

NEWCASTLE DISEASE VIRUSES AND USES THEREOF

[0001] This application claims priority to U.S. Provisional Application No. 61/782,994, filed on March 14, 2013, which is incorporated by reference herein in its entirety.

[0002] This invention was made, in part, with Government support under award numbers 5T32CA009207-35 and HHSN26620070010C from the National Institutes of Health. The Government has certain rights in this invention.

1. INTRODUCTION

[0003] Described herein are chimeric Newcastle disease viruses engineered to express an agonist of a co-stimulatory signal of an immune cell and compositions comprising such viruses. Also described herein are chimeric Newcastle disease viruses engineered to express an antagonist of an inhibitory signal of an immune cell and compositions comprising such viruses. The chimeric Newcastle disease viruses and compositions are useful in the treatment of cancer. In addition, described herein are methods for treating cancer comprising administering Newcastle disease viruses in combination with an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell.

2. BACKGROUND

[0004] Newcastle Disease Virus (NDV) is a member of the Avulavirus genus in the Paramyxoviridae family, which has been shown to infect a number of avian species (Alexander, DJ (1988). Newcastle disease, Newcastle disease virus -- an avian paramyxovirus. Kluwer Academic Publishers: Dordrecht, The Netherlands. pp 1-22). NDV possesses a single-stranded RNA genome in negative sense and does not undergo recombination with the host genome or with other viruses (Alexander, DJ (1988). Newcastle disease, Newcastle disease virus -- an avian paramyxovirus. Kluwer Academic Publishers: Dordrecht, The Netherlands. pp 1-22). The genomic RNA contains genes in the order of 3'-NP-P-M-F-HN-L-5', described in further detail below. Two additional proteins, V and W, are produced by NDV from the P gene by alternative mRNAs that are generated by RNA editing. The genomic RNA also contains a leader sequence at the 3' end.

[0005] The structural elements of the virion include the virus envelope which is a lipid bilayer derived from the cell plasma membrane. The glycoprotein, hemagglutinin-neuraminidase (HN) protrudes from the envelope allowing the virus to contain both hemagglutinin (*e.g.*, receptor binding / fusogenic) and neuraminidase activities. The fusion glycoprotein (F), which also interacts with the viral membrane, is first produced as an inactive precursor, then cleaved post-translationally to produce two disulfide linked polypeptides. The active F protein is involved in penetration of NDV into host cells by facilitating fusion of the viral envelope with the host cell plasma membrane. The matrix protein (M), is involved with viral assembly, and interacts with both the viral membrane as well as the nucleocapsid proteins.

[0006] The main protein subunit of the nucleocapsid is the nucleocapsid protein (NP) which confers helical symmetry on the capsid. In association with the nucleocapsid are the P and L proteins. The phosphoprotein (P), which is subject to phosphorylation, is thought to play a regulatory role in transcription, and may also be involved in methylation, phosphorylation and polyadenylation. The L gene, which encodes an RNA-dependent RNA polymerase, is required for viral RNA synthesis together with the P protein. The L protein, which takes up nearly half of the coding capacity of the viral genome is the largest of the viral proteins, and plays an important role in both transcription and replication. The V protein has been shown to inhibit interferon- α and to contribute to the virulence of NDV (Huang *et al.* (2003). Newcastle disease virus V protein is associated with viral pathogenesis and functions as an Alpha Interferon Antagonist. *Journal of Virology* **77**: 8676-8685).

