparent association and dissociation kinetic rate constants (k_a and k_d values) were determined on an SPRi reader (MX96, Cytiva, Salt Lake City, UT) in a
 10 running buffer of PBS-Tween 0.01%. Anti-human PD-L1 antibodies were covalently conjugated on a Carboxymethyl dextran hydrogel 50L chip (XanTec bioanalytics GmbH, Dusseldorf, Germany) on a CFM (Cytiva). Freshly mixed activating reagents (150 μ l of 0.4 M EDC and 150 μ l of 0.1 M sulfo-NHS in 5 ml of H₂O) were used to activate the surface of the SPR substrate for 7 minutes. Antibodies at 10 mg/ml, in acetic acid buffer pH 4.5, were
 15 used for printing for 15 minutes. The printed chip was then quenched on an SPRi reader (MX96, Cytiva) with 1 M ethanolamine for 15 minutes. For kinetics analysis, purified recombinant His tagged human PD-L1 (0, 2.05, 5.12, 12.8, 32, 80, 200, 500 nM) was injected sequentially. For each concentration, there was 5 minutes of association followed by 10 minutes of dissociation. Data were processed and analyzed in SPR Inspection Tool and
 20 Scrubber softwares (Biosensor Tools LLC, Salt Lake City, UT). The kinetic data were referenced with the interstitial reference spots and double-referenced to a buffer cycle, and then fit globally to a 1:1 binding model to determine their parent association and dissociation kinetic rate constants (k_a and k_d values). The ratio k_d/k_a was used to derive the K_D value of each antigen/mAb interaction, *i.e.* $K_D = k_d/k_a$.

25 *Affinity Maturation of anti-PD-1 Antibodies*

Affinity matured anti-PD-1 antibodies derived from PD-1 antibody mAb25 were generated via construction of a mutant library, phage display panning, and screening of monoclonal antibodies. The library contained mutations in the heavy chain, where synthetic diversity in CDRH1, CDRH2, and CDRH3 was introduced, while the light chain sequence
 30 was held constant to maintain compatibility with single light chain constructs. The library went through 4 rounds of phage display panning rounds aimed at increasing affinity for human PD-1 and gaining mouse cross-reactivity. In each round, an off-rate competition step was employed after initial binding to biotinylated antigens (*i.e.*, 1 hour incubation with excess unlabeled antigen or parental IgG). After the final round of panning, clones were picked,

their sequences analyzed, and unique Fab clones were assayed via Octet SPR binding kinetics and cell-binding equilibrium assays to identify lead candidates. Lead candidates were converted from Fab to human IgG and further characterized.

The resulting anti-PD-1 antibodies from different selection rounds were plotted on k_d/k_a double log plots. Apparent association and dissociation kinetic rate constants (k_a and k_d values) were determined on an SPRi reader (MX96, Cytiva, Salt Lake City, UT) in a running buffer of PBS-Tween 0.01%. Anti-human PD-1 antibodies were covalently conjugated on a Carboxymethyl dextran hydrogel 50L chip (XanTec bioanalytics GmbH, Dusseldorf, Germany) on a CFM (Cytiva). Freshly mixed activating reagents (150 μ l of 0.4 M EDC and 150 μ l of 0.1 M sulfo-NHS in 5 ml of H₂O) were used to activate the surface of the SPR substrate for 7 minutes. Antibodies at 10 mg/ml, in acetic acid buffer pH 4.5, were used for printing for 15 minutes. The printed chip was then quenched on an SPRi reader (MX96, Cytiva) with 1 M ethanolamine for 15 minutes. For kinetics analysis, purified recombinant His tagged human PD-1 (0, 2.05, 5.12, 12.8, 32, 80, 200, 500 nM) was injected sequentially. For each concentration, there was 5 minutes of association followed by 10 minutes of dissociation. Data were processed and analyzed in SPR Inspection Tool and Scrubber softwares (Biosensor Tools LLC, Salt Lake City, UT). The kinetic data were referenced with the interstitial reference spots and double-referenced to a buffer cycle, and then fit globally to a 1:1 binding model to determine their apparent association and dissociation kinetic rate constants (k_a and k_d values). The ratio k_d/k_a was used to derive the K_D value of each antigen/mAb interaction, *i.e.* $K_D = k_d/k_a$.

