

MULTIVALENT PD-L1 BINDING COMPOUNDS FOR TREATING CANCER**BACKGROUND OF THE INVENTION****Technical Field**

[0002] This invention relates to methods and materials for treating cancer. The invention provides methods and materials for making multivalent PD-L1 binding compounds. This invention also provides methods and materials for expressing PD-1 polypeptides in cells of a mammal having cancer. In particular, the invention relates to compositions comprising PD-1 polypeptides, the PD-1 polypeptides may be in the form of one or more multivalent programmed death-ligand 1 (PD-L1) binding compounds. In some cases, a multivalent PD-L1 binding compound can include two or more programmed cell death protein 1 (PD-1) polypeptides (and/or fragments thereof having the ability to bind PD-L1). The compositions comprising PD-L1 binding compounds are administered to a mammal having cancer in an amount effective to treat the cancer and, optionally, are administered in combination with one or more cancer therapies.

[0003] PD-L1, also called B7-H1, is an immune checkpoint protein that regulates the immune system through its binding of the PD-1 receptor. In the tumor microenvironment, overexpression of PD-L1 on tumor cells helps suppress antitumor immunity (Dong *et al.*, *Nat Med.* 8:793-800, (2002); Hatanishi *et al.*, *Int. J. Clin. Oncol.* 21:462-473 (2016); Dong *et al.*, *Nat. Med.* 5:1365-1369 (1999); Chen *et al.*, *J. Clin. Invest.* 125:3384-3391 (2015); He *et al.*, *Sci. Rep.* 5:13110 (2015); Chen *et al.*, *Clin. Cancer Res.* 18:6580-6587 (2012); Ohaegbulam *et al.*, *Trends Mol. Med.* 21:24-33 (2015); and Postow *et al.*, *J. Clin. Oncol.* 33:1974-1982 (2015)).

[0004] Antigen-presenting cells (APC) take up antigens released from cancer cells and present them to T cells. Cancer cells can also present antigens to activated T cells in the context of the major histocompatibility complex. Upon T cell activation, PD-1 receptors are expressed on T cells and inhibit immune responses by engagement of PD-L1 and PD-L2 ligands on APC and PD-L1 on cancer cells. Therefore, monoclonal antibody (mAb)-mediated specific blockade of the PD-1/PD-

L1/PD-L2 pathway can enhance anti-tumor immunity. In addition to T cells and APC, PD-1 and PD-L1 can be induced on other immune cells.

[0005] In the cancer disease state, the interaction of the PD-L1 which is present on a PD-L1 positive cancer cell with the PD-1 which is present on a T-cell can reduce T-cell function signals to prevent the immune system from attacking the PD-L1 positive cancer cell. Soluble PD-1 (sPD-1) can act as a decoy by binding to PD-L1 present on PD-L1 positive cancer cells. For example, when sPD-1 is bound to PD-L1 present on a PD-L1 positive cancer cell, the PD-L1 is not free to interact with a PD-1 present on a T-cell, thereby allowing the T-cell to function by attacking the PD-L1 positive cancer cell.

BRIEF SUMMARY OF THE INVENTION

[0006] This invention provides methods and materials for making one or more multivalent PD-L1 binding compounds. This invention provides compositions comprising PD-L1 binding compounds comprising PD-1 polypeptides, the PD-1 polypeptides may be in the form of one or more multivalent PD-L1 binding compounds. The PD-L1 binding compounds can act as a decoy by binding to PD-L1 present on PD-L1 positive cancer cells.

[0007] This invention provides methods and materials comprising PD-1 polypeptides, the PD-1 polypeptides may be in the form of one or more multivalent PD-L1 binding compounds which are administered to a mammal having cancer to treat the mammal. In some cases, a multivalent PD-L1 binding compound includes two or more amino acid segments that can bind PD-L1 (e.g., PD-1 polypeptides and/or fragments thereof). A multivalent PD-L1 binding compound includes two or more PD-1 polypeptides (or fragments thereof having the ability to bind PD-L1) such that the compound can bind two or more PD-L1 polypeptides. This invention also provides methods of making PD-L1 binding compounds and compositions comprising recombinant Ads comprising at least one amino acid segment comprising

a PD-1 polypeptide which forms a PD-L1 binding compound or a multivalent PD-L1 binding compound described herein.

[0008] In an embodiment, mouse PD-1 polypeptides and/or human PD-1 polypeptides fused to a scaffold polypeptide can form a polypeptide conjugate (*e.g.*, a polypeptide conjugate including a plurality of (*e.g.*, two or more) associated amino acid chains) such that when the scaffold polypeptide fused to one or more PD-1 polypeptides is in a polypeptide conjugate, the polypeptide conjugate can form a multivalent PD-L1 binding compound that includes two or more PD-1 polypeptides.

[0009] In an embodiment, a polypeptide conjugate can include three amino acid chains, where the scaffold polypeptide can be a sigma-1 polypeptide derived from *Mammalian orthoreovirus 3*, and where the polypeptide conjugate can include three PD-1 polypeptides.

[00010] In an embodiment, a polypeptide conjugate can include four amino acid chains, where the scaffold polypeptide can be a streptavidin polypeptide, and where the polypeptide conjugate can include four PD-1 polypeptides.

[00011] This invention also provides compositions comprising recombinant Ads comprising nucleic acids encoding PD-1 polypeptides which bind PD-L1. In an embodiment, a mouse PD-1 polypeptide and/or a human PD-1 polypeptide is fused to a Vitamin K-dependent gamma-carboxyglutamic domain of a factor X single-chain antibody polypeptide (a GLA or GLA-EGF domain of an FX polypeptide) and is present on adenoviral hexon polypeptides such that when the polypeptide is present on two or more viral hexon polypeptides present on the capsid of a virus particle, the virus particle can form a multivalent PD-L1 binding compound that includes from about 240 to about 720 PD-1 polypeptides.

[00012] This invention provides methods and materials for treating cancer by administering one or more recombinant adenoviruses (Ads) expressing a PD-1 protein and/or amino acid segments which bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof). The recombinant Ads expressing PD-1 may be

administered in combination with a cancer immunotherapy according to a therapeutic treatment regime.

[00013] Using multivalent PD-L1 binding compounds (e.g., as compared to monomeric PD-1 polypeptides) can increase the efficacy of PD-1 to neutralize PD-L1 present on PD-L1 positive cancer cells, can prevent PD-L1 positive cancer cells from escaping the immune system, and/or can allow anti-cancer agents (e.g., cancer immunotherapies) to more effectively target PD-L1 positive cancer cells. In some cases, adenovirus presentation of PD-1 polypeptides can be used to retarget therapeutic Ads to PD-L1 on a cell for nucleic acid delivery or to kill PD-L1-expressing cells by oncolytic cell death.

[00014] In an embodiment, a PD-L1 binding compound also can include a targeting molecule. A targeting and cell fusion molecule can be a viral polypeptide such as a measles virus (MV) hemagglutinin (H) polypeptide, a MV fusion (F) polypeptide, or a vesicular stomatitis virus (VSV) glycoprotein (G) polypeptide.

[00015] The PD-L1 binding compound also can include one or more therapeutic polypeptides. The therapeutic polypeptide is selected from a 4-1BB ligand (4-1BBL) polypeptide, a OX40 ligand (OX40L) polypeptide, a CD40 ligand (CD40L) polypeptide, or a granulocyte-macrophage colony-stimulating factor (GM-CSF) polypeptide. The therapeutic polypeptide may also be a polypeptide which activates glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein (GITR) signaling.

[00016] The PD-L1 binding compound also can include a detectable polypeptide. The detectable polypeptide can be a green fluorescent protein (GFP) or a luciferase polypeptide.

[00017] The invention encompasses recombinant vectors for the expression of PD-L1 binding compounds comprising nucleic acids encoding PD-1 polypeptides, the PD-1 polypeptides may be expressed with one or more targeting polypeptides,

and/or one or more therapeutic polypeptides, and/or proteins (e.g., collagen, elastin, laminin, and fibrinogen). The PD-1 polypeptides may be expressed as fusion proteins with heterologous polypeptides. The nucleic acids encoding PD-1 polypeptides, as well as the nucleic acids encoding targeting polypeptides, therapeutic polypeptides and proteins, may be comprised in expression vectors, optionally comprising expression cassettes, which allow for expression of the polypeptides in prokaryotic or eukaryotic cells. The vector can be a viral vector. The viral vector can be an Ad, an adeno-associated virus (AAV), or a lentivirus. The viral vector may be an Ad selected from Ad657, Ad6/57/6, and variants thereof. The Ad may be a Conditionally Replicating Ad (CRAd). The viral vector can be an oncolytic viral vector.

[00018] In another aspect, the invention encompasses viral vectors for the expression of PD-L1 binding compounds comprising nucleic acids encoding an amino acid chain including a PD-1 polypeptide and a scaffold polypeptide as described herein (e.g., a polypeptide conjugate that can include a plurality of amino acid chains, where each amino acid chain includes a PD-1 polypeptide and a scaffold polypeptide; and where the plurality of amino acid chains can form the polypeptide conjugate).

[00019] In another aspect, the invention encompasses methods for treating a mammal having cancer. The methods can include, or consist essentially of, administering a composition comprising a PD-L1 binding compound to a mammal having cancer.

[00020] The method of the invention comprises administering an effective amount of compositions comprising PD-L1 binding compounds described herein. In an embodiment, the PD-L1 binding compounds are administered in combination with one or more cancer therapeutics to the mammal, whereby the number of cancer cells present in the mammal is reduced. The one or more cancer therapeutics may include an immunotherapy which targets PD-1 or PD-L1. The immunotherapy may

be selected from nivolumab, pembrolizumab, atezolizumab, avelumab, cemiplimab, and durvalumab.

[00021] In another aspect, the invention relates to methods for treating a mammal having cancer comprising, or consisting essentially of, administering a composition comprising a multivalent PD-L1 binding compound to a mammal having cancer. The multivalent PD-L1 binding compound may comprise a polypeptide conjugate described herein (e.g., a polypeptide conjugate including a plurality of amino acid chains, where each amino acid chain includes a PD-1 polypeptide and a scaffold polypeptide). The cancer may be a prostate cancer, breast cancer, ovarian cancer, lung cancer (e.g., a non-small cell lung cancer), hepatocellular carcinoma, pancreatic cancer, kidney cancer, melanoma, brain cancer, colon cancer, lymphoma, myeloma, lymphocytic leukemia, or myelogenous leukemia. The administering can include systemic or local administration (e.g. intravenous, intratumoral, intramuscular, intraorgan, intralymph node administration).

[00022] An aspect of the invention relates to multivalent programmed cell death protein ligand 1 (PD-L1) binding compounds comprising a plurality of amino acid chains, wherein each amino acid chain comprises at least one programmed cell death protein 1 (PD-1) polypeptide.

[00023] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the multivalent PD-L1 binding compound is a polypeptide conjugate comprising a scaffold polypeptide selected from Ig polypeptides, sigma-1 polypeptides and streptavidin polypeptides.

[00024] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the multivalent PD-L1 binding compound is a polypeptide conjugate comprising more than one scaffold polypeptide.

[00025] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the PD-1 polypeptide is a human PD-1 or a murine PD-1.

[00026] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the plurality of amino acid chains comprise a therapeutic polypeptide, a targeting polypeptide or an antigenic polypeptide.

[00027] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the targeting polypeptide is selected from a measles virus hemagglutinin (MVH) polypeptide, a measles virus fusion (MVF) polypeptide, and a vesicular stomatitis virus glycoprotein (VSVG) polypeptide.

[00028] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the therapeutic polypeptide is selected from a 4-1BB ligand (4-1BBL) polypeptide, a OX40 ligand (OX40L) polypeptide, a CD40 ligand (CD40L) polypeptide, a granulocyte-macrophage colony-stimulating factor (GM-CSF) polypeptide and a GITR agonist.

[00029] A further aspect of the invention relates to such a multivalent PD-L1 binding compound wherein each amino acid chain comprising at least one programmed cell death protein 1 (PD-1) polypeptide is associated with a recombinant Adenovirus (Ad) and the plurality of amino acid chains are present on a coat polypeptide of the recombinant Ad.

[00030] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the recombinant Ad comprises capsid hexon polypeptides of an Ad strain Ad6 and at least one capsid hexon hypervariable region (HVR) polypeptide from Ad strain Ad57.

[00031] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the capsid hexon polypeptides of an Ad strain Ad6 comprise HVR polypeptides 1-7 from Ad strain Ad57.

[00032] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the capsid hexon polypeptides of an Ad strain Ad6 comprise HVR polypeptides 2-6 from Ad strain Ad57.

[00033] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the programmed cell death protein 1 (PD-1) polypeptide is human PD-1.

[00034] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein the PD-1 polypeptide is fused to a Vitamin K-dependent gamma-carboxyglutamic domain of a factor X single-chain antibody polypeptide (a GLA domain of an FX polypeptide).

[00035] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein each amino acid chain comprises a targeting molecule selected from a measles virus hemagglutinin (MVH) polypeptide, a measles virus fusion (MVF) polypeptide, and a vesicular stomatitis virus glycoprotein (VSVG) polypeptide.

[00036] A further aspect of the invention relates to such a multivalent PD-L1 binding compound, wherein each amino acid chain comprises one or more therapeutic polypeptides selected from a 4-1BB ligand (4-1BBL) polypeptide, a OX40 ligand (OX40L) polypeptide, a CD40 ligand (CD40L) polypeptide, and a granulocyte-macrophage colony-stimulating factor (GM-CSF) polypeptide.

[00037] A further aspect of the invention relates to such a pharmaceutical composition comprising the multivalent PD-L1 binding compound and a pharmaceutically acceptable carrier.

[00038] A further aspect of the invention relates to a method of treating cancer in a subject in need thereof, comprising administering the multivalent PD-L1 binding compound.

[00039] A further aspect of the invention relates to such a method further comprising administering one or more cancer therapeutics to the mammal.

[00040] A further aspect of the invention relates to such a method wherein the cancer therapeutic is an immunotherapy which targets PD-1.