[0007] Naturally-occurring NDV has been reported to be an effective oncolytic agent in a variety of animal tumor models (Sinkovics, JG, and Horvath, JC (2000). Newcastle disease virus (NDV): brief history of its oncolytic strains. *J Clin Virol* **16**: 1-15; Zamarin *et al.*, 2009; Mol Ther **17**: 697; Elankumaran *et al.*, 2010; J Virol **84**: 3835; Schirrmacher *et al.*, 2009; Methods Mol Biol **542**: 565; Bart *et al.*, 1973; Nat New Biol **245**: 229). Naturally-occurring strains of NDV have been used in multiple clinical trials against advanced human cancers (Sinkovics, JG, and Horvath, JC (2000). Newcastle disease virus (NDV): brief history of its oncolytic strains. *J Clin Virol* **16**: 1-15; Lorence *et al.* (2007). Phase 1 clinical experience using intravenous administration of PV701, an oncolytic Newcastle disease virus. *Curr Cancer Drug Targets* **7**: 157-167; Hotte *et al.* (2007). An optimized clinical regimen for the oncolytic virus PV701. *Clin Cancer Res* **13**: 977-985; Freeman *et al.* (2006). Phase I/II trial of intravenous NDV-HUJ

oncolytic virus in recurrent glioblastoma multiforme. *Mol Ther* **13**: 221-228; Pecora *et al.* (2002). Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers. *J Clin Oncol* **20**: 2251-2266; Csatory *et al.* (2004). MTH-68/H oncolytic viral treatment in human high-grade gliomas. *J Neurooncol* **67**: 83-93). However, the success of naturally-occurring strains of NDV in these clinical trials for advanced human cancers was only marginal (Hotte *et al.* (2007). An optimized clinical regimen for the oncolytic virus PV701. *Clin Cancer Res* **13**: 977-985; Freeman *et al.* (2006). Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme. *Mol Ther* **13**: 221-228; Pecora *et al.* (2002). Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers. *J Clin Oncol* **20**: 2251-2266). As such, there remains a need for NDV-based therapies useful in the treatment of cancer, especially advanced cancer.

3. SUMMARY

[0008] In one aspect, presented herein are chimeric Newcastle disease viruses (NDVs) engineered to express an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell. In a specific embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an agonist of a co-stimulatory signal of an immune cell, wherein the agonist is expressed. In a specific embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an antagonist of an inhibitory signal of an immune cell, wherein the antagonist is expressed.

[0009] In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an agonist of a co-stimulatory signal of an immune cell and a mutated F protein that causes the NDV to be highly fusogenic, wherein the agonist and the mutated F protein are expressed. In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an agonist of a co-stimulatory signal of an immune cell and a mutated F protein with a mutated cleavage site, wherein the agonist and the mutated F protein are expressed. In a specific embodiment, the chimeric NDVs expressing the mutated F protein have increased fusogenic activity relative to the corresponding virus expressing the counterpart F protein without the mutations to the cleavage site. In another specific embodiment, the modified F protein is incorporated into the virion.

[0010] In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an antagonist of an inhibitory signal of an immune cell and a mutated F protein that causes the NDV to be highly fusogenic, wherein the antagonist and the mutated F protein are expressed. In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes antagonist of an inhibitory signal of an immune cell and a mutated F protein with a mutated cleavage site, wherein the antagonist and the mutated F protein are expressed. In a specific embodiment, the chimeric NDVs expressing the mutated F protein have increased fusogenic activity relative to the corresponding virus expressing the counterpart F protein without the mutations to the cleavage site. In another specific embodiment, the modified F protein is incorporated into the virion.

[0011] In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an agonist of a co-stimulatory signal of an immune cell and a cytokine (*e.g.*, interleukin (IL)-2), wherein the agonist and the cytokine are expressed. In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an agonist of a co-stimulatory signal of an immune cell, a cytokine (*e.g.*, IL-2) and a mutated F protein that causes the NDV to be highly fusogenic, wherein the agonist, the cytokine and the mutated F protein are expressed. In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an agonist of a co-stimulatory signal of an immune cell, a cytokine (*e.g.*, IL-2) and a mutated F protein with a mutated cleavage site, wherein the agonist, the cytokine and the mutated F protein are expressed. In a specific embodiment, the chimeric NDVs expressing the mutated F protein with the mutated cleavage site are highly fusogenic. In another specific embodiment, the mutated F protein is incorporated into the virion.