Nonspecific T cell + K562-PD-L1 tumor target cell assay

T cells were isolated from previously frozen PBMCs (peripheral blood mononuclear cells) using a negative selection kit and activated using IMMUNOCULT™ anti-CD3/CD28 T cell activator in X-VIVO 15 media supplemented with 10 % FBS ("X-10"). After 3 days, the cells were switched to X-10 media containing 5 ng/ml IL-2 and 2.5 ng/ml IL-7 ("hX-10"). Every 2-3 days the cells were fed fresh hX-10. After 10 days of activation, the IMMUNOCULT™-expanded T cells were CELLTRACE™ Violet-labeled and co-cultured with K562 cells that were stably transduced to express human CD32B and PD-L1 ("K32P") in 96 well round-bottom plates with 50,000 T cells and 25,000 K32P cells per well. Antibodies (Bispecific 3, KEYTRUDA, and an isotype control) were added at final concentrations between 10 and 0.001 nM done in 10-fold dilutions along with 0.25 μ g/ml anti-CD3 (clone OKT3). After 3 days, supernatants were collected for measuring IFN γ cytokine production via MSD plates, and cells were stained for flow cytometry, and then run

on a BD LSRFORTESSA™ cytometer to look at T cell activation and target cell killing. Target cell killing is measured by counting the number of live K32P targets in the experimental wells compared to the number of K32P targets in a set of no CD3 control wells. All data was then analyzed in GraphPad Prism. As shown in **Figures 9A-9B**, Bispecific 3 induced higher amounts of IFN γ and killing of K32P target cells as compared to both the isotype control antibody and KEYTRUDA.

CMV antigen recall assay

Day 12 expanded CMV antigen specific CD8+ T cells, from an HLA A02:01 donor, were thawed and rested overnight in hX-10 media containing 2 ug/ml DNase I. The following day, the cells were collected, and dead cells were removed using Ficoll-separation. The remaining cells were then co-cultured with K562 cells expressing HLA-A02:01, CMV protein pp65-IRES-GFP, and PD-L1 ("KACP", GFP+) and K562 cells expressing HLA-A02:01 ("KA", GFP-) in 96 well round-bottom plates with 25,000 CMV T cells, 50,000 KACP cells, and 50,000 KA cells per well. Antibodies (Bispecific 3, KEYTRUDA, a combination of mAb1 and mAb28, and an isotype control) were added at final concentrations between 10 and 0.0001 nM. This dosing included 10-fold dilutions between 10 and 0.1 nM and then 2-fold dilutions until 0.0001 nM. After 2 days of co-culture, specific killing of KACP tumor targets via flow cytometry were analyzed. Specific killing is defined ratiometrically as the ratio of live GFP+ KACP cells to GFP- KA cells, normalized to the ratio of these cells when no T cells are present. As shown in **Figure 10A**, Bispecific 3 increased the specific killing of tumor antigen target cells. The increase mediated by Bispecific 3 was always higher than what was seen for KEYTRUDA, while at high doses the combination of mAb1 and mAb28 killed an equal number of KACP cells. Critically, at the low doses of antibody (0.001 through 0.01 nM) Bispecific 3 showed an increase in killing of KACP cells as compared to both KEYTRUDA and the combination of mAb1 and mAb28, indicating that Bispecific 3 can be used to mediate antigen-specific killing of target cells at lower doses.

In a separate experiment, the effect of Bispecific 3 on Raji cell-specific killing was examined in a manner similar to that outlined above for K562 cell-specific killing assay. Briefly, CMV antigen specific CD8+ T cells were co-cultured with Raji cells expressing HLA-A02:01, CMV protein pp65-IRES-GFP, and PD-L1 ("RACP", GFP+) and Raji cells expressing HLA-A02:01 ("RA", GFP-) in 96 well round-bottom plates with 25,000 CMV T cells, 50,000 RACP cells, and 50,000 RA cells per well. Antibodies (Bispecific 3,

KEYTRUDA, a combination of mAb1 and mAb28, and an isotype control) were added at final concentrations between 10 and 0.0001 nM. This dosing included 10-fold dilutions between 10 and 0.1 nM and then 2-fold dilutions until 0.0001 nM. After 2 days of co-culture, specific killing of RACP tumor targets via flow cytometry were analyzed. Specific killing is defined ratiometrically as the ratio of live GFP+ RACP cells to GFP- RA cells, normalized to the ratio of these cells when no T cells are present. As shown in **Figure 10B**, Bispecific 3 increased the specific killing of tumor antigen target cells. The increase mediated by Bispecific 3 was noticeably higher at 0.001 nM than what was seen for either KEYTRUDA or the combination of mAb1 and mAb28, while at high doses Bispecific 3 and the combination of mAb1 and mAb28 caused comparable killing of target cells. These data indicate that Bispecific 3 can be used to mediate antigen-specific killing of target cells at lower doses.