[00041] A further aspect of the invention relates to such a method wherein the immunotherapy is selected from the group consisting of nivolumab, pembrolizumab, atezolizumab, avelumab, cemiplimab, and durvalumab.

[00042] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[00043] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[00044] Figure 1A shows schematics of exemplary engineered multivalent PD-L1 binding compounds. A multivalent PD-L1 binding compound comprising a PD-1 polypeptide fused to an Ig polypeptide which is a dimeric polypeptide conjugate that includes two amino acid chains that each include one PD-1 polypeptide. A multivalent PD-L1 binding compound that is a trimeric polypeptide conjugate comprising three amino acid chains that each include one PD-1 polypeptide fused to a sigma polypeptide. A multivalent PD-L1 binding compound that is a tetrameric polypeptide conjugate comprises four amino acid chains that each include one PD-1 polypeptide fused to a streptavidin polypeptide.

[00045] An amino acid chain including one PD-1 polypeptide fused to a GLA domain of an FX polypeptide. The fusion proteins can be constructed with either entity on the N-terminus or the C-terminus. The fusion protein which is bound to the surface of an adenovirus particle generates a multivalent PD-L1 binding compound.

[00046] Figure 1B shows engineered PD-L1 binding compounds by introduction of PD-1 motifs into natural biopolymers like collagen, laminin, fibronectin, or elastin. Fusion of PD-1 to protein monomers and expression in cells will allow their assembly into multivalent polymers displaying many PD-L1-binding motifs.

[00047] Figure 2A shows nucleic acid vectors that can encode amino acid chains that can be used to generate multivalent PD-L1 binding compounds. Figure 2A shows schematics of a nucleic acid vector that can encode an amino acid chain that includes one murine PD-1 (mPD-1) polypeptide fused to an IgG polypeptide (top left), a nucleic acid vector that can encode an amino acid chain that includes one human PD-1 (hPD-1) polypeptide fused to an IgG polypeptide (top right), and an exemplary cloning strategy (bottom) for replacing nucleic acid encoding the IgG with nucleic acid encoding a GLA domain of a FX polypeptide to generate a nucleic acid vector that can encode an amino acid chain that includes a PD-1 polypeptide fused to a GLA domain of an FX polypeptide, or for replacing nucleic acid encoding the IgG with nucleic acid encoding a sigma-1 polypeptide to generate a nucleic acid vector

that can encode an amino acid chain that includes a PD-1 polypeptide fused to a sigma-1 polypeptide.

[00048] Figure 2B contains images of gels showing expression of an amino acid chain comprising a PD-1 polypeptide. Lane 1: Molecular Weight Marker (base pairs; bp); Lane 2: a PD-1 polypeptide fused to a GLA domain of an FX polypeptide; Lane 3: Molecular Weight Marker (bp); Lane 4: an amino acid chain including a PD-1 polypeptide fused to a sigma-1 polypeptide.

[00049] Figure 3 shows schematics of a nucleic acid vector that can encode an amino acid chain that includes a hPD-1 polypeptide fused to a GLA domain of an FX polypeptide (top left), a nucleic acid vector that can encode an amino acid chain that includes a hPD-1 polypeptide fused to a sigma-1 polypeptide (top right), and an exemplary cloning strategy (bottom) for replacing the nucleic acid encoding the hPD-1 polypeptide with nucleic acid encoding a mPD-1 polypeptide.

[00050] Figure 4 shows schematics of exemplary nucleic acid vectors that can encode amino acid chains that can be used to generate multivalent PD-L1 binding compounds.

[00051] Figure 5 shows schematics of exemplary adenoviral (Ad) vectors that can encode amino acid chains that can be used to generate multivalent PD-L1 binding compounds.

[00052] Figure 6 shows a schematic of a genome of an Ad having nucleic acid encoding an immune checkpoint polypeptide that can activate T cells (4-1BBL), nucleic acid encoding one or more PD-1 polypeptides (PD-1-X, where X is a dimeric, trimeric, tetrameric, or polymeric binding scaffold), and nucleic acid encoding an antigen such as a cancer antigen or an infectious disease antigen (HPV epitope). Also shown is a restriction site map of the Ad genome.

[00053] Figure 7 shows an alignment of Ad5, 6, and 57 showing variation in hexon and E3 regions. (A) A Pustell DNA alignment of the genomes of Ad6 and Ad57. Boxes indicate hexon and E3 regions where variation is highest between the two viruses. (B) ClustalW amino acid alignment of the hypervariable region in hexon proteins from Ad5, Ad6, and Ad57.

[00054] Figure 8 shows the construction of Ad657 by replacement of the Ad6 HVRs with Ad57 HVRs. Abbreviation: HVRs, hypervariable regions.

[00055] Figure 9 is a schematic of a replication competent Ad (RC-Ad), wherein E1 expression is controlled by the native E1 promoter; a variant CRAAd –Probasin-E1A (Ad-PB), wherein E1 expression is controlled by prostate-specific probasin promoter; CRAAd-dl1101, wherein p300 pathway binding ablated, susceptible to IFN pathway in normal cells; CRAAd-dl1107, wherein pRB binding ablated allows virus to kill cancer cells with RB pathway disruptions, but is repressed in RB+ normal cells; CRAAd-dl1101/07, wherein p300 pathway binding ablated, susceptible to IFN pathway pRB binding ablated allows virus to kill cancer cells with RB pathway disruptions, but is repressed in RB+ normal cells.

[00056] Figure 10 shows amino acid sequences of the N-terminal portion of the wild-type E1A polypeptide and the E1A N-terminus of the CRAAd variants, dl1101, dl1107 and dl1101/1107.

[00057] Figure 11 shows as schematic of different E3 immune evasion genes in Ad species C exemplar Ad6 and Ad species D exemplar Ad26. Both Ads express size and sequence variants of E3 12.5K, 6.7K, 19K, 10.4K (RID α), 14.5K (RID β), and 14.7K genes, as well as a depiction of the functions of these E3 encoded proteins.

[00058] Figure 12 depicts sites on Ad HVRs which may be modified, for example, by PEGylation or "BAPylation" with biotin acceptor peptides (BAPs).

[00059] Figure 13 is a schematic showing Ad therapeutic cycles. A) A schematic of serotype-switching with Ads. B) A schematic of an exemplary therapeutic cycle where Ad6 and Ad657 can be used for multiple rounds of treatment by serotype-switching in combination with covalent polymer conjugation.

[00060] Figure 14 shows conjugation of polyethylene glycol (PEG) to Ad657-HVR1-C. A) SDS-PAGE of Ad proteins with and without PEGylation. Arrows show size increases due to chemical attachment of PEG to hexon. B) Effects of targeted PEGylation by maleimide-PEG and non-targeting NHS-PEG on virus infection.

[00061] Figure 15 shows chimeric HVR constructs that combine different HVRs from different Ad serotypes to modulate natural interactions with cells and blood factors improve pharmacology combined with insertion of cell binding and cell detargeting peptides in different HVRs to change cell entry and cell avoidance. In this example, a single cysteine amino acid is inserted into the HVR1 and HVR5 of Ad657 to modulate pharmacology and allow targeted conjugation of polymers like polyethylene glycol or other moieties like imaging agents like fluorophores.

[00062] Figure 16 shows plasmid maps for representative CRAbs and peptide combinations. Shown are hexons as well as insertions of cell targeting peptides into individual HVRs.

[00063] Figure 17 shows plasmid maps for representative CRAbs and peptide combinations. Shown are hexons as well as insertions of cell targeting peptides into individual HVRs.

[00064] Figure 18 shows plasmid maps for representative CRAbs and peptide combinations. Shown are hexons as well as insertions of cell targeting peptides into individual HVRs.

[00065] Figure 19. PD-1 PD-L1 Blockade Assay - Fusion protein was purified from cells infected with pAd6/57/6-dl1107-DE3-RSV-hPD-1-HA-Ig-I on a protein A

column. This protein was titrated along side positive control, anti-PD-1 monoclonal antibody from Promega (Madison, WI, USA).

[00066] Figure 20. Tumor Growth Assessment - B16-CAR melanoma cells were injected subcutaneously in C57BL/6 mice and tumors were injected with 3e11 viral particles of the indicated vectors. CRAAd+PD-L1 decoy is pAd6/57/6-dl1107- Δ E3-RSV-hPD-1-HA-Ig-I. CRAAd+PD-L1 decoy + immune simulator represent tumors that were co-injected with pAd6/57/6-dl1107- Δ E3-RSV-hPD-1-HA-Ig-I and a second adenovirus expressing 4-1BBL.

DETAILED DESCRIPTION OF THE INVENTION

[00067] This invention provides methods and compositions for treating cancer comprising PD-1 polypeptides. The PD-1 polypeptides may be in the form of multivalent PD-L1 binding compounds.

[00068] In some cases, a multivalent PD-L1 binding compound comprises two or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof). For example, a multivalent PD-L1 binding compound that includes two or more PD-1 polypeptides (and/or fragments thereof having the ability to bind PD-L1) can bind two or more PD-L1 polypeptides. In some cases, a multivalent PD-L1 binding compound can include a single amino acid chain that includes two or more amino acid segments that can bind PD-L1.

[00069] In some cases, a multivalent PD-L1 binding compound can include a polypeptide conjugate including a plurality of (*e.g.*, two or more) amino acid chains that each include one or more amino acid segments that can bind PD-L1. For example, an amino acid chain that includes one or more amino acid segments that can bind PD-L1 can be a fusion polypeptide that includes one or more PD-1 polypeptides fused to a scaffold polypeptide that can form a polypeptide conjugate with one or more other polypeptides that include one or more amino acid segments that can bind PD-L1 (*e.g.*, a polypeptide conjugate including a plurality of associated amino acid chains). When the scaffold polypeptide fused to one or more PD-1

polypeptides is in a polypeptide conjugate, the polypeptide conjugate can include two or more PD-1 polypeptides.

[00070] In an embodiment, a virus particle comprising a PD-1 polypeptide may be in the form of a multivalent PD-L1 binding compound wherein two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more amino acid segments that can bind PD-L1.

[00071] In an embodiment, a PD-L1 binding compound comprises a recombinant virus which is genetically modified to express the PD-1 polypeptide, wherein the PD-1 polypeptide is displayed on the coat of the virus. In a further embodiment, the recombinant virus is genetically modified to express the PD-1 polypeptide in combination with one or more polypeptides which are heterologous to the recombinant virus backbone, for example, therapeutic polypeptides, targeting molecules/polypeptides and antigenic polypeptides.

[00072] In an embodiment, a PD-L1 binding compound comprises a recombinant Ad (i.e., Ad657, Ad6/57/6 and variants thereof) which is modified in one or more hexon hypervariable regions (HVRs) of the virus capsid protein and comprises at least one PD-1 polypeptide. In an embodiment, the recombinant Ad is further modified to comprise heterologous polypeptides such as targeting polypeptides, therapeutic polypeptides and/or antigens. The PD-1 polypeptides, as well as the heterologous polypeptides, are expressed upon viral replication. Upon virus assembly, the expressed polypeptides may be displayed on the surface of the virus as a component of the virus capsid structure.

[00073] In a further embodiment, a PD-L1 binding compound comprises a recombinant Ad which is genetically modified to express the PD-1 polypeptide as a fusion protein with one or more heterologous polypeptides, wherein the fusion protein is displayed on the capsid of the virus particle. Heterologous polypeptides may include a GLA domain from a FX polypeptide, CAR polypeptides, CD46 polypeptides, desmoglein polypeptides, integrin polypeptides, single-chain antibody

polypeptides, camelid antibody polypeptides, capsid polypeptides, and envelope binding polypeptides.

[00074] In some cases, an amino acid chain that includes one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide can be a fusion polypeptide including one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide. In an embodiment, the fusion protein of PD-1 and a heterologous polypeptide may be expressed in a host cell, purified and admixed with an Ad such that the fusion protein is bound to the surface of the Ad via covalent attachment of the heterologous polypeptide to the Ad. When a first fusion polypeptide including one or more PD-1 polypeptides fused to a polypeptide that can bind to a viral coat polypeptide is bound to a first viral coat polypeptide on a virus particle and a second fusion polypeptide including one or more PD-1 polypeptides fused to a polypeptide that can bind to a viral coat polypeptide is bound to a second viral coat polypeptide on that virus particle, the virus particle will include two or more PD-1 polypeptides.

[00075] In some cases, a PD-L1 binding compound can include a virus particle where one or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof). The PD-1 polypeptides may be expressed during viral replication and assembly and displayed on the coat of the virus. In an embodiment, the Ad (*i.e.*, Ad657, Ad6/57/6 and variants thereof) is modified in an HVR region to express the PD-1 polypeptide in combination with one or more heterologous polypeptides selected from therapeutic polypeptides, targeting polypeptides and antigenic polypeptides. The PD-1 polypeptide may be expressed as a fusion protein with one or more heterologous polypeptides. For example, a virus particle is genetically modified to express two or more amino acid chains that each include at least one PD-1 polypeptide and a heterologous polypeptide, wherein the amino acid chains are displayed on the surface of the virus particle as a component of the virus capsid thereby forming a multivalent PD-L1 binding compound. In some cases, a virus particle is genetically modified to express two or

more amino acid chains that each include a PD-1 polypeptide and is modified to express one or more amino acid chains that comprise a heterologous polypeptide, wherein the amino acid chains are displayed on the surface of the virus particle as a component of the virus capsid thereby forming a multivalent PD-L1 binding compound. In some cases, an amino acid chain includes two or more PD-1 polypeptides and a heterologous polypeptide that are displayed on the surface of the virus particle.

[00076] In an embodiment, an amino acid chain that comprises one or more PD-1 polypeptides can be a fusion protein that comprises one or more PD-1 polypeptides fused to a heterologous polypeptide. The heterologous polypeptide may bind to the surface of an Ad to coat the virus particle. In an embodiment, the fusion proteins are expressed in a host cell, purified and admixed with an Ad such that the fusion protein coats the surface of the Ad viral particle thereby forming a multivalent PD-L1 binding compound.