[0012] In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an antagonist of an inhibitory signal of an immune cell of an immune cell and a cytokine (*e.g.*, IL-2), wherein the antagonist and the cytokine are expressed. In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an antagonist of an inhibitory signal of an immune cell, a cytokine (*e.g.*, IL-2) and a mutated F protein that causes the NDV to be highly fusogenic, wherein the antagonist, the cytokine and the mutated F protein are expressed. In another embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an antagonist of an inhibitory

signal of an immune cell, a cytokine (e.g., IL-2) and a mutated F protein with a mutated cleavage site, wherein the antagonist, the cytokine and the mutated F protein are expressed. In a specific embodiment, the chimeric NDVs expressing the mutated F protein with the mutated cleavage site are highly fusogenic. In another specific embodiment, the mutated F protein is incorporated into the virion.

[0013] In a specific embodiment, the agonist of a co-stimulatory signal of an immune cell is an agonist of a co-stimulatory receptor expressed by an immune cell. Specific examples of co-stimulatory receptors include glucocorticoid-induced tumor necrosis factor receptor (GITR), Inducible T-cell costimulator (ICOS or CD278), OX40 (CD134), CD27, CD28, 4-1BB (CD137), CD40, CD226, cytotoxic and regulatory T cell molecule (CRTAM), death receptor 3 (DR3), lymphotoxin-beta receptor (LTBR), transmembrane activator and CAML interactor (TACI), B cell-activating factor receptor (BAFFR), and B cell maturation protein (BCMA). In a specific embodiment, the agonist of a co-stimulatory receptor expressed by an immune cell is an antibody (or an antigen-binding fragment thereof) or ligand that specifically binds to the co-stimulatory receptor. In one embodiment, the antibody is a monoclonal antibody. In another embodiment, the antibody is an sc-Fv. In a specific embodiment, the antibody is a bispecific antibody that binds to two receptors on an immune cell. In one embodiment, the bispecific antibody binds to a receptor on an immune cell and to another receptor on a cancer cell. In specific embodiments, the antibody is a human or humanized antibody. In certain embodiments, the ligand or antibody is a chimeric protein comprising a NDV F protein or fragment thereof, or NDV HN protein or fragment thereof. Methods for generating such chimeric proteins are known in the art. *See, e.g.*, U.S. Patent Application Publication No. 2012-0122185, the disclosure of which is herein incorporated by reference in its entirety. Also see Park et al., PNAS 2006; 103:8203-8 and Murawski et al., J Virol 2010; 84:1110-23, the disclosures of which is herein incorporated by reference in their entireties. In certain embodiments, the ligand or antibody is expressed as a chimeric F protein or NDV F-fusion protein, wherein the chimeric F protein or NDV F-fusion protein comprises the cytoplasmic and transmembrane domains or fragments thereof of the NDV F glycoprotein and the extracellular domain comprises the ligand or antibody. In some embodiments, the ligand is expressed as a chimeric HN protein or NDV HN-fusion protein, wherein the chimeric HN protein or NDV HN-fusion protein comprises the transmembrane and extracellular domains or fragments thereof of the NDV HN glycoprotein and

the extracellular domain comprises the ligand or antibody. In a specific embodiment, the ligand or antibody is expressed as a chimeric protein, such as described in Section 7, Example 2, *infra*.