Staphylococcus aureus Enterotoxin A ("SEA") assay

Previously frozen PBMCs were thawed and incubated with 0.1 mg/ml DNase I in PBS for 15 minutes, passed through a 40 μ m nylon mesh filter, and then plated in X-10 media at 100,000 cells per well of a 96 well round-bottom plate. Antibodies (Bispecific 3, KEYTRUDA, a combination of mAb1 and mAb28, and an isotype control) were added at final concentrations between 10 and 0.0001 nM, along with 10 ng/ml SEA. After 3 days of co-culture, IL-2 cytokine production was analyzed. As shown in **Figure 11**, Bispecific 3 induced more IL-2 than KEYTRUDA at all tested doses. Importantly, Bispecific 3 induced increased IL-2 production starting at lower concentrations of antibody as compared to both KEYTRUDA and the combination of mAb1 and mAb28.

PD-1 Expression Determination Assays

Previously frozen PBMCs were thawed and incubated with 0.1 mg/ml DNase I in PBS for 15 minutes, passed through a filter, and then plated at 1×10^6 cells/ml in hX-10 with 0.25 μ g/ml anti-CD3 (clone OKT3) and 0.25 μ g/ml anti-CD28 (clone CD28.2) for 3 days. After 3 days, the cells were adjusted to 2×10^6 cells/ml in hX-10 by spinning down the cells and removing excess media. The cells were then resuspended and plated at 1×10^6 cells/well (0.5 ml) in a 48 well plate. Antibodies (Bispecific 3, KEYTRUDA, a combination of mAb1 and mAb28, Atezolizumab, or isotype control) were then added to each well to get a final volume of 1 ml/well hX-10, with final concentrations of 0.01 nM or 1 nM antibody. After an overnight culture, the cells were collected into 1.5 ml Eppendorf tubes and lysed in 100 μ l of Lysis buffer containing a protease inhibitor cocktail. After spinning to clear the supernatants

of particulates, cleared supernatant was stored at -80°C until use for western blots. For Western blots, samples were adjusted to include 1X LDS sample buffer and 1X reducing agent, heated at 70°C for 10 minutes, then 20 ul per well was loaded onto 4-12% Bis-Tris gels. After the gel ran, it was transferred to nitrocellulose membrane, blocked with TBS-0.1% Tween-20 (TBST) containing 5% dry milk for 1 hour at room temperature, washed in TBST, and then incubated overnight at 4°C with anti-PD-1 (clone D4W2J, Cell Signaling Technologies) or anti-β-Actin (Clone 13E5, Cell Signaling Technologies) antibodies in TBST containing 5% bovine serum albumin. The following day, the membranes were washed in TBST, incubated with a HRP-conjugated anti-Rabbit IgG antibody, washed in TBST, then developed with SUPERSIGNAL™ Pico substrate. Chemiluminescent and white light images were collected on the Amersham Imager 600 and superimposed to generate the images shown. As shown in **Figure 12A**, Bispecific 3 is unique in its ability to cause the loss of cellular PD-1 expression by internalization and/or subsequent degradation of PD-1 and/or shedding.