[00077] In some cases, a multivalent PD-L1 binding compound described herein can have increased affinity for PD-L1 (*e.g.*, as compared to a monomeric PD-1 polypeptide). In some cases, a multivalent PD-L1 binding compound described herein can have increased avidity for PD-L1 (*e.g.*, as compared to a monomeric PD-1 polypeptide).

[00078] This invention provides methods and materials for making compositions comprising PD-1 polypeptides and multivalent PD-L1 binding compounds, as well as methods and materials for making recombinant viruses comprising nucleic acid molecules that encode an amino acid chain that can be used to generate multivalent PD-L1 binding compounds described herein.

[00079] Multivalent PD-L1 binding compounds described herein (*e.g.*, a fusion polypeptide that includes one or two or more PD-1 polypeptides, or a polypeptide conjugate that includes two or more amino acid chains that each include one or more PD-1 polypeptides, or a virus particle where viral coat proteins comprise one or more

amino acid chains that include one or more PD-1 polypeptides, or a virus particle where one or more PD-1 polypeptides are bound to the surface of the virus particle) can include any appropriate number of amino acid segments that can bind PD-L1 (e.g., PD-1 polypeptides and/or fragments thereof that can bind PD-L1).

[00080] In some cases, a multivalent PD-L1 binding compound described herein can include two or more (e.g., 2, 3, 4, 5, 6, 240, 720, or more) amino acid segments that can bind PD-L1. In some cases, a multivalent PD-L1 binding compound described herein can include from about two to about 720 amino acid segments that can bind PD-L1. For example, when a multivalent PD-L1 binding compound described herein is a single amino acid chain that includes two or more amino acid segments that can bind PD-L1, the multivalent PD-L1 binding compound can include about 2, 3, 4, 5, 6, 7, or 8 PD-1 polypeptides (or fragments thereof that bind PD-L1). For example, when a multivalent PD-L1 binding compound described herein is a polypeptide conjugate including a plurality of amino acid chains that each include one or more amino acid segments that can bind PD-L1, the multivalent PD-L1 binding compound can include about 2, 3, 4, 5, 6, 7, or 8 PD-1 polypeptides (or fragments thereof that bind PD-L1).

[00081] For example, when a multivalent PD-L1 binding compound described herein is a virus particle where two or more viral coat polypeptides (e.g. capsid polypeptides) are modified to include one or more amino acid chains that include one or more amino acid segments that can bind PD-L1, the multivalent PD-L1 binding compound can include from about 240 PD-1 polypeptides to about 720 PD-1 polypeptides (or fragments thereof that bind PD-L1). In cases where a multivalent PD-L1 binding compound described herein includes two amino acid segments that can bind PD-L1 (or a virus particle where one or more viral coat polypeptides are modified to include one or more amino acid chains that include two or more amino acid segments that can bind PD-L1), the multivalent PD-L1 binding compound can be referred to as a dimeric or divalent PD-L1 binding compound. In cases where a multivalent PD-L1 binding compound described herein includes three amino acid segments that can bind PD-L1, the multivalent PD-L1 binding compound can be

referred to as a trimeric or trivalent PD-L1 binding compound. In cases where a multivalent PD-L1 binding compound described herein includes four amino acid segments that can bind PD-L1, the multivalent PD-L1 binding compound can be referred to as a tetrameric or tetravalent PD-L1 binding compound.

[00082] Multivalent PD-L1 binding compounds described herein can include any appropriate amino acid segment(s) that can bind PD-L1.

[00083] In an embodiment, an amino acid segment that can bind PD-L1 can include a PD-1 polypeptide. In some cases, an amino acid segment that can bind PD-L1 can include any fragment of a PD-1 polypeptide provided that the fragment retains the ability to bind PD-L1. In some cases, a PD-1 polypeptide can be a sPD-1 polypeptide. A PD-1 polypeptide can be from any appropriate animal. In some cases, a PD-1 polypeptide can be from a mammal. For example, a PD-1 polypeptide can be an hPD-1 polypeptide. For example, a PD-1 polypeptide can be a mPD-1 polypeptide. A PD-1 polypeptide can include any appropriate PD-1 polypeptide sequence. In some cases, a PD-1 polypeptide can be modified (*e.g.*, for higher affinity interactions with PD-L1). Exemplary PD-1 polypeptide sequences (and the nucleic acids encoding such polypeptides) can be as set forth in the National Center for Biotechnology Information (NCBI) databases at, for example, Accession No. AI928135 (Version AI928135.1), Accession No. AY238517 (Version AY238517.1), and Accession No. CR988122 (Version CR988122.1), Accession No. U64863 (Version U64863.1), Accession No. NM_008798 (Version NM_008798.2), and Accession No. NP_032824 (Version NP_032824.1). In some cases, a PD-1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO:5. In some cases, a PD-1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO:6.

[00084] In some cases, an amino acid segment that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof) can have a sequence that deviates from a wild type PD-1 polypeptide sequence (*e.g.*, a wild type PD-1 polypeptide having the amino acid sequence set forth in SEQ ID NO:5 or SEQ ID NO:6), sometimes

referred to as a variant sequence. For example, a PD-1 polypeptide sequence (and/or a fragment thereof having the ability to bind PD-L1) can have at least 80% sequence identity to SEQ ID NO:5 or SEQ ID NO:6. In some embodiments, an amino acid segment that can bind PD-L1 can have at least 85% sequence identity, 90% sequence identity, 95% sequence identity, or at least 99% sequence identity to SEQ ID NO:5 or SEQ ID NO:6. Percent sequence identity is calculated by determining the number of matched positions in aligned polypeptide sequences, dividing the number of matched positions by the total number of aligned amino acids, respectively, and multiplying by 100. A matched position refers to a position in which identical amino acids occur at the same position in aligned sequences. The total number of aligned amino acids refers to the minimum number of amino acids in a PD-1 polypeptide that are necessary to align the second sequence, and does not include alignment (e.g., forced alignment) with non-PD-1 sequences, such as those fused to a PD-1 polypeptide (e.g., amino acids from an IgG polypeptide, a streptavidin polypeptide, sigma-1, or a GLA domain of an FX polypeptide that is fused to a PD-1 polypeptide). The total number of aligned amino acids may correspond to the entire PD-1 sequence or may correspond to fragments of the full-length PD-1 sequence. Sequences can be aligned using the algorithm described by Altschul *et al.* (*Nucleic Acids Res.*, 25:3389-3402 (1997)) as incorporated into BLAST (basic local alignment search tool) programs, available at ncbi.nlm.nih.gov on the World Wide Web. BLAST searches or alignments can be performed to determine percent sequence identity between a PD-1 polypeptide and any other sequence or portion thereof using the Altschul *et al.* algorithm. BLASTN is the program used to align and compare the identity between nucleic acid sequences, while BLASTP is the program used to align and compare the identity between amino acid sequences. When utilizing BLAST programs to calculate the percent identity between a PD-1 sequence and another sequence, the default parameters of the respective programs are used.

[00085] In some cases, a multivalent PD-L1 binding compound can include a polypeptide conjugate including a plurality of amino acid chains that each include one or more amino acid segments that can bind PD-L1 (e.g., PD-1 polypeptides

and/or fragments thereof). As used herein, a plurality of amino acid chains that each include one or more amino acid segments that can bind PD-L1 refers to two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, or more) amino acid chains that each include one or more amino acid segments that can bind PD-L1. For example, an amino acid chain that includes one or more PD-1 polypeptides can be a fusion polypeptide that includes one or more PD-1 polypeptides fused to a scaffold polypeptide that can form a polypeptide conjugate with one or more other polypeptides that include one or more amino acid segments that can bind PD-L1 (*e.g.*, a polypeptide conjugate including a plurality of associated amino acid chains). When the scaffold polypeptide fused to one or more PD-1 polypeptides is in a polypeptide conjugate, the polypeptide conjugate can include two or more PD-1 polypeptides. In some cases, a polypeptide conjugate including a plurality amino acid chains that each include one or more PD-1 polypeptides can be a homomeric PD-1 polypeptide (*e.g.*, can include two or more of the same amino acid chains that each include one or more PD-1 polypeptides). In some cases, a polypeptide conjugate including a plurality amino acid chains that each include one or more PD-1 polypeptides can be a heteromeric PD-1 polypeptide (*e.g.*, can include two or more different amino acid chains that each include one or more PD-1 polypeptides).

[00086] An amino acid chain including one or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof) present in a polypeptide conjugate described herein (*e.g.*, a polypeptide conjugate including a plurality amino acid chains that each include one or more PD-1 polypeptides) can include one or more amino acid segments that can bind PD-L1 fused to any appropriate scaffold polypeptide. For example, an amino acid chain including one or more PD-1 polypeptides present in a polypeptide conjugate described herein can be a fusion polypeptide that includes one or more PD-1 polypeptides fused to any appropriate scaffold polypeptide. In some cases, a scaffold polypeptide can have the ability to form a polypeptide conjugate (*e.g.*, a polypeptide conjugate including a plurality of associated amino acid chains). For example, two or more amino acid chains including one or more PD-1 polypeptides fused to a scaffold polypeptide can form a polypeptide conjugate that includes two or more PD-1 polypeptides. When

two or more amino acid chains including one or more PD-1 polypeptides fused to a scaffold polypeptide form a polypeptide conjugate, the two or more amino acid chains can be bonded (e.g., by a covalent bond or an ionic bond). A scaffold polypeptide can be any appropriate polypeptide that can form a polypeptide conjugate with another polypeptide. A scaffold polypeptide can naturally form a polypeptide conjugate or can be engineered to form a polypeptide conjugate. Examples of scaffold polypeptides that can form a polypeptide conjugate can include, without limitation, Ig polypeptides (e.g., IgG polypeptides such as IgG2 polypeptides), sigma-1 polypeptides, and streptavidin polypeptides.

[00087] An amino acid chain including one or more amino acid segments that can bind PD-L1 (e.g., PD-1 polypeptides and/or fragments thereof) that can be present in a polypeptide conjugate described herein (e.g., a polypeptide conjugate including a plurality amino acid chains that each include one or more PD-1 polypeptides) can include any appropriate amino acid sequence. For example, an amino acid chain including one or more PD-1 polypeptides fused to a scaffold polypeptide can include any appropriate amino acid sequence. Exemplary amino acid sequences of amino acid chains including one or more PD-1 polypeptides and a scaffold polypeptide include, without limitation, those amino acid sequences set forth in SEQ ID NO: 7, 10, 11, 12, 13, 16, 17, 18, 19, 20, 21, 24, 25, 26, or 27. In some cases, the amino acid sequence of an amino acid chain including one or more PD-1 polypeptides and a scaffold polypeptide can have a sequence that deviates from one of the amino acid sequences set forth in SEQ ID NO: 7, 10, 11, 12, 13, 16, 17, 18, 19, 20, 21, 24, 25, 26, or 27, sometimes referred to as a variant sequence. For example, an amino acid sequence of a fusion polypeptide containing one or more PD-1 polypeptides and a scaffold polypeptide can have at least 80% sequence identity to any one of the amino acid sequences set forth in SEQ ID NO: 7, 10, 11, 12, 13, 16, 17, 18, 19, 20, 21, 24, 25, 26, or 27. In some embodiments, an amino acid sequence of a fusion polypeptide containing one or more PD-1 polypeptides and a scaffold polypeptide can have at least 85% sequence identity, 90% sequence identity, 95% sequence identity, or at least 99% sequence identity to any one of the amino acid sequences set forth in SEQ ID NO: 7, 10, 11, 12, 13, 16, 17, 18, 19, 20,

21, 24, 25, 26, or 27. Percent sequence identity is calculated by determining the number of matched positions in aligned polypeptide sequences, dividing the number of matched positions by the total number of aligned amino acids, respectively, and multiplying by 100. A matched position refers to a position in which identical amino acids occur at the same position in aligned sequences. Sequences can be aligned using the algorithm described by Altschul *et al.* (*Nucleic Acids Res.*, 25:3389-3402 (1997)) as incorporated into BLAST (basic local alignment search tool) programs, available at ncbi.nlm.nih.gov on the World Wide Web. BLAST searches or alignments can be performed to determine percent sequence identity between a polypeptide and any other sequence or portion thereof using the Altschul *et al.* algorithm. BLASTN is the program used to align and compare the identity between nucleic acid sequences, while BLASTP is the program used to align and compare the identity between amino acid sequences. When utilizing BLAST programs to calculate the percent identity between a polypeptide sequence and another sequence, the default parameters of the respective programs are used. In some cases, an amino acid chain including one or more PD-1 polypeptides that can be present in a polypeptide conjugate described herein (*e.g.*, a polypeptide conjugate including a plurality amino acid chains that each include one or more PD-1 polypeptides) can be as set forth in Example 7.

[00088] In some cases, an amino acid chain including one or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof) can be fused to a scaffold polypeptide that can form a polypeptide conjugate including two associated amino acid chains (*e.g.*, an IgG polypeptide such as an IgG2 polypeptide). For example, two amino acid chains each including one PD-1 polypeptide fused to an IgG2 polypeptide can form a polypeptide conjugate that includes two PD-1 polypeptides. Exemplary amino acid sequences of amino acid chains including one PD-1 polypeptide fused to an IgG2 polypeptide include, without limitation, those amino acid sequences set forth in SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:21.

[00089] In some cases, an amino acid chain including one or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof) can be fused to a scaffold polypeptide that can form a polypeptide conjugate including three associated amino acid chains (*e.g.*, a sigma-1 polypeptide). For example, three amino acid chains each including one PD-1 polypeptide fused to a sigma-1 polypeptide can form a polypeptide conjugate that includes three PD-1 polypeptides. Exemplary amino acid sequences of amino acid chains including one PD-1 polypeptide fused to a sigma-1 polypeptide include, without limitation, those amino acid sequences set forth in SEQ ID NO:10, SEQ ID NO:16, or SEQ ID NO:24.