In a subsequent assay, previously frozen PBMCs were thawed and incubated with 0.1 mg/ml DNase I in PBS for 15 minutes, passed through a filter, and then plated at 1×10^6 cells/ml in hX-10 with 0.25 µg/ml anti-CD3 (clone OKT3) and 0.25 µg/ml anti-CD28 (clone CD28.2) for 3 days. After 3 days, the cells were adjusted to 2×10^6 cells/ml in hX-10 by spinning down the cells and removing excess media. The cells were then resuspended and plated at 1×10^6 cells/well (0.5 ml) in a 48 well plate. Antibodies (Bispecific 3, KEYTRUDA, a combination of mAb 1 and mAb 28, Atezolizumab, Atezolizumab and KEYTRUDA, Bispecific 3 with 50 nM mAb 1, or isotype control) were then added to each well to get a final volume of 1 ml/well hX-10 with final concentration of 0.1 nM, 1 nM, or 10 nM antibody. After an overnight culture, the cells were collected into 1.5 ml Eppendorf tubes and lysed in 100 ul of Lysis buffer containing a protease inhibitor cocktail. After spinning to clear the supernatants of particulates, cleared supernatant was stored at -80°C until use for western blots. For western blots, samples were adjusted to include 1X LDS sample buffer and 1X reducing agent, heated at 70°C for 10 minutes, then 10 ul per well was loaded onto 4-12% Bis-Tris gels. After the gel ran, it was transferred to nitrocellulose membrane, blocked with TBS-0.1% Tween-20 (TBST) containing 5% dry milk for 1 hour at room temperature, washed in TBST, and then incubated overnight at 4C with anti-PD-1 (clone D4W2J, Cell Signaling Technologies) or anti-β-Actin (Clone 13E5, Cell Signaling Technologies) antibodies TBST containing 5% bovine serum albumin. The following day, the membranes were washed in TBST, incubated with a HRP-conjugated anti-Rabbit IgG antibody, washed

in TBST, then developed with SUPERSIGNAL™ Pico substrate. Chemiluminescent and white light images were collected on the Amersham Imager 600 and superimposed to generate the images shown. As shown in **Figures 12B-12C**, Bispecific 3 is unique in its ability to cause the loss of cell-surface PD-1 expression through internalization and/or subsequent degradation of PD-1 and/or shedding of PD-1. Additionally, when the anti-PD-L1 antibody, mAb1, was added at 50 nM to wells containing Bispecific 3, the ability of Bispecific 3 to drive loss of cell-surface PD-1 expression by PD-1 internalization and/or subsequent degradation of PD-1 and/or shedding was lost. This suggests that both arms of Bispecific 3 should be engaged to drive loss of cell-surface PD-1 expression by internalization and/or degradation of PD-1 and/or shedding.

Figure 12D shows that treatment with Bispecific 3 increases the amount of PD-1 in the supernatant when both binding arms of the bispecific are engaged concurrently. This effect is lost when the PD-L1 targeting arm is blocked by mAb1, the parent PD-L1 arm antibody. This suggests that Bispecific 3 increases shedding of PD-1 into the supernatant.

The effect of valency versus the sequence of the binding arms was next investigated. A new bispecific was generated (Bispecific 5) having a first N-terminal Fab that binds PD-L1 derived from the VH and VL sequences of mAb1, and a second N-terminal Fab that binds PD-1 derived from the VH and VL sequences of mAb28. In other words, while they share the same VH and VL sequences for binding to PD-1 and PD-L1, Bispecific 5 has one monovalent arm binding PD-L1 and one monovalent arm binding PD-1, as compared to Bispecific 3, which has bivalent arms binding PD-L1 and bivalent arms binding PD-1.

40×10^6 PBMCs were treated with 0.25 $\mu\text{g/ml}$ anti-CD3 and 0.25 $\mu\text{g/ml}$ anti-CD28 for 3 days in a T75 flask in hX-10 media containing IL-2 and IL-7. Cells were incubated for 3 days in the flask with no manipulation. On Day 3, the cells were collected without washing away any of the old media. Cells were plated in wells of a 48-well plate, at a final volume of 1 ml, with 1×10^6 cells/well and antibodies at 0.01 nM, 0.1 nM, 1 nM, and 10 nM. After an overnight incubation, cells were collected into 1.5 ml tubes, with a wash to collect all cells from the well, then lysed and used for western blotting. Western blots were run for PD-1 and Actin. **Figure 12E** demonstrates that the valency of the binding arms influences the degree of the loss of PD-1 expression. As shown, loss of PD-1 expression starts to occur at higher doses of Bispecific 5 (monovalent binding arms, total valency= 2 or bivalent) versus Bispecific 3 (bivalent binding arms, total valency=4 or tetravalent), suggesting that the increased valency of Bispecific 3 is responsible for this difference.

Next, the effect(s) of ADAM/MMP inhibition was examined using Batimastat, a broad-spectrum inhibitor of multiple MMPs and ADAMs, which are sheddases or proteases responsible for cleaving proteins off the plasma membrane of cells. Briefly, 40×10^6 PBMCs were treated with $0.25 \mu\text{g/ml}$ anti-CD3 and $0.25 \mu\text{g/ml}$ anti-CD28 for 3 days in a T75 flask in hX-10 media containing IL-2 and IL-7. Cells were incubated for 3 days in the flask with no manipulation. On Day 3, the cells were collected without washing away any of the old media. Cells were plated in wells of a 48-well plate, at a final volume of 1 ml, with 1×10^6 cells/well. At least $\frac{1}{2}$ hour before antibody addition, Batimastat or DMSO vehicle were added at increasing concentrations of $0 \mu\text{M}$, $1.25 \mu\text{M}$, $2.5 \mu\text{M}$, $5 \mu\text{M}$, and $10 \mu\text{M}$ and pre-incubated at 37°C to look at the consequence of ADAM/MMP inhibition. Isotype control and Bispecific 3 were then added at 1 nM. After an overnight incubation, cells were collected into 1.5 ml tubes, with a wash to collect all cells from the well, then lysed and used for western blotting. Western blots were run for PD-1 and Actin. **Figure 12F** demonstrates that pretreatment with Batimastat, a broad-spectrum inhibitor of multiple MMPs and ADAMs, greatly reduces the amount of cell-associated PD-1 loss, suggesting that PD-1 loss or shedding is due to cleavage by an MMP or ADAM protease. **Figure 12G** suggests that Bispecific 3 drives loss of cell-surface PD-1 expression primarily when it binds to PD-1 and PD-L1 that are in the trans configuration, *i.e.*, are being expressed by different cells. Given that PD-1 and PD-L1 can be expressed on the same cell, it was investigated whether Bispecific 3 binding to PD-1 and PD-L1 in *cis* results in loss of PD-1 expression or PD-1 shedding, or whether the binding by Bispecific 3 needs to be in *trans*, with Bispecific 3 bridging a first cell, such as a tumor cell expressing PD-L1, and a second cell, such as aT effector cell expressing PD-1. Jurkat cells expressing only PD-1, only PD-L1, or both PD-1 and PD-L1 were used in experiments. Briefly, a total of 0.5×10^6 Jurkat cells expressing PD-1, Jurkat cells expressing PD-1 and PD-L1, or a 1:1 mix of PD-1-only or PD-L1 only-expressing Jurkat cells were treated with isotype control or Bispecific 3 at 0.01, 0.1 and 1 nM. After an overnight incubation, cells were collected into 1.5 ml tubes, with a wash to collect all cells from the well, then lysed and used for western blotting. Western blots were run to determine PD-1 and Actin levels.

In vivo function of Bispecific 3

Day 13 expanded CMV antigen specific CD8⁺ T cells from an HLA A02:01 donor were collected and then mixed with K562 cells expressing HLA-A02:01, CMV protein pp65-IRES-GFP, and PD-L1 ("KACP") at a ratio of 2:1 KACP:CMV T cell and Matrigel such that 100 μl included 1X Matrigel, 5×10^6 KACP cells, and 2.5×10^6 CMV T cells. NSG mice were

implanted subcutaneously on their flank with 100 μ L of prepared Matrigel-KACP-CMV T cell mixture per mouse. Antibody dosing was started on the day of implantation and given at equimolar amounts (200 μ g for each monoclonal, 333 μ g for Bispecific 3) and mice were re-dosed with antibody every 3 days. Treatment groups included a control group that was given KACP tumor cells in Matrigel alone ("No T cell transfer"), and groups given isotype, KEYTRUDA, mAb1 and mAb28 (mAb1+mAb28), or Bispecific 3. There were 5 mice per group, and mice were monitored daily, with tumors measured twice a week. The protocol is schematically depicted in **Figure 13A**. As shown in **Figure 13B**, the No T cell transfer group had tumors that grew more aggressively than any group containing T cells. In this model, KEYTRUDA gave no benefit in delaying KACP tumor growth as compared to the isotype control. Both Bispecific 3 and the combination mAb1 and mAb28 groups had significant delays in tumor growth as compared to both the isotype and KEYTRUDA groups. At day 24, there was a significant divergence between the Bispecific 3 group and the group treated with a combination of mAb1 and mAb28, with Bispecific 3 causing a delay in tumor growth as compared to the combination.