[00090] In some cases, an amino acid chain including one or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof) can be fused to a scaffold polypeptide that can form a polypeptide conjugate including four associated amino acid chains (*e.g.*, a streptavidin polypeptide). For example, four amino acid chains each including one PD-1 polypeptide fused to a streptavidin polypeptide can form a polypeptide conjugate that includes four PD-1 polypeptides. Exemplary amino acid sequences of amino acid chains including one PD-1 polypeptide fused to a streptavidin polypeptide include, without limitation, those amino acid sequences set forth in SEQ ID NO:11 or SEQ ID NO:17.

[00091] In some cases, a multivalent PD-L1 binding compound can include a virus particle where two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof). For example, a virus particle can be modified by coating the virus particle with two or more amino acid chains that each include one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide. An amino acid chain including one or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof) that can be used to coat a virus particle to generate a multivalent PD-L1 binding compound (*e.g.*, a virus particle where two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more PD-1 polypeptides) can include one or more amino acid segments that can bind PD-L1

fused to any appropriate polypeptide that can bind to a viral coat polypeptide. In some cases, a polypeptide that can bind to a viral coat polypeptide can covalently bind to one or more viral coat polypeptides. In some cases, a polypeptide that can bind to a viral coat polypeptide can bind to a viral coat polypeptide with high affinity (e.g., from about 0.25 μ M to about 100 μ M). A polypeptide that can bind to a viral coat polypeptide can naturally bind to a viral coat polypeptide or is engineered to bind to a viral coat polypeptide. A polypeptide that can bind to a viral coat polypeptide can bind to any appropriate viral coat polypeptide.

[00092] Examples of viral coat polypeptides include, without limitation, capsid polypeptides (e.g., hexon polypeptides, fiber polypeptides, penton polypeptides), and adenovirus IX polypeptides. A polypeptide that can bind to a viral coat polypeptide can bind to a viral coat polypeptide present on any appropriate type of virus particle. A virus particle can be a replication competent (RC) virus particle (e.g., helper-dependent (HD) virus particles and single-cycle (SC) virus particles), a virus particle can be a replication-defective (RD) virus particle, or a virus can be a conditionally replicating virus particle (CRA_d). In some cases, a virus particle can be an oncolytic virus particle.

[00093] In some cases, a virus particle can be a viral vector. Examples of virus particles that can be coated with one or more amino acid chains that include one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide include, without limitation, Ads, adeno-associated viruses (AAVs), lentiviruses, enteroviruses, reoviruses, poxviruses, measles virus, and herpes viruses.

[00094] Examples of polypeptides which may be present on a viral coat polypeptide include, without limitation, a GLA domain polypeptide (e.g., a GLA domain from a FX polypeptide), CAR polypeptides, CD46 polypeptides, desmoglein polypeptides, integrin polypeptides, single-chain antibody polypeptides, camelid antibody polypeptides, capsid polypeptides, and envelope binding polypeptides.

[00095] An amino acid chain including one or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof) that can be used to coat a virus particle to generate a multivalent PD-L1 binding compound (*e.g.*, a virus particle where one or two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more PD-1 polypeptides) can include any appropriate amino acid sequence. For example, a virus particle can be contacted with a composition comprising two or more amino acid chains that each include one or more PD-1 polypeptides and a polypeptide that can covalently bind to a viral coat polypeptide such that the amino acid chains coat the virus particle. In some cases, an amino acid chain that includes one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide can be a fusion polypeptide including one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide. An amino acid chain that includes one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide can include any appropriate amino acid sequence. Exemplary amino acid sequences of amino acid chains including one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide include, without limitation, those amino acid sequences set forth in SEQ ID NO: 8, 9, 14, 15, 22, or 23. In some cases, the amino acid sequence of an amino acid chain including one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide can have a sequence that deviates from one of the amino acid sequences set forth in SEQ ID NO: 8, 9, 14, 15, 22, or 23 sometimes referred to as a variant sequence. For example, an amino acid sequence of a fusion polypeptide containing one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide can have at least 80% sequence identity to any one of the amino acid sequences set forth in SEQ ID NO: 8, 9, 14, 15, 22, or 23. In some embodiments, an amino acid sequence of a fusion polypeptide containing one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide can have at least 85% sequence identity, 90% sequence identity, 95% sequence identity, or at least 99% sequence identity to any one of the amino acid sequences set forth in SEQ ID NO: 8, 9, 14, 15, 22, or 23. Percent sequence identity is calculated by determining the number of matched positions in aligned polypeptide sequences, dividing the number of matched positions by the total

number of aligned amino acids, respectively, and multiplying by 100. A matched position refers to a position in which identical amino acids occur at the same position in aligned sequences. Sequences can be aligned using the algorithm described by Altschul *et al.* (*Nucleic Acids Res.*, 25:3389-3402 (1997)) as incorporated into BLAST (basic local alignment search tool) programs. In some cases, an amino acid chain including one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide can be as set forth in the Examples.

[00096] In some cases, an amino acid chain including one or more amino acid segments that can bind PD-L1 (*e.g.*, PD-1 polypeptides and/or fragments thereof) fused to a polypeptide that can bind to a viral coat polypeptide (*e.g.*, a GLA domain of a FX polypeptide) can be used to coat (*e.g.*, to modify one or more viral coat polypeptides) a virus particle to form a coated virus particle having two or more amino acid chains including one or more amino acid segments that can bind PD-L1 which is bound to one or more viral coat polypeptides present on the surface of the virus particle.

[00097] For example, one or more amino acid chains each including one PD-1 polypeptide fused to a GLA domain of a FX polypeptide can be bound to two or more viral hexon polypeptides and/or other viral coat proteins to coat a virus particle with two or more amino acid chains each including one PD-1 polypeptide fused to a GLA domain of a FX polypeptide. When a virus particle is an Ad, 240 hexon polypeptide homo-trimers (*e.g.*, polypeptide conjugates including three hexon polypeptides) can be present on the virus particle (see, *e.g.*, Chen *et al.*, *Human gene therapy*, 21:739-749 (2010)). In some cases, a fusion polypeptide including one PD-1 polypeptide and a GLA domain of a FX polypeptide can be used to coat an Ad particle to generate a multivalent PD-L1 binding compound that includes about 240 PD-1 polypeptides. For example, when a fusion polypeptide including one PD-1 polypeptide fused to a GLA domain of a FX polypeptide is bound to each hexon polypeptide trimer on an Ad virus particle, the virus particle will include 240 PD-1 polypeptides. In some cases, a fusion polypeptide including one PD-1 polypeptide and a GLA domain of a FX polypeptide can be used to coat an Ad particle to

generate a multivalent PD-L1 binding compound that may include between about 240 PD-1 polypeptides and about 720 PD-1 polypeptides. For example, when a fusion polypeptide including one PD-1 polypeptide fused to a GLA domain of a FX polypeptide is bound to each hexon polypeptide on an Ad virus particle, the virus particle will include 720 PD-1 polypeptides.

[00098] In some cases, multivalent PD-L1 binding compounds described herein (e.g., a fusion polypeptide that includes two or more PD-1 polypeptides, or a polypeptide conjugate that includes one or two or more amino acid chains that each include one or more PD-1 polypeptides, or a virus particle where two or more viral coat polypeptides include one or more amino acid chains that include one or more PD-1 polypeptides, or a virus particle where one or more viral coat polypeptides include one or more amino acid chains that include two or more PD-1 polypeptides) also can include one or more additional molecules/polypeptides.

[00099] Examples of molecules that can be included in a multivalent PD-L1 binding compound described herein include, without limitation, targeting molecules (e.g., targeting polypeptides and targeting nucleic acid sequences), antigens, therapeutic molecules, and detectable polypeptides. In some cases, when a multivalent PD-L1 binding compound is a polypeptide conjugate including a plurality of amino acid chains that each include one or more PD-1 polypeptides and/or PD-1 polypeptide fragments having the ability to bind PD-L1, and the polypeptide conjugate includes one or more additional molecules, the one or more additional molecules can be included in at least one (e.g., 1, 2, 3, 4, or more) amino acid chain including one or more PD-1 polypeptides and/or PD-1 polypeptide fragments having the ability to bind PD-L1 that is present in the polypeptide conjugate. For example, when a multivalent PD-L1 binding compound is a polypeptide conjugate including a plurality of amino acid chains that each include one or more PD-1 polypeptides and/or PD-1 polypeptide fragments having the ability to bind PD-L1, and the polypeptide conjugate includes one or more additional molecules, the one or more additional molecules can be included in each amino acid chain including one or more PD-1 polypeptides and/or PD-1 polypeptide fragments having the ability to bind PD-

L1 that is present in the polypeptide conjugate. In some cases, when a multivalent PD-L1 binding compound is a virus particle where two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more PD-1 polypeptides and/or PD-1 polypeptide fragments having the ability to bind PD-L1 (or a virus particle where one or more viral coat polypeptides are include one or more amino acid chains that include two or more amino acid segments that include one or more PD-1 polypeptides and/or PD-1 polypeptides fragments having the ability to bind PD-L1), the one or more additional molecules can be included in at least one (e.g., 1, 2, 3, 4, or more) amino acid chain including one or more PD-1 polypeptides and/or PD-1 polypeptide fragments used to modify a viral coat polypeptide on a virus particle. For example, when a multivalent PD-L1 binding compound is a virus particle where two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more PD-1 polypeptides and/or PD-1 polypeptide fragments having the ability to bind PD-L1 (or a virus particle where one or more viral coat polypeptides are modified to include one or more amino acid chains that include two or more amino acid segments that include one or more PD-1 polypeptides and/or PD-1 polypeptides fragments having the ability to bind PD-L1), the one or more additional molecules can be included in each amino acid chain including one or more PD-1 polypeptides and/or PD-1 polypeptide fragments used to modify a viral coat polypeptide on a virus particle.

[000100] When PD-L1 binding compounds described herein also include one or more targeting molecules (e.g., targeting polypeptides and targeting nucleic acid sequences), a targeting molecule can be any appropriate targeting molecule. In some cases, a targeting molecule can be a polypeptide (e.g., a targeting polypeptide). In some cases, a targeting molecule can be nucleic acid sequence (e.g., a targeting nucleic acid sequence). In some cases, a targeting molecule can attract a T-cell to a PD-L1 positive cell (e.g., a PD-L1 positive cancer cell). For example, when a multivalent PD-L1 binding compound that includes a targeting molecule is bound to PD-L1 on a PD-L1 positive cell, the targeting molecule on the multivalent PD-L1 binding compound can attract T cells to the PD-L1 positive cell. For example, when a multivalent PD-L1 binding compound includes a targeting

molecule, the targeting molecule on the multivalent PD-L1 binding compound can direct the multivalent PD-L1 binding compound to a target location (e.g., a target tissue) within a mammal. Examples of targeting molecules that can be included in a multivalent PD-L1 binding compound described herein include, viral polypeptides, antigens (e.g., peptide antigens), miRNA targeting sequences (e.g., tissue-specific miRNA targeting sequences), antibodies, growth factors, and carbohydrates. When a targeting polypeptide is a viral polypeptide, the viral polypeptide can be derived from any appropriate virus (e.g., measles virus (MV), canine distemper virus (CDV), vesicular stomatitis virus (VSV), gibbon ape leukemia virus (GALV), Zika virus, human immunodeficiency virus (HIV), and human endogenous retrovirus (HERV)). When a targeting polypeptide is a viral polypeptide, the viral polypeptide can be any appropriate viral polypeptide (e.g., a hemagglutinin (H) polypeptide such as a detargeted H polypeptide, a fusion (F) polypeptide, a viral glycoprotein (G) polypeptide, envelope (env; e.g., HERV env), syncytin-1, and syncytin-2. In some cases, a viral polypeptide can be a MV H polypeptide. In some cases, a viral polypeptide can be a MV F polypeptide. In some cases, a viral polypeptide can be a VSV G polypeptide.

[000101] When PD-L1 binding compounds described herein also include one or more therapeutic molecules, a therapeutic molecule can be any appropriate therapeutic molecule. In some cases, a therapeutic molecule can be a therapeutic polypeptide. In some cases, a therapeutic molecule can reduce or eliminate PD-L1 inactivation of one or more T cells. In some cases, a therapeutic molecule can activate and/or accumulate one or more pharmaceutical agents (e.g., anti-cancer drugs). Examples of therapeutic molecules that can be included in a multivalent PD-L1 binding compound described herein include, a 4-1BB ligand (4-1BBL) polypeptide, an OX40 ligand (OX40L) polypeptide, a granulocyte-macrophage colony-stimulating factor (GM-CSF) polypeptide, anti-PD-1 antibodies, nucleic acid encoding anti-PD-1 antibodies, thymidine kinase, cytosine deaminase, and iodide symporter. In some cases, a therapeutic molecule can be a therapeutic antibody. The therapeutic antibody may be an agonist antibody which targets GITR. Signaling through GITR enhances T-cell proliferation and effector functions and protects T

cells from activation-induced cell death, which in turn increases the frequency of memory T cells. Combining GITR agonists with the PD-L1 binding compounds of the invention may demonstrate a strong synergy.

[000102] When PD-L1 binding compounds described herein also include one or more detectable polypeptides, a detectable polypeptide can be any appropriate detectable polypeptide. In some cases, a detectable polypeptide can be detected (*e.g.*, by a clinician) to determine the location of one or more PD-L1 binding compounds described herein. In some cases, a detectable polypeptide can be detected (*e.g.*, by a clinician) to monitor the persistence of one or more multivalent PD-L1 binding compounds described herein. Examples of detectable polypeptides that can be included in a PD-L1 binding compound described herein are selected from fluorescent polypeptides (*e.g.*, green fluorescent protein (GFP), luciferase polypeptides, peptide labels, and sodium iodide symporter.