Example 6: *In vivo* studies for pharmacological investigation of Bispecific 3

Co-inoculation model of K562-A2-CMV-PD-L1 target cells and CMV T-cells in NSG mice

In two separate studies with the protocol schematically depicted in **Figure 13A**, NSG female mice were co-injected subcutaneously (s.c.) with 100 μ L Matrigel containing 5×10^6 K562-A2-CMV-PD-L1 ("KACP") cells and either 2.5×10^6 (**Figures 13B and 14A**; effector: target ratio = 0.5:1) or 5×10^6 (**Figure 14B**; effector: target ratio = 1:1) CMV-specific T cells expanded *in vitro* from the same donor. Mice were blind-grouped the day of inoculation in 5 groups of 5 mice per group, or 10 mice per group respectively. To determine the anti-tumor activity of the human T cells, in both studies the first group of mice were injected only with tumor cells. The second group received human IgG1 isotype control antibodies (0.2 mg Q3Dx5). The third group received KEYTRUDA (0.2 mg Q3Dx5), the fourth group was treated with Bispecific 3 (0.333 mg Q3Dx5), and the fifth group received a combination of the anti-PD-1 (mAb28) and anti-PD-L1 (mAb1) antibodies (0.2 mg each Q3Dx5). All antibodies were injected i.p. and dosing started on implantation day. Anti-tumor activity was determined by tumor growth monitored by tumor volume measurements, body weight loss, and overall survival. Tumor size and body weight were measured 2-3 times per week, with mice euthanized when tumors were approaching 2000 mm³ or mice lost 20% of body weight. Data was analyzed and graphed using Graph Pad Prism software. As illustrated in **Figures**

14A and 14B, Bispecific 3 displayed increased anti-tumor efficacy as compared to the different monoclonal antibodies tested.

Transplantable syngeneic mouse models

Breast cancer EMT-6 cells (5×10^4 /mouse) were implanted in the mammary fat pad of BALB/c female mice. When tumors were established (tumor vol approximately 50 mm^3), mice were grouped (n=8) and treated with human IgG1 isotype control (0.2 mg Q3Dx3) or Bispecific 3 (0.333 mg Q3Dx3). Antibodies were delivered by i.p. injections.

Using a similar study design, bladder cancer MB-49 cells (5×10^5 /mouse) were injected s.c. in female C57BL/6 mice. When tumors were established (tumor volume approximately 75 mm^3) mice were grouped (n=8) and treated with human IgG1 isotype control (0.2 mg Q3Dx3) or Bispecific 3 (0.333 mg Q3Dx3). Antibodies were delivered by i.p. injections.

For both studies, tumor size and body weight were measured 2-3 times per week, with mice euthanized when tumors were approaching 2000 mm^3 or mice lost 20% of body weight. Results were plotted, graphed, and analyzed by Graph Pad Prism software. Bispecific 3 showed greater anti-tumor efficacy in the EMT-6 breast cancer cell model (Figure 15A) and in the MB-49 cell model (Figure 15B) as compared to the isotype control-treatment.

Engineered transplantable syngeneic models in transgenic mice

Mouse colon cancer MC-38 cells engineered to express human PD-L1 (MC-38-hPD-L1) were injected s.c. in C57BL/6 female mice in which the extracellular domains of PD-1 and PD-L1 were replaced with human PD-1 and PD-L1, while the transmembrane and signaling domains of the receptor-ligand pair were not modified. The genetic knock-in of human PD-1 and PD-L1 allowed for testing our bispecific PD-1xPD-L1 antibodies head to head with KEYTRUDA, which does not interact with mouse PD-1, and thus, cannot be evaluated in syngeneic mouse models. Mice with established MC-38-hPD-L1 tumors were grouped (n=8) and treated i.p. with isotype control (0.2 mg Q3Dx3), KEYTRUDA (0.2 mg Q3Dx3), or Bispecific 3 (0.333 mg Q3Dx3). Treatment with Bispecific 3 controlled MC-38-hPD-L1 tumor growth significantly better than isotype control- or KEYTRUDA treatments (Figure 16A). In addition, Bispecific 3 treatment resulted in an increase in survival in the MC-38-hPD-L1 tumor mice as compared to the isotype control- or KEYTRUDA-treated mice (Figure 16B).