[000103] This document also provides nucleic acids (*e.g.*, nucleic acid vectors) that can encode amino acid chains described herein (*e.g.*, an amino acid chain that includes one or more PD-1 polypeptides fused to a scaffold polypeptide, or an amino acid chain that includes one or more PD-1 polypeptides fused to a polypeptide that can bind to a viral coat polypeptide) that can be used to generate multivalent PD-L1 binding compounds described herein (*e.g.*, a fusion polypeptide that includes two or more PD-1 polypeptides, or a polypeptide conjugate that includes two or more amino acid chains that each include one or more PD-1 polypeptides, or a virus particle where one or two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more PD-1 polypeptides, or a virus particle where one or more viral coat polypeptides are modified to include one or more amino acid chains that include two or more PD-1 polypeptides). In some cases, nucleic acid can encode an amino acid chain that can be used to generate a polypeptide conjugate including two or more amino acid chains that each include one or more PD-1 polypeptides. For example, nucleic acid can encode an amino chain including one or more PD-1 polypeptides and a scaffold polypeptide. In some cases, nucleic acid can encode an amino acid chain that can be used to generate a virus particle

where one or more viral coat polypeptides comprise one or more amino acid chains that include one or more PD-1 polypeptides. For example, nucleic acid can encode an amino chain including one or more PD-1 polypeptides and a polypeptide that can bind to a viral coat polypeptide.

[000104] Nucleic acid (*e.g.*, nucleic acid vectors) encoding amino acid chains described herein (*e.g.*, an amino acid chain that includes one or more PD-1 polypeptides fused to a scaffold polypeptide, or an amino acid chain that includes one or more PD-1 polypeptides fused to a polypeptide that can bind to a viral coat polypeptide) that can be used to generate multivalent PD-L1 binding compounds described herein can be a nucleic acid selected from DNA (*e.g.*, a DNA construct), RNA (*e.g.*, mRNA), or a combination thereof. In some cases, nucleic acid encoding an amino acid chain described herein can be a vector (*e.g.*, an expression vector or a viral vector). A vector can be a RC vector (*e.g.*, HD vectors and SC vectors) or a vector can be a RD vector. When a vector is a viral vector, the viral vector can be derived from any appropriate type of virus. In some cases, a viral vector can be derived from an oncolytic virus. Examples of viruses from which viral vectors can be derived include, without limitation, Ads, adeno-associated viruses (AAVs), lentiviruses, enteroviruses, reoviruses, poxviruses, measles virus, and herpes viruses.

[000105] In some cases, nucleic acid encoding an amino acid chain described herein also can include one or more regulatory elements (*e.g.*, to regulate expression of the amino acid chain). Examples of regulatory elements that can be included in nucleic acid encoding an amino acid chain described herein include, without limitation, promoters (*e.g.*, constitutive promoters, tissue/cell-specific promoters, and inducible promoters such as chemically-activated promoters and light-activated promoters), enhancers, nucleic acid sequences (*e.g.*, suicide genes) whose expression can induce apoptosis, necrosis, and other forms of cell death.

[000106] In some cases, nucleic acids (*e.g.*, nucleic acid vectors) encoding amino acid chains described herein (*e.g.*, an amino acid chain that includes one or

more PD-1 polypeptides fused to a scaffold polypeptide, or an amino acid chain that includes one or more PD-1 polypeptides fused to a polypeptide that can bind to a viral coat polypeptide) that can be used to generate multivalent PD-L1 binding compounds described herein (*e.g.*, a fusion polypeptide that includes two or more PD-1 polypeptides, or a polypeptide conjugate that includes two or more amino acid chains that each include one or more PD-1 polypeptides, or a virus particle where two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more PD-1 polypeptides, or a virus particle where one or more viral coat polypeptides are modified to include one or more amino acid chains that include two or more PD-1 polypeptides) can produce two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) amino acid chains described herein.

[000107] In some cases, nucleic acids (*e.g.*, nucleic acid vectors) encoding amino acid chains described herein (*e.g.*, an amino acid chain that includes one or more PD-1 polypeptides fused to a scaffold polypeptide, or an amino acid chain that includes one or more PD-1 polypeptides fused to a polypeptide that can bind to a viral coat polypeptide) that can be used to generate multivalent PD-L1 binding compounds described herein (*e.g.*, a fusion polypeptide that includes two or more PD-1 polypeptides, or a polypeptide conjugate that includes two or more amino acid chains that each include one or more PD-1 polypeptides, or a virus particle where two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more PD-1 polypeptides, or a virus particle where one or more viral coat polypeptides are modified to include one or more amino acid chains that include two or more PD-1 polypeptides) can continuously produce one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) amino acid chains described herein.

[000108] In some cases, nucleic acids (*e.g.*, nucleic acid vectors) encoding amino acid chains described herein (*e.g.*, an amino acid chain that includes one or more PD-1 polypeptides fused to a scaffold polypeptide, or an amino acid chain that includes one or more PD-1 polypeptides fused to a polypeptide that can bind to a viral coat polypeptide) that can be used to generate multivalent PD-L1 binding compounds described herein (*e.g.*, a fusion polypeptide that includes two or more

PD-1 polypeptides, or a polypeptide conjugate that includes two or more amino acid chains that each include one or more PD-1 polypeptides, or a virus particle where two or more viral coat polypeptides are modified to include one or more amino acid chains that include one or more PD-1 polypeptides, or a virus particle where one or more viral coat polypeptides are modified to include one or more amino acid chains that include two or more PD-1 polypeptides) can produce one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) amino acid chains described herein for the duration of the persistence of the nucleic acid (*e.g.*, until the nucleic acid is degraded).

[000109] In some cases, amino acid chains encoded by nucleic acid described herein can be used to generate multivalent PD-L1 binding compounds described herein. For example, two or more amino acid chains including one or more PD-1 polypeptides fused to a scaffold polypeptide can assemble (*e.g.*, can self-assemble) into a polypeptide conjugate including two or more amino acid chains that each include one or more PD-1 polypeptides to generate a polypeptide conjugate described herein. When two or more amino acid chains including one or more PD-1 polypeptides fused to a scaffold polypeptide assemble into a polypeptide conjugate including two or more amino acid chains that each include one or more PD-1 polypeptides to generate a polypeptide conjugate, the amino acid chains can assemble *in vivo* or *in vitro*. For example, one or more amino acid chains including one or more PD-1 polypeptides fused to a polypeptide that can bind to a viral coat polypeptide can bind to two or more viral coat polypeptides present on the surface of a virus particle to coat to generate a coated virus particle described herein. When one or more amino acid chains including one or more PD-1 polypeptides fused to a polypeptide that can bind to a viral coat polypeptide coat virus particle to generate a coated virus particle, the virus particle can be coated *in vivo* or *in vitro*.

[000110] In some cases, multivalent PD-L1 binding compounds described herein can be purified. A "purified" polypeptide, protein or nucleic acid refers to a polypeptide or nucleic acid that constitutes the major component in a mixture of components, *e.g.*, 30% or more, 40% or more, 50% or more, 60% or more, 70% or more, 80% or more, 90% or more, 95% or more, or 99% or more by weight. For

example, a purified multivalent PD-L1 binding compound can constitute about 30% or more by weight of a composition containing one or more multivalent PD-L1 binding compounds. Polypeptides may be purified by methods including, but not limited to, affinity chromatography and immunosorbent affinity column. For example, a purified nucleic acid encoding an amino acid chain that can be used to generate a multivalent PD-L1 binding compound described herein can constitute about 30% or more by weight of a composition containing one or more amino acid chains that can be used to generate a multivalent PD-L1 binding compound described herein. Nucleic acid may be purified by methods including, but not limited to, phenol-chloroform extraction and column purification (e.g., mini-column purification).

[000111] Alternatively, the invention also provides multivalent PD-1 binding compounds that include two or more amino acid segments that can bind PD-1 (e.g., PD-L1 polypeptides and/or fragments thereof) as well as methods for making and using multivalent PD-1 binding compounds that include two or more amino acid segments that can bind PD-1 (e.g., PD-L1 polypeptides and/or fragments thereof). In some cases, a multivalent PD-1 binding compound can include two or more amino acid segments that can bind PD-1 (e.g., PD-L1 polypeptides and/or fragments thereof). For example, a multivalent PD-1 binding compound that includes two or more PD-L1 polypeptides (and/or fragments thereof having the ability to bind PD-1) can bind two or more PD-1 polypeptides. In some cases, a multivalent PD-1 binding compound can include a single amino acid chain that includes two or more amino acid segments that can bind PD-1.

[000112] Also provided herein are methods and materials for treating cancer, for example, administering a composition containing one or more multivalent PD-L1 binding compounds described herein. The one or more multivalent PD-L1 binding compounds may be administered to a subject in combination with therapeutic polypeptides. In an embodiment a multivalent PD-L1 binding compound comprises a therapeutic polypeptide which is administered to a subject for treating cancer. In another embodiment a multivalent PD-L1 binding compound is co-administered with an Ad expressing a therapeutic polypeptide to a subject for treating cancer.

[000113] The invention encompasses virus particles wherein two or more viral coat polypeptides comprise one or more amino acid chains that include one or more PD-1 polypeptides. The virus particle may be a recombinant virus particle comprising PD-1 polypeptides, including PD-1 fusion proteins, in the capsid protein of the virus particle. The virus particle may be a virus particle which has been coated with PD-1 polypeptides, for example, PD-1 fusion proteins.

[000114] In an embodiment, where the PD-L1 binding compound comprises a recombinant virus, the virus is a recombinant Ad, for example, Ad strain Ad657 or Ad strain Ad6/57/6, and variants thereof. The recombinant Ad may be modified to be a conditionally replicating Ad (CRAAd). Such recombinant Ads are described in US Application Serial No. 16/690,733, the disclosure of which is incorporated herein in its entirety by reference as if fully set forth herein.

[000115] In an embodiment, the invention encompasses a recombinant, conditionally replicating Ad virus particle which comprises one or more amino acid chains that each include one or two or more PD-1 polypeptides, or nucleic acid encoding amino acid chains that can be used to generate multivalent PD-L1 binding compounds.

[000116] In some cases, a recombinant Ad described herein (e.g., a recombinant Ad having oncolytic anti-cancer activity such as a recombinant Ad657) can include an Ad genome containing one or more nucleic acid deletions. A nucleic acid deletion can be a full deletion (e.g., deletion of a nucleic acid encoding a polypeptide) or a partial deletion (e.g., deletion of one or more nucleotides within a nucleic acid encoding a polypeptide). A nucleic acid deletion can reduce or eliminate transcription and translation of a polypeptide encoded by the deleted nucleic acid. In some cases, a nucleic acid encoding a polypeptide associated with production of infectious progeny can be deleted. Examples of nucleic acids that can be deleted and/or modified in a recombinant Ad described herein may encode E1 (e.g., E1A and E1B), E2, E3, E4, pIII A, fiber, E1B, and include viral enhancers and promoters.

For example, a recombinant Ad described herein (e.g., a recombinant Ad having oncolytic anti-cancer activity such as a recombinant Ad657) can include an Ad genome containing a deletion of one or more nucleotides within a nucleic acid encoding an E1 polypeptide. In some cases, a recombinant Ad described herein can include one or more substitutions in a nucleic acid encoding an E1 polypeptide.

[000117] In particular embodiments, a recombinant Ad described herein is modified to comprise a probasin promoter comprising, for example, a nucleic acid of SEQ ID NO:41, is modified to comprise a dl1101 deletion in a nucleic acid encoding an E1 polypeptide; a recombinant Ad described herein is modified to comprise a dl1107 deletion in a nucleic acid encoding an E1 polypeptide; a recombinant Ad described herein is modified to comprise a dl1101 deletion and a dl1107 deletion. The dl1101 modification to E1a prevents binding to p300 and to make virus susceptible to Interferon (INF) repressions. The dl1107 mutation in E1a prevents its binding to pRB to block virus replication in cells with intact pRB pathways. See Figure 10 for N-terminal amino acid sequences of the E1A polypeptide, for example, wild-type Ad E1A, and CRAAd-657-dl1101, CRAAd-657-dl1107 and CRAAd-657-dl1101/1107 variants.

[000118] Schematic of mutations in Ad6, Ad657 and variants thereof involving mutations in the E1 protein to convert the virus to a conditionally-replicating Ad (CRAAd) is shown in Figure 9 and Figure 10. These include dl1101 and/or the dl1107 that block binding to p300 and pRB, respectively.

[000119] Figure 10 shows the N-terminal amino acid sequences of E1A in a wild-type Ad, as well as Ad variants E1A dl1101, E1A dl1107 and E1A dl1101/1107.

[000120] Also shown is the replacement of the Ad E1 promoter with the prostate-specific promoter probasin-E1 DNA sequence (SEQ ID NO:41) to generate the CRAAd, Ad-PB (Figure 9). The probasin promoter is androgen dependent, so will work in androgen-sensitive tumors like LNCaP, but not in androgen-resistant tumors like DU145.

[000121] The replication competent Ads, as well as the CRAbs, of the invention are genetically modified by methods known to those skilled in the art to express one or more therapeutic polypeptides. The therapeutic polypeptides may be immune stimulatory polypeptides, for example, CD40L, 4-1BBL, OX40, GM-CSF, and combinations thereof.

[000122] Moreover, in an embodiment, the replication competent Ads, as well as the CRAbs, of the invention are genetically modified by methods known to those skilled in the art to express both PD-1 polypeptides and one or more therapeutic polypeptides as fusion proteins.

[000123] The replication competent Ads, as well as the CRAbs, of the invention may be genetically modified by methods known to those skilled in the art to comprise modified fiber/knob capsid proteins. The Ad fiber protein is a complex of three apparently identical subunits which mediates the initial attachment step. The native Ad6 fiber protein (SEQ ID NO:35) binds CAR.

[000124] In a further aspect of the invention, fiber-modified recombinant Ads having different fiber proteins or modifications in the fiber/knob proteins which are not native to the parental Ad were generated. Recombinant Ads, including CRAbs, comprising capsid proteins from different Ad strains and heterologous fiber/knob proteins were generated, for example, recombinant Ads comprising a heterologous Ad35 fiber polypeptide or Chimpanzee C68 fiber polypeptide, +/- a K7 peptide.

[000125] A chimeric Ad, AdF35 fiber chimera, has the amino acid sequence of SEQ ID NO:36 and is shorter than Ad5 and Ad6 fiber proteins and retargets virus to CD46.

[000126] A fiber-modified recombinant Ad, comprising K7 Fiber having the sequence of SEQ ID NO:37, targets virus to heparin sulfate proteoglycans and negative charges on cells.

[000127] A recombinant, chimeric Ad, 6/FC68 Fiber comprising the sequence of SEQ ID NO:38, is a chimeric Ad having a fiber protein from chimpanzee adenovirus C68. The fiber protein is shorter than Ad5 or Ad6 fiber proteins and binds CAR.

[000128] A recombinant, chimeric Ad, 6/FC68-K7 Fiber comprising the sequence of SEQ ID NO:39, is a chimeric Ad having a fiber protein from chimpanzee adenovirus C68. The fiber protein is shorter than Ad5 or Ad6 fiber proteins. The 6/FC68-K7 Fiber binds CAR and is retargeted to heparin sulfate and negative charges.

[000129] A recombinant, chimeric Ad, 6/FC68-HI-K7 Fiber comprising the sequence of SEQ ID NO:40, is a chimeric Ad having a fiber protein from chimpanzee adenovirus C68. The fiber protein is shorter than Ad5 or Ad6 fiber proteins. The 6/FC68-HI-K7 Fiber binds CAR and is retargeted to heparin sulfate and negative charges.

[000130] In some cases, a recombinant Ad described herein (e.g., a recombinant Ad having oncolytic anti-cancer activity such as a recombinant Ad657) can include an Ad genome containing one or more nucleic acid insertions. For example, a nucleic acid insertion can include a nucleic acid encoding a polypeptide, for example a therapeutic polypeptide. A nucleic acid can be inserted into any appropriate location within a genome of a recombinant Ad described herein. In some cases, a nucleic acid encoding a polypeptide can be inserted into a HVR (e.g., HVR 5 loop) of a genome of a recombinant Ad described herein. For example, when a nucleic acid encoding a polypeptide is inserted into a HVR of a genome of a recombinant Ad described herein, the nucleic acid encoding a polypeptide can express one or more polypeptides, and the expressed polypeptide(s) can be incorporated into the capsid of the recombinant Ad. In cases where a nucleic acid encoding a polypeptide is inserted into a HVR region of a genome of a recombinant Ad described herein, the recombinant Ad can present from about 1 to about 720 polypeptides encoded by the inserted nucleic acid on its surface. A nucleic acid insertion can be nucleic acid encoding any appropriate polypeptide. In some cases,

a nucleic acid insertion can encode a therapeutic polypeptide, a polypeptide antigen or a cytokine.

[000131] In general, Ads may be modified to include CRAAd modifications described herein.

[000132] In an embodiment, a recombinant Ad is a conditionally replicating Ad with a dl1101 modification to E1a, which prevents binding to p300 and makes the virus susceptible to IFN repression, and a dl1107 mutation in E1a to prevent its binding to pRB to block virus replication in cells with intact pRB pathways, and an E3A deletion. The CRAAd expresses human PD-1 fused to human immunoglobulin.

[000133] In an embodiment, a recombinant Ad (for example Ad657) is a conditionally replicating Ad with a dl1101 modification to E1a, which prevents binding to p300 and makes virus susceptible to IFN repression, and an E3A deletion. The CRAAd expresses human PD-1 fused to human Factor X (GLA) on the surface of the virus particle, wherein the CRAAd is retargeted to cells expressing PD-L1 on the surface of the cell.

[000134] In an embodiment, a recombinant Ad is a conditionally replicating Ad (for example Ad657) with a dl1101 modification to E1a, which prevents binding to p300 and makes virus susceptible to IFN repression, and a dl1107 mutation in E1a to prevent its binding to pRB to block virus replication in cells with intact pRB pathways, and an E3A deletion. The CRAAd expresses human PD-1 fused to human Factor X (GLA) on the surface of the virus particle, wherein the CRAAd is retargeted to cells expressing PD-L1 on the cell surface.

[000135] The virus particles having PD-1 polypeptides bound to the surface of the virus particle can be used for treating a mammal having cancer (*e.g.*, a cancer including one or more PD-L1 positive cancer cells). The virus particles may be administered to a subject in an amount effective for the treatment of cancer and,

optionally, may be administered in combination with cancer therapies, including immunotherapies and chemotherapeutic agents.

[000136] In an aspect of the invention, a composition containing one or more multivalent PD-L1 binding compounds or nucleic acid encoding an amino acid chain that can be used to generate multivalent PD-L1 binding compounds described herein and, optionally, one or more cancer therapies, can be administered to a mammal having cancer to treat the mammal. A multivalent PD-L1 binding compound described herein can bind to PD-L1 on PD-L1 positive cancer cells. Binding of a PD-1 polypeptides can neutralize PD-L1 function, and can thereby prevent PD-L1 positive cells (*e.g.*, PD-L1 positive cancer cells) from escaping the immune system and/or can allow anti-cancer agents (*e.g.*, cancer immunotherapies) to more effectively target PD-L1 positive cells. In cases where nucleic acid encoding a multivalent PD-L1 binding compound described herein is an oncolytic Ad vector encoding a multivalent PD-L1 binding compound described herein, the oncolytic Ad can infect cell and can drive T cell responses directed to the infected cell. An oncolytic Ad can infect any appropriate type of cell. In some cases, an oncolytic Ad can infect a PD-L1 positive cell (*e.g.*, a PD-L1 positive cancer cell). In some cases, an oncolytic Ad can infect non-dividing cells (*e.g.*, kidney cells).

[000137] In some cases, a composition comprising or consisting essentially of one or more PD-L1 binding compounds described herein can be used for treating a mammal having cancer (*e.g.*, a cancer including one or more PD-L1 positive cancer cells). For example, a composition containing one or more multivalent PD-L1 binding compounds and, optionally, one or more cancer therapeutics, can be administered to a mammal having cancer to treat the mammal.

[000138] Alternatively, the methods and materials described herein can be used for treating other diseases or disorders associated with PD-L1 positive cells. In some cases, a composition containing one or more PD-L1 binding compounds described herein can be used for treating a mammal having an infectious disease (*e.g.*, an infectious disease including one or more PD-L1 positive macrophages such

as cancer infiltrating macrophages). Examples of infectious diseases that can include one or more PD-L1 positive cells include, without limitation, HIV, hepatitis, and malaria. In some cases, a composition containing one or more PD-L1 binding compounds described herein can be used for treating a mammal having an autoimmune disease (*e.g.*, an autoimmune disease including one or more PD-L1 positive macrophages such as cancer infiltrating macrophages).

[000139] Any appropriate mammal having cancer can be treated as described herein. For example, humans and other primates such as monkeys having cancer can be treated with a composition containing one or more PD-L1 binding compounds described herein and, optionally, can be treated in combination with one or more cancer treatments to reduce the severity of the cancer, to reduce a symptom of the cancer, and/or to reduce the number of cancer cells present within the mammal within the human or other primate. In some cases, dogs, cats, horses, cows, pigs, sheep, mice, and rats having cancer can be treated with a composition containing one or more multivalent PD-L1 binding compounds described herein, viruses comprising PD-L1 binding compounds, or nucleic acid encoding an amino acid chain that can be used to generate multivalent PD-L1 binding compounds described herein, and, optionally, can be treated with one or more cancer treatments as described herein.

[000140] When treating a mammal (*e.g.*, a human) having a cancer as described herein, the cancer can be any appropriate cancer. A cancer can include one or more PD-L1 positive cancer cells. A cancer can include one or more solid tumors. A cancer can be a blood cancer. Examples of cancers that can be treated as described herein include, without limitation, breast cancer, colorectal cancer, kidney cancer, lung cancer (*e.g.*, non-small cell lung cancer), ovarian cancer, melanoma, brain cancer, sarcoma, prostate cancer, pancreatic cancer, head and neck cancer, liver cancer, retinoblastoma, lymphoma, and leukemia.

[000141] In some cases, a mammal can be identified as having a cancer (*e.g.*, a cancer including one or more PD-L1 positive cancer cells). Any appropriate method

can be used to identify a mammal having cancer. For example, imaging techniques and biopsy techniques can be used to identify mammals (*e.g.*, humans) having cancer.

[000142] Once identified as having a cancer, a mammal can be administered a composition containing one or more PD-L1 binding compounds described herein, and, optionally, can be treated with one or more cancer therapeutics and/or cancer treatments. The one or more cancer therapeutics can include any appropriate cancer treatments. In some cases, a cancer treatment can include surgery. In some cases, a cancer treatment can include radiation therapy. In some cases, a cancer treatment can include administration of a pharmacotherapy such as a chemotherapy, hormone therapy, targeted therapy, and/or cytotoxic therapy. Examples of cancer treatments include, without limitation, administration of one or more receptor tyrosine kinase inhibitors (*e.g.*, erlotinib), administration of one or more PD1/PD-L1 inhibitors (*e.g.*, nivolumab, pembrolizumab, atezolizumab, avelumab, cemiplimab, and durvalumab), administration of one or more immunotherapies such as immunotherapies that can target PD1/PD-L1 (*e.g.*, nivolumab, pembrolizumab, atezolizumab, avelumab, cemiplimab, durvalumab, alemtuzumab, ipilimumab, ofatumumab, and rituximab), administration of one or more antibodies which target GITR, administration of one or more platinum compounds (*e.g.*, a cisplatin or carboplatin), administration of one or more taxanes (*e.g.*, paclitaxel, docetaxel, or an albumin bound paclitaxel such as nab-paclitaxel), administration of altretamine, administration of capecitabine, administration of cyclophosphamide, administration of etoposide (vp-16), administration of gemcitabine, administration of ifosfamide, administration of irinotecan (cpt-11), administration of liposomal doxorubicin, administration of melphalan, administration of pemetrexed, administration of topotecan, administration of vinorelbine, administration of one or more luteinizing-hormone-releasing hormone (LHRH) agonists (such as goserelin and leuprolide), administration of one or more anti-estrogen therapies (such as tamoxifen), administration of one or more aromatase inhibitors (such as letrozole, anastrozole, and exemestane), administration of one or more angiogenesis inhibitors (such as bevacizumab), administration of one or more poly(ADP)-ribose polymerase (PARP)

inhibitors (such as olaparib, rucaparib, and niraparib), administration of external beam radiation therapy, administration of brachytherapy, administration of radioactive phosphorus, and administration of any combinations thereof. In cases where a mammal having cancer is treated with a composition containing one or more multivalent PD-L1 binding compounds or nucleic acid encoding an amino acid chain that can be used to generate multivalent PD-L1 binding compounds described herein, and is treated with one or more cancer treatments, the composition containing one or more cancer treatments can be administered at the same time or independently. For example, the composition containing one or more multivalent PD-L1 binding compounds or nucleic acid encoding an amino acid chain that can be used to generate multivalent PD-L1 binding compounds described herein can be administered first, and the one or more cancer treatments administered second, or *vice versa*.

[000143] In some cases, a composition comprising one or more PD-L1 binding compounds described herein can be formulated into a pharmaceutically acceptable composition for administration to a mammal having cancer. For example, a therapeutically effective amount of one or more multivalent PD-L1 binding compounds or nucleic acid encoding an amino acid chain that can be used to generate multivalent PD-L1 binding compounds described herein can be formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. The pharmaceutical composition may further comprise a therapeutic polypeptide and/or a recombinant virus which is genetically modified to express a therapeutic polypeptide. A pharmaceutical composition comprising, or consisting essentially of a multivalent PD-L1 binding compound, and optionally a therapeutic polypeptide, can be formulated for administration in solid or liquid form including, without limitation, sterile solutions, suspensions, sustained-release formulations, tablets, capsules, pills, powders, nano-particles, and granules. Pharmaceutically acceptable carriers, fillers, and vehicles that may be used in a pharmaceutical composition described herein include, without limitation, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial

glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

[000144] A pharmaceutical composition comprising or consisting essentially of one or more multivalent PD-L1 binding compounds as described herein, and optionally a therapeutic polypeptide, are formulated for oral or parenteral (including subcutaneous, intramuscular, intravenous, intratumoral, and intradermal) administration.

[000145] When being administered orally, a pharmaceutical composition comprising one or more PD-L1 binding compounds or nucleic acid encoding an amino acid chain that can be used to generate multivalent PD-L1 binding compounds described herein can be in the form of a pill, tablet, or capsule.

[000146] Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions that can contain anti-oxidants, buffers, bacteriostatic agents, and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets. Such injection solutions can be in the form, for example, of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated using, for example, suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation can be a sterile injectable solution or suspension in a non-toxic

parenterally-acceptable diluent or solvent, for example, as a solution in 1, 3-butanediol. Examples of acceptable vehicles and solvents that can be used include, without limitation, mannitol, water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils can be used as a solvent or suspending medium. In some cases, a bland fixed oil can be used such as synthetic mono- or di-glycerides. Fatty acids, such as oleic acid and its glyceride derivatives can be used in the preparation of injectables, as can natural pharmaceutically-acceptable oils, such as olive oil or castor oil, including those in their polyoxyethylated versions. In some cases, these oil solutions or suspensions can contain a long-chain alcohol diluent or dispersant.

[000147] Specific embodiments disclosed herein may be further limited in the claims using "consisting of" or "consisting essentially of" language. When used in the claims, whether as filed or added per amendment, the transition term "consisting of" excludes any element, step, or ingredient not specified in the claims. The transition term "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s).

[000148] In some cases, a pharmaceutically acceptable composition comprising one or more PD-L1 binding compounds described herein can be administered locally or systemically. For example, a composition containing a compound that inhibits the function of an intracellular PD-L1 domain can be administered locally by injection into or near a cancer (*e.g.*, a tumor) in a mammal (*e.g.*, a human). For example, a composition containing a compound that inhibits the function of an intracellular PD-L1 domain can be administered systemically by oral administration or by injection (*e.g.*, subcutaneous, intramuscular, intravenous, intratumoral, and intradermal injection) to a mammal (*e.g.*, a human).