In a separate study, C57BL/6 human PD-1/PD-L1 transgenic female mice were injected s.c. with 1×10^5 B16F10-hPD-L1 cells, an extremely aggressive mouse melanoma cell line. Mice were grouped (n=8) as soon as the melanoma was visible (day 4 after tumor

cell inoculation) in 5 groups which received the following treatment. Group 1 received isotype control human IgG1 (0.2 mg Q3Dx3), group 2 Avelumab (0.2 mg Q3Dx3), group 3 KEYTRUDA (0.2 mg Q3Dx3), group 4 Bispecific 3 (0.333 Q3Dx3), and group 5 combination of KEYTRUDA and Avelumab (0.2 mg each Q3Dx3). For both studies tumor size and body weight were measured 2-3 times per week, and mice euthanized when tumors were approaching 2000 mm³ or mice lost 20% of body weight. Survival was recorded and analyzed. Data were plotted, graphed, and analyzed by Graph Pad Prism software. As illustrated in **Figures 17A and 17B**, Bispecific 3 was significantly more effective in delaying tumor growth in the B16F10-hPD-L1 mice as compared to the other treatment groups tested. In addition, B16F10-hPD-L1 mice treated with Bispecific 3 survived longer on average than mice receiving the other tested treatments (**Figure 17C and Figure 17E**).

Example 7: Bispecific 3 has monoclonal-like DLP's and parental-like binding

Figures 18A-18D demonstrate that Bispecific 3 has drug-like properties (DLP's) similar to a well-behaved monoclonal antibody and maintains parental PD-1 and PD-L1 binding. **Figure 18A** shows that Bispecific 3 shows similar binding to CHO cells expressing human PD-1 as parental clone mAb28 (top), and to CHO cells expressing human PD-L1 as parental clone mAb1 (bottom). **Figure 18B** shows that Bispecific 3 shows similar binding to CHO cells expressing cynomolgus PD-1 as parental clone mAb28 (top), and to CHO cells expressing cynomolgus PD-L1 as parental clone mAb1 (bottom). **Figure 18C** shows that Bispecific 3 shows similar binding to CHO cells expressing mouse PD-1 as parental clone mAb28 (top), and to CHO cells expressing mouse PD-L1 as parental clone mAb1 (bottom). **Figure 18D** shows a size-exclusion chromatography trace of Bispecific 3 after Protein A chromatography (top) demonstrating a single peak with greater than 98% purity and a differential scanning fluorimetry (DSF) trace of Bispecific 3 (bottom) demonstrating that the molecule has high thermal stability.

SEQUENCES

SEQ ID NO: 114 (GenBank accession number NP_005009.2, UniProt Q15116 – full-length human PD-1 precursor)

```
mqipqapwpv vwavlqlgwr pgwfldspdr pwnpptfspa llvvtgedna tftcsfsnts
esfvlnwyrn spsnqtdkla afpedrsqpg qdcfrfrvtql pngrdfhmsv vrarrndsgt
ylcgaislap kaqikeslra elrvterrae vptahpspsp rpagqfqtlv vgvvggllgs
```

lvlllvwvlav icsraargti garrtgqplk edpsavpvfs vdygeldfqw rektpeppvp
cvpeqteyat ivfpgmgts sparrgsadg prsaqplrpe dghcswpl

SEQ ID NO: 115 (GenBank accession number NP_054862.1, UniProt Q9NZQ7 – human PD-L1)

5 mrifavfifm tywhllnaft vtvpkdlyvv eygsnmtiec kfpvekqldl aalivyweme
dkniiqfvhg eedlkvqhss yrqrarllkd qlslgnaalq itdvklqdag vyrcmisygg
adykritvkv napynkinqr ilvvdptse heltcqaegy pkaeviwts dhqvlsgktt
ttnskreekl fnvtstlrin tttneifyct frfldpeenh taelvipelp lahppnerth
lvilgaillc lgvaltfifr lrkgrmmdvk kcgiaqdtnsk kgsdthleet

10

SEQ ID NO: 116 (GenBank accession number NP_079515.2, UniProt Q9BQ51 – human PD-L2)