[000149] Effective doses can vary depending on the severity of the cancer, the route of administration, the age and general health condition of the subject, excipient usage, the possibility of co-usage with other therapeutic treatments such as use of other agents, and the judgment of the treating physician.

[000150] An effective amount of a composition containing one or more PD-L1 binding compounds described herein can be any amount that can reduce the severity of the cancer, to reduce a symptom of the cancer, and/or to reduce the number of cancer cells present within the mammal without producing significant toxicity to the mammal. The effective amount can remain constant or can be adjusted as a sliding scale or variable dose depending on the mammal's response to treatment. Various factors can influence the actual effective amount used for a particular application. For example, the frequency of administration, duration of treatment, use of multiple treatment agents, route of administration, and severity of the condition (*e.g.*, cancer) may require an increase or decrease in the actual effective amount administered.

[000151] The frequency of administration can be any frequency that sensitizes cancer cells in the mammal to one or more cancer treatments (*e.g.*, one or more cancer immunotherapies) without producing significant toxicity to the mammal. For example, the frequency of administration can be from about once a week to about three times a day, or from about twice a month to about six times a day, or from about twice a week to about once a day. The frequency of administration can remain constant or can be variable during the duration of treatment. A course of treatment with a composition containing one or more PD-L1 binding compounds described herein can include rest periods. For example, a composition containing one or more multivalent PD-L1 binding compounds or nucleic acid encoding an amino acid chain that can be used to generate multivalent PD-L1 binding compounds described herein can be administered daily over a two-week period followed by a two-week rest period, and such a regimen can be repeated multiple times. As with the effective amount, various factors can influence the actual frequency of administration used for a particular application. For example, the effective amount, duration of treatment, use of multiple treatment agents, route of administration, and severity of the condition (*e.g.*, cancer) may require an increase or decrease in administration frequency.

[000152] An effective duration for administering a composition containing one or more PD-L1 binding compounds described herein can be any duration that can reduce the severity of the cancer, to reduce a symptom of the cancer, and/or to reduce the number of cancer cells present within the mammal without producing significant toxicity to the mammal. Thus, the effective duration can vary from several days to several weeks, months, or years. In general, the effective duration for the treatment of cancer can range in duration from six months to one year. Multiple factors can influence the actual effective duration used for a particular treatment. For example, an effective duration can vary with the frequency of administration, effective amount, use of multiple treatment agents, route of administration, and severity of the condition being treated.

[000153] In certain instances, a course of treatment and the severity of one or more symptoms related to the condition being treated (*e.g.*, cancer) can be monitored. Any appropriate method can be used to determine whether or not the sensitivity of cancer cells in the mammal to one or more cancer treatments (*e.g.*, one or more cancer immunotherapies) is increased. For example, the responsiveness of cancer (*e.g.*, based on the size and/or number of tumors) can be assessed using office imaging techniques at different time points.

[000154] The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1. Construction of Recombinant Adenoviruses: Insertion of Individual HVRs from Different Ad Serotypes with the Insertion of Cell Targeting/Detargeting Peptides or Novel Amino Acids.

[000155] Recombinant adenoviruses were constructed by recombinant DNA technology utilizing methods known to those skilled in the art. A recombinant Ad (Ad657) is derived from a first Ad strain Ad6 (*e.g.*, can include a genome of a first Ad

strain) and may include capsid hexon HVRs from a second Ad strain, Ad57, see Figure 8. These embodiments have been applied generally in the context of Ads which combine different capsid hexon HVRs from different Ads (i.e., shuffling HVRs). Figure 7 shows an alignment of Ad5, Ad6, and Ad57 showing variation in hexon regions. For example, HVR1 of Ad strain Ad6 with HVRs 2-7 of Ad strain Ad57 or HVR1 and 7 of Ad strain Ad6 with HVRs 2-6 of Ad strain Ad57. In a further embodiment, an Ad6/56/6 virus has HVRs 1 and 7 from Ad strain Ad6 and HVRs 2-6 from Ad strain Ad657.

[000156] In an aspect of the invention, chimeric Ads were generated which have an Ad6 HVR1 and Ad57 HVRs 2-7, the chimera, referred to as Ad6/57 HVR chimera, comprises the hexon having the amino acid sequence of SEQ ID NO:28.

[000157] In yet another aspect of the invention, chimeric Ads were generated which have Ad6 HVR1 and 7 and Ad57 HVRs 2-6, the chimera, referred to as Ad6/57/6 HVR chimera, comprises the hexon having the amino acid sequence of SEQ ID NO:29.

[000158] To obtain recombinant Ad strain Ad657, a nucleic acid encoding Ad57 HVRs 1-7 was synthesized and inserted into the Ad6 hexon in a plasmid with an FRT-Zeocin®-FRT cassette between pVI and hexon. This was recombined into various pAd6 plasmids to generate Ad657 and variants thereof. The amino acid sequence of the Ad657 hexon is set forth in SEQ ID NO:30.

[000159] With respect to variants of Ad657, the Ad57 HVR sequence was synthesized with HVR1 modified with a cysteine, flexibility amino acids, and restriction sites to allow insertions of other peptides. This was inserted into the Ad6 hexon in a plasmid with an FRT-Zeocin®-FRT cassette between pVI and hexon. This was recombined into various pAd6 plasmids to generate Ad657 variants with cysteine in HVR1, the variant referred to as Ad657-HVR1-XXA comprises the hexon having the amino acid sequence of SEQ ID NO:42.

[000160] With respect to variants of Ad657, the Ad57 HVR sequence was synthesized with HVR5 modified with a cysteine, flexibility amino acids, and restriction sites to allow insertions of other peptides. This was inserted into the Ad6 hexon in a plasmid with an FRT-Zeocin®-FRT cassette between pVI and hexon. This was recombined into various pAd6 plasmids to generate Ad657 variants with cysteine in HVR5, the variant referred to as Ad657-HVR5-XXA comprises the hexon having the amino acid sequence of SEQ ID NO:43.

[000161] With respect to variants of Ad657, the Ad57 HVR sequence was synthesized with HVR1 modified with a cysteine, flexibility amino acids, and restriction sites to allow insertions of other peptides. This was inserted into the Ad6 hexon in a plasmid with an FRT-Zeocin®-FRT cassette between pVI and hexon. This was recombined into various pAd6 plasmids to generate Ad657 variants without cysteine in HVR1, but with restriction sites allowing peptide insertions into HVR1, the variant referred to as Ad657-HVR1-XA comprises the hexon having the amino acid sequence of SEQ ID NO:44.

[000162] With respect to variants of Ad657, the Ad57 HVR sequence was synthesized with HVR5 modified with a cysteine, flexibility amino acids, and restriction sites to allow insertions of other peptides. This was inserted into the Ad6 hexon in a plasmid with an FRT-Zeocin®-FRT cassette between pVI and hexon. This was recombined into various pAd6 plasmids to generate Ad657 variants without cysteine in HVR5, but with restriction sites allowing peptide insertions into HVR5, the variant referred to as Ad657-HVR5-XA comprises the hexon having the amino acid sequence of SEQ ID NO:45.

[000163] With respect to variants of Ad657, the Ad657 HVR1-XA sequence was modified by insertion of a biotin acceptor peptide into HVR1. This was recombined into various pAd6 plasmids to generate Ad657 variants a BAP in HVR1, the variant referred to as Ad657-HVR1-PSTCD comprises the hexon having the amino acid sequence of SEQ ID NO:46.

[000164] The insertion of a biotin acceptor peptide detargets the virus variants from the liver, allows the virus to be retargeted with avidin or streptavidin and biotinylated ligands, and allows the virus to be purified on monomeric avidin or streptavidin columns.

[000165] With respect to variants of Ad657, the Ad657 HVR1-XA sequence was modified by insertion of a biotin acceptor peptide (BAP) into HVR1. This was recombined into various pAd6 plasmids to generate Ad657 variants a BAP in HVR1, the variant referred to as Ad657-HVR5-PSTCD comprises the hexon having the amino acid sequence of SEQ ID NO:47.

[000166] With respect to variants of Ad657, the Ad657 HVR5-XA sequence was modified by insertion of a synthetic V1/V2 loop from HIV envelope into HVR5, the variant referred to as Ad657-HVR5-V1/V2 comprises the hexon having the amino acid sequence of SEQ ID NO:48.

[000167] The insertion of a synthetic V1/V2 loop from HIV envelope allows display of this antigen to serve as a vaccine as well as retargeting by binding to proteins that interact with HIV envelope.

[000168] With respect to variants of Ad657, the Ad657 HVR5-XA sequence was modified by insertion of synthetic peptides from human papilloma virus (HPV) into HVR5, the variant referred to as Ad657-HVR5-HPV comprises the hexon having the amino acid sequence of SEQ ID NO:49.

[000169] The insertion of synthetic peptides from human papilloma virus allows display of HPV peptides as antigens for vaccine purposes as well as for retargeting by binding to proteins that interact with HPV peptides.

[000170] In another aspect of the invention, chimeric Ads were generated which have an Ad6 HVR1 and Ad57 HVRs 2-7, the chimera, referred to as Ad6/57 HVR chimera, comprises the hexon having the amino acid sequence of SEQ ID NO:50.

[000171] In yet another aspect of the invention, chimeric Ads were generated which have Ad6 HVR1 and 7 and Ad57 HVRs 2-6, the chimera, referred to as Ad6/57/6 HVR chimera, comprises the hexon having the amino acid sequence of SEQ ID NO:51.

[000172] The plasmid maps of Figures 16-18 show the combination of the insertion of individual HVRs from different Ad serotypes with the insertion of cell targeting/detargeting peptides, therapeutic polypeptides, or novel amino acids such as cysteine into the hexon for targeted chemical modification and shielding.

[000173] In certain embodiments, cell binding peptides are inserted into HVR 1 or HVR 5, which embodiments serve as examples of inserting these and other peptides in any of the HVRs of an Ad.

Example 2. Targeted Chemical Conjugation of Cysteine-modified Hexon-modified Ad57-HVR5C.

[000174] Figure 13 is a depiction of Ad variants showing the combination of insertion of individual HVRs from different Ad serotypes with the insertion of novel amino acids such as cysteine into the hexon for targeted chemical modification and shielding.

[000175] This example demonstrates the ability to target polymer and other chemical modifications to cysteines inserted into an Ad hexon (Figure 15). Untargeted PEG inactivates virus infection whereas cysteine-targeting PEGylation retains virus functions.

[000176] In an aspect of the invention, the use of polymers or inserted peptides/proteins to detarget, retarget, and shield from antibodies, proteins, cells is contemplated. Figures 12 and 14 depict sites of Ad HVRs which may be modified, for example, by PEGylation or "BAPylation". Another example shows insertion of a biotin acceptor peptide (BAP) is inserted into these HVRs allowing for vector retargeting with avidin or streptavidin and biotinylated ligands or with avidin- or streptavidin fusion

proteins. BAP insertion also allows the viruses to be purified on monomeric avidin or streptavidin columns for vector production. Likewise, Ad57-HVR1-XXA and XA shows the example of inserting a cysteine into this site to allow targeted chemical modification with maleimide or other cysteine-reactive agents.

[000177] In an embodiment, the different Ad serotypes and/or variants comprise polymer shielding to allow multi dosing of Ad6 and Ad657 variants. An exemplary therapeutic cycle where Ad6 and Ad657 can be used for multiple rounds of treatment by serotype-switching in combination with covalent polymer conjugation is shown (Figure 13).

[000178] Ad657-HVR1C expressing GFP^{Luciferase} was produced from cells and purified on CsCl gradients. The virus was covalently modified with 5 kDa polyethylene glycol (PEG). The virus was treated with either NHS-PEG that reacts randomly with amines/lysines on viral proteins or with maleimide PEG that reacts specifically with cysteine that was inserted into HVR1 using the XXA shuttle plasmid. These unmodified or modified viruses were then purified by a final CsCl spin followed by desalting. The indicated virus were separated on SDS-PAGE gels, stained with SyproRuby, and visualized by imaging (Figure 14). This shows that NHS-PEGylation randomly modifies many viral proteins as demonstrated by increases in the apparent mass of the proteins (indicated by arrows). In contrast, targeted maleimide PEG reaction with the cysteine in HVR1 modifies only hexon and does not damage other viral capsomer proteins. The effects of PEGylation on virus function is evaluated.

Example 3. Conditionally Replicating Ads (CRAds).

[000179] Schematic of mutations in Ad6, Ad657 and variants thereof involving mutations in the E1 protein to convert the virus to a conditionally-replicating Ad (CRAd) is shown in Figure 9, Figure 10 and Figure 11. These include dl1101 and/or the dl1107 that block binding to p300 and pRB, respectively.

[000180] Figure 10 shows the N-terminal amino acid sequences of E1A in a wild-type Ad, as well as Ad variants E1A dl1101, E1A dl1107 and E1A dl1101/1107.

[000181] Also shown is the replacement of the Ad E1 promoter with the prostate-specific promoter probasin and the E1 DNA sequence of SEQ ID NO:31 to generate the CRA_d, Ad-PB (Figure 9). The probasin promoter is androgen dependent, so will work in androgen-sensitive tumors like LNCaP, but not in androgen-resistant tumors like DU145.

[000182] In an embodiment, a conditionally replicative Ad657 virus having a dl1101 modification to E1, and a dl1107 mutation in E1a, and an E3A deletion, is constructed to express human PD-1 fused to human immunoglobulin as a PD-L1 decoy protein. The virus also expresses GFP_{Luciferase} from a CMV promoter. Such conditionally replicative Ad657 virus variant is referred to as CrAd6dl1101/1107DE3ADP-hPD-1-Ig-GL.

Example 4. Retargeted and Detargeted Recombinant Adenovirus.

[000183] *In vitro*, Ads bind and enter cells through the combined interactions of its fiber and penton base proteins with cell surface receptors. The trimeric fiber binds the coxsackie-adenovirus receptor (CAR), and cells that lack CAR are relatively resistant to infection unless they also express α_v integrins that can be bound by an RGD motif on the penton base.