15 miflllmlsl elqlhqiaal ftvtvpkely iiehgsnvtl ecnfdtgshv nlgaitaslq
kvendtsphr eratllleeql plgkasfhip qvqvrdeggy qciiiygvaw dykyltlkvk
asyrkinthi lkvpemdeve ltcqatgypl aevswpnvsv pantsshrtp eglyqvtsvl
rlkpppgrnf scvfwnthvr eltlasidlg sqmeprthpt wllhifipfc iiafifiatv
ialrkqlcqk lysskdttkr pvtttkretn sai

WE CLAIM:

1. A multispecific antigen-binding construct comprising at least two antigen-binding arms, wherein:

a first arm binds PD-1 and comprises a heavy chain variable region and a light chain variable region, the heavy chain variable region of the first arm comprising a CDRH1 of SEQ ID NO: 77, a CDRH2 of SEQ ID NO: 71, and a CDRH3 of SEQ ID NO: 75, and

a second arm binds a PD-1 ligand and comprises a heavy chain variable region and a light chain variable region, the heavy chain variable region of the second arm comprising a CDRH1 of SEQ ID NO: 1, a CDRH2 of SEQ ID NO: 7, and a CDRH3 of SEQ ID NO: 8;

wherein the light chain variable region of the first arm and the light chain variable region of the second arm each comprise a CDRL1 of SEQ ID NO: 9, a CDRL2 of SEQ ID NO: 5, and a CDRL3 of SEQ ID NO: 10; and

wherein the multispecific antigen-binding construct blocks the interaction of PD-1 and the PD-1 ligand.

2. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO: 90.

3. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 90.

4. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the second arm comprises an amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO: 55.

5. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the second arm comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 55.
6. The multispecific antigen-binding construct of claim 1, wherein the first and the second antigen-binding arms each comprise a common light chain variable region.
7. The multispecific antigen-binding construct of claim 1, wherein the construct comprises at least two arms that bind PD-1 and at least two arms that bind PD-L1.
8. The multispecific antigen-binding construct of claim 1, wherein the construct comprises a bivalent antibody specific for PD-1, and a bivalent antibody specific for PD-L1, wherein the bivalent antibody specific for PD-1 and the bivalent antibody specific for PD-L1 are linked.
9. The multispecific antigen-binding construct of claim 1, wherein the heavy chain of the first arm has an amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO: 100; and wherein the light chain of the first arm and the second arm has an amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO: 101.
10. The multispecific antigen-binding construct of claim 1, wherein heavy chain of the first arm has an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 100, and wherein the light chain of the first arm and the second arm have an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 101.
11. The multispecific antigen-binding construct of claim 1, wherein the heavy chain of the first arm has an amino acid sequence of SEQ ID NO: 100, and wherein the light chain of

the first arm and the second arm have an amino acid sequence of SEQ ID NO: 101.

12. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 90.

13. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the first arm comprises an amino acid sequence that is at least 99% identical to the amino acid sequence of SEQ ID NO: 90.

14. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the first arm comprises the amino acid sequence of SEQ ID NO: 90.

15. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the second arm comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 55.

16. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the second arm comprises an amino acid sequence that is at least 99% identical to the amino acid sequence of SEQ ID NO: 55.

17. The multispecific antigen-binding construct of claim 1, wherein the heavy chain variable region of the second arm comprises the amino acid sequence of SEQ ID NO: 55.

18. The multispecific antigen-binding construct of claim 1, wherein the heavy chain of the first arm has an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 100, and wherein the light chain of the first arm and the second arm have an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 101.

19. The multispecific antigen-binding construct of claim 1, wherein the heavy chain of the first arm has an amino acid sequence that is at least 99% identical to the amino acid sequence of SEQ ID NO: 100, and wherein the light chain of the first arm and the second arm have an amino acid sequence that is at least 99% identical to the amino acid sequence of SEQ ID NO: 101.

20. The multispecific antigen-binding construct of claim 1, wherein the first arm binds PD-1 expressed by an immune cell.

21. The multispecific antigen-binding construct of claim 1, wherein the second arm binds a PD-1 ligand expressed by a second cell.

22. The multispecific antigen-binding construct of claim 21, wherein the second cell is a tumor cell and the PD-1 ligand is PD-L1.