[000184] The Ad fiber protein is a complex of three apparently identical subunits which mediates the initial attachment step. The native Ad6 fiber protein comprises the amino acid sequence set forth in SEQ ID NO:35 and binds CAR.

[000185] In a further aspect of the invention, fiber-modified recombinant Ads having different fiber proteins which are not native to the parental Ad were generated. Recombinant Ads, including CRA_ds, comprising capsid proteins from different Ad strains were generated, for example, recombinant Ads comprising a heterologous Ad35 fiber polypeptide or Chimpanzee C68 fiber polypeptide, +/- a K7 peptide.

[000186] A chimeric Ad, AdF35 fiber chimera, has the amino acid sequence of SEQ ID NO:36 and is shorter than Ad5 and Ad6 fiber proteins and retargets virus to CD46.

[000187] A fiber-modified recombinant Ad, comprising K7 Fiber having the sequence of SEQ ID NO:37, targets virus to heparin sulfate proteoglycans and negative charges on cells.

[000188] A recombinant, chimeric Ad, 6/FC68 Fiber comprising the sequence of SEQ ID NO:38, is a chimeric Ad having a fiber protein from chimpanzee adenovirus C68. The fiber protein is shorter than Ad5 or Ad6 fiber proteins and binds CAR.

[000189] A recombinant, chimeric Ad, 6/FC68-K7 Fiber comprising the sequence of SEQ ID NO:39, is a chimeric Ad having a fiber protein from chimpanzee adenovirus C68. The fiber protein is shorter than Ad5 or Ad6 fiber proteins. The 6/FC68-K7 Fiber binds CAR and is retargeted to heparin sulfate and negative charges.

[000190] A recombinant, chimeric Ad, 6/FC68-HI-K7 Fiber comprising the sequence of SEQ ID NO:40, is a chimeric Ad having a fiber protein from chimpanzee adenovirus C68. The fiber protein is shorter than Ad5 or Ad6 fiber proteins. The 6/FC68-HI-K7 Fiber binds CAR and is retargeted to heparin sulfate and negative charges.

[000191] Blood factor X (FX) binds with nanomolar affinity to the hexons of species C adenovirus and, consequently, enables species C adenovirus to efficiently transduce liver hepatocytes after IV injection.

[000192] The following CRAAd viruses are constructed which express human Factor X (GLA) fused to human PD-1. Wild-type and high affinity mouse and human PD-1 domains were fused to the N- or C-termini of human FX GLA-EGF domain and their expression was driven by either RSV or CMV promoter. In some cases these

fusion protein expression cassettes were inserted into the E3 domain of the indicated viruses. In others, they were inserted between the fiber and E4 genes of the viruses.

[000193] Conditionally replicative Ad657 with a dl1101 modification to E1a prevent its binding to p300 and to become susceptible to IFN, an E3A deletion, with an RSV expression cassette inserted into the E3 deletion. This virus expresses human Factor X (GLA) fused to human PD-1 and an adenovirus retargeting protein to PD-L1+ cells (i.e., CrAd657d1101DE3ADP*hPD1-GLA-GL-L).

[000194] Conditionally replicative Ad657 with a dl1101 modification to E1a prevent its binding to p300 and to make virus susceptible to IFN repression and a dl1107 mutation in E1a to prevent its binding to pRB to block virus replication in cells with intact pRB pathways, with deleted E3, and comprises an RSV expression cassette inserted into the E3 deletion. This virus expresses human Factor X (GLA) fused to human PD-1 (i.e., CrAd657*1101/1107DE3BSTRSVhPD1-GLA*IgIZI). During viral replication, the PD-1 will be expressed in a cell as a monomer which is capable of binding PD-L1 when released from the cell. The PD-1 can also be bound to the surface of an Ad to retarget the Ad to PD-L1-expressing cells.

[000195] Conditionally replicative Ad6/57/6 with a dl1107 mutation in E1a, and an E3A deletion with an RSV expression cassette inserted into the E3 deletion, is constructed to express human PD-1 fused to human immunoglobulin as a PD-L1 decoy protein (i.e., pAd/57/6-dl1107-ΔE3-RSV-hPD-1-HA-Ig-I). See Figure 16.

[000196] The fusion protein was purified from cells infected with pAd6/57/6-dl1107-DE3-RSV-hPD-1-HA-Ig-I on a protein A column. This protein was titrated along side positive control anti-PD-1 monoclonal antibody in the PD-1/PD-L1 Blockade Assay from Promega (Madison, WI, USA). The high affinity human hPD-1-HA-Ig fusion protein inhibits PD-L1. See Figure 19.

Example 5. Construction of recombinant Ads for expression of PD-L1 binding compounds in combination with heterologous polypeptides.

[000197] A conditionally replicative Ad virus (CRAd657 or CRAd 6/57/6) is modified to express PD-1 polypeptides in combination with one or more heterologous polypeptides (e.g., therapeutic polypeptides and/or targeting polypeptides). In one embodiment, the RSV-PD-1 fusion cassette is inserted into an E3 deletion of the virus and the therapeutic transgenes are inserted as CMV or MCMV or RSV expression cassettes in between fiber and E4 genes according to methods known to those skilled in the art. See Figure 17 and Figure 18.

Example 6. Tumor Growth Assessment of CRAd Expressing High Affinity Human hPD-1-HA-Ig Fusion Protein Alone or in Combination with Immune Therapy Payloads.

[000198] B16-CAR melanoma cells are injected subcutaneously in C57BL/6 mice and resulting tumors are injected with 3×10^{11} viral particles of the multivalent PD-L1 binding compounds of the invention. CRAd+PD-L1 decoy is pAd6/57/6-dl1107- Δ E3-RSV-hPD-1-HA-Ig-I. CRAd+PD-L1 decoy + immune simulator are co-injected into tumors using pAd6/57/6-dl1107- Δ E3-RSV-hPD-1-HA-Ig-I and a second adenovirus expressing 4-1BBL. CRAd expressing high affinity human hPD-1-HA-Ig fusion protein delays tumor growth by itself or in combination with immune therapy payloads. See Figure 20.

[000199] In an embodiment, the CRAd657 or CRAd6/57/6 is modified to express PD-1 polypeptides in combination with one or more therapeutic polypeptides. The therapeutic polypeptide may be selected from GM-CSF, 4-1BBL, CD40L and OX40, and combinations thereof. In one aspect, the PD-1 polypeptide is expressed with the therapeutic polypeptide as a fusion protein. In a further aspect, a RSV-PD-1 fusion cassette is inserted into an E3 deletion of the virus and the therapeutic transgenes are inserted as CMV or MCMV or RSV expression cassettes in between the virus fiber and E4 genes. See Figures 17 and 18.

Example 7. Construction of Multivalent PD-L1 Binding Compounds.

[000200] Fusion proteins including a PD-1 polypeptide and either a scaffold polypeptide or a GLA domain of an FX polypeptide are generated from genes that are PCR cloned with mutations and fusion junctions from wild-type, mutant, or

synthetic genes according to methods known to those skilled in the art. Fusion proteins are assembled by cloning in expression vectors, culturing under conditions which allow expression and assembly of proteins and, optionally, purifying the proteins from the cellular milieu.

[000201] The fusion proteins may be produced by plasmid transfection or infection of cells with vectors expressing PD-1 polypeptides and/or fragments thereof fused to a polypeptide that can bind to a viral coat polypeptide, followed by purification with PD-1 or a fusion partner on antibody columns or on PD-L1 columns or on biotin columns.

[000202] In an embodiment, Ads may be contacted with the purified PD-1 fusion proteins under conditions which allow for coating of the Ad surface with the fusion protein. For example, the GLA domain of an FX polypeptide binds to the surface of the Ad such that the PD-1 is exposed on the surface of the Ad to bind to PD-L1 on the surface of a cell. An adenovirus wherein the fusion partner is bound to the Ad surface and the PD-1 is free to bind PD-L1 on a cell, provides a multimeric PD-L1 binding compound.

[000203] In an embodiment, nucleic acids encoding the fusion proteins are inserted into plasmids or viral vectors or are inserted in mRNA production vectors and these are transfected or infected *in vitro* into cell cultures or infected *in vivo* in animals. Samples are assayed for protein expression with antibodies against PD-1 and/or fusion protein partners.

[000204] Nucleic acid (e.g., DNA, mRNA, and viral vectors) encoding the fusion proteins are delivered into normal or cancer-bearing animals and changes in peripheral blood cells, lymph node cells, spleen cell, and tumor cell populations are assessed by staining with CD3, CD4, CD8, PD-1, PD-L1, and T cell activation markers.

[000205] Nucleic acid (e.g., DNA, mRNA) or viral vectors encoding the fusion proteins are delivered into cancer-bearing animals and effects on tumor size, metastases, dissemination, and survival were assessed.

Other Embodiments.

[000206] It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

Claims

1. A multivalent programmed death ligand 1 (PD-L1) binding compound comprising,

a recombinant Adenovirus (Ad) comprising capsid hexon polypeptides of an Adenovirus strain Ad6 and at least one capsid hexon hypervariable region (HVR) polypeptide from Adenovirus strain Ad57,

wherein the recombinant Ad comprises a plurality of amino acid chains bound to the surface of the recombinant Ad,

wherein each amino acid chain comprises at least one programmed cell death protein 1 (PD-1) polypeptide.
2. The PD-L1 binding compound of Claim 1, wherein the capsid hexon polypeptides of the Adenovirus strain Ad6 comprise HVR polypeptides 1-7 from Adenovirus strain Ad57.
3. The PD-L1 binding compound of Claim 1, wherein the capsid hexon polypeptides of the Adenovirus strain Ad6 comprise HVR polypeptides 2-7 from Adenovirus strain Ad57.
4. The PD-L1 binding compound of Claim 1, wherein the recombinant Ad is a conditionally-replicating Adenovirus (CRAd) which has been modified in an E1A gene encoding an E1A polypeptide, wherein the CRAd exhibits amino acid substitutions in the E1A polypeptide relative to wild-type E1A polypeptide of an Ad strain.
5. The PD-L1 binding compound of Claim 4, wherein the recombinant Ad is a conditionally-replicating Adenovirus (CRAd) wherein the N-terminal portion of the E1A polypeptide comprises an amino acid sequence set forth in SEQ ID NO:32, SEQ ID NO:33 or SEQ ID NO:34.

6. The PD-L1 binding compound of Claim 1, wherein the PD-1 polypeptide is in the form of a fusion protein with a heterologous polypeptide selected from targeting polypeptides, therapeutic polypeptides and/or antigens.
7. The PD-L1 binding compound of Claim 1, wherein the PD-1 polypeptide is a human PD-1 or a murine PD-1.
8. The PD-L1 binding compound of Claim 1, wherein the PD-1 polypeptide exhibits higher affinity interactions with PD-L1 relative to a wild-type PD-1 protein.
9. The PD-L1 binding compound of Claim 1, wherein the PD-1 polypeptide is a fragment of a human PD-1 polypeptide, wherein the fragment of the human PD-1 polypeptide comprises one or more amino acid changes relative to a wild type PD-1, which changes cause an increased affinity for human PD-L1.
10. The PD-L1 binding compound of Claim 1, wherein the recombinant Ad comprises an expression cassette comprising a promoter operably linked to nucleic acids encoding a PD-1 polypeptide which allows for expression of the PD-1 polypeptide in eukaryotic cells.
11. The PD-L1 binding compound of Claim 1, wherein the recombinant Ad comprises nucleic acids which encode one or more therapeutic polypeptides.
12. The PD-L1 binding compound of Claim 11, wherein the recombinant Ad comprises nucleic acids which encode an antibody.
13. The PD-L1 binding compound of Claim 1, wherein the recombinant Ad comprises an expression cassette comprising a promoter operably linked to nucleic acids encoding a therapeutic polypeptide which allows for expression of therapeutic polypeptides in eukaryotic cells.

14. The PD-L1 binding compound of Claim 13, wherein the therapeutic polypeptides are selected from a 4-1BB ligand polypeptide, a OX40 ligand polypeptide, a CD40 ligand polypeptide, and a granulocyte-macrophage colony-stimulating factor polypeptide.
15. The PD-L1 binding compound of Claim 6, wherein the PD-1 polypeptide is fused to a heterologous polypeptide which is a Vitamin K-dependent gamma-carboxyglutamic domain of a factor X single-chain antibody polypeptide (a GLA domain of an FX polypeptide).
16. A pharmaceutical composition comprising the multivalent PD-L1 binding compound of any one of Claims 1 to 15 and a pharmaceutically acceptable carrier.
17. Use of the multivalent PD-L1 binding compound of any one of Claim 1 to 15 in the preparation of a medicament for treating cancer in a subject, wherein cancer cells of the cancer are positive for PD-L-1 expression.
18. The use of claim 17, wherein the subject is identified as having a cancer selected from the group consisting of breast cancer, lung cancer, melanoma and renal cancer.
19. The use of Claim 17 or Claim 18, further comprising one or more cancer therapeutics to be administered to the subject.
20. The use of Claim 19, wherein the cancer therapeutic is an immunotherapy which targets PD-1/PD-L1 pathways.
21. The use of Claim 20, wherein the immunotherapy is selected from the group consisting of nivolumab, pembrolizumab, atezolizumab, avelumab, cemiplimab, and durvalumab.

22. Use of

a multivalent programmed death ligand 1 (PD-L1) binding compound comprising a recombinant Adenovirus (Ad) comprising capsid hexon polypeptides of an Adenovirus strain Ad6 and at least one capsid hexon hypervariable region (HVR) polypeptide from Adenovirus strain Ad57,

wherein the recombinant Ad comprises a plurality of amino acid chains displayed on the surface of the recombinant Ad, and

wherein each amino acid chain comprises at least one programmed cell death protein 1 (PD-1) polypeptide,

in the preparation of a medicament for treating cancer in a subject, wherein cancer cells of the cancer are positive for PD-L1 expression.