[0014] In a specific embodiment, the antagonist of an inhibitory signal of an immune cell is an antagonist of an inhibitory receptor expressed by an immune cell. Specific examples of inhibitory receptors include cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4 or CD52), programmed cell death protein 1 (PD1 or CD279), B and T-lymphocyte attenuator (BTLA), killer cell immunoglobulin-like receptor (KIR), lymphocyte activation gene 3 (LAG3), T-cell membrane protein 3 (TIM3), adenosine A2a receptor (A2aR), T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), and CD160. In a specific embodiment, the antagonist of an inhibitory receptor expressed by an immune cell is an antibody (or an antigen-binding fragment thereof) that specifically binds to the co-stimulatory receptor. In one embodiment, the antibody is a monoclonal antibody. In another embodiment, the antibody is an sc-Fv. In specific embodiments, the antibody is a human or humanized antibody. In another specific embodiment, the antagonist of an inhibitory receptor is a soluble receptor or antibody (or an antigen-binding fragment thereof) that specifically binds to a ligand of the inhibitory receptor. In certain embodiments, the antibody is a chimeric protein comprising a NDV F protein or fragment thereof, or NDV HN protein or fragment thereof. *See, e.g.*, U.S. Patent Application Publication No. 2012-0122185, Park *et al.*, PNAS 2006; 103: 8203-8, and Murawski *et al.*, J. Virol 2010; 84:1110-23, which are each incorporated herein by reference in their entirety. In certain embodiments, the antibody is expressed as a chimeric F protein or NDV F-fusion protein, wherein the chimeric F protein or NDV-F-fusion protein comprises the cytoplasmic and transmembrane domains or fragments thereof of the NDV F glycoprotein and the extracellular domain comprises the antibody. In some embodiments, the antibody is expressed as a chimeric HN protein or NDV HN-fusion protein, wherein the chimeric HN protein or NDV HN-fusion protein comprises the transmembrane and intracellular domains or fragments thereof of the NDV HN glycoprotein and the extracellular domain comprises the antibody.

[0015] In another aspect, presented herein are methods for propagating the NDVs described herein (*e.g.*, chimeric NDVs described herein). The NDVs described herein (*e.g.*, chimeric NDVs described herein) can be propagated in any cell, subject, tissue or organ susceptible to a NDV infection. In one embodiment, the NDVs described herein (*e.g.*, chimeric NDVs described

herein) may be propagated in a cell line. In another embodiment, the NDVs described herein (*e.g.*, chimeric NDVs described herein) may be propagated in cancer cells. In another embodiment, the NDVs described herein (*e.g.*, chimeric NDVs described herein) may be propagated in an embryonated egg. In certain embodiments, presented herein are isolated cells, tissues or organs infected with an NDV described herein (*e.g.*, a chimeric NDV described herein). *See, e.g.*, Section 5.4, *infra*, for examples of cells, animals and eggs to infect with an NDV described herein (*e.g.*, a chimeric NDV described herein). In specific embodiments, presented herein are isolated cancer cells infected with an NDV described herein (*e.g.*, a chimeric NDV described herein). In certain embodiments, presented herein are cell lines infected with an NDV described herein (*e.g.*, a chimeric NDV described herein). In other embodiments, presented herein are embryonated eggs infected with an NDV described herein (*e.g.*, a chimeric NDV described herein).

[0016] In another aspect, presented herein are compositions comprising an NDV described herein (*e.g.*, a chimeric NDV described herein). In a specific embodiment, presented herein are pharmaceutical compositions comprising an NDV described herein (*e.g.*, a chimeric NDV described herein) and a pharmaceutically acceptable carrier. In another embodiment, presented herein are pharmaceutical compositions comprising cancer cells infected with an NDV described herein (*e.g.*, a chimeric NDV described herein), and a pharmaceutically acceptable carrier. In specific embodiments, the cancer cells have been treated with gamma radiation prior to incorporation into the pharmaceutical composition. In specific embodiments, the cancer cells have been treated with gamma radiation before infection with the NDV (*e.g.*, chimeric NDV). In other specific embodiments, the cancer cells have been treated with gamma radiation after infection with the NDV (*e.g.*, chimeric NDV). In another embodiment, presented herein are pharmaceutical compositions comprising protein concentrate from lysed NDV-infected cancer cells (*e.g.*, chimeric-NDV infected cancer cells), and a pharmaceutically acceptable carrier.

[0017] In another aspect, presented herein are methods for producing pharmaceutical compositions comprising an NDV described herein (*e.g.*, a chimeric NDV described herein). In one embodiment, a method for producing a pharmaceutical composition comprises: (a) propagating an NDV described herein (*e.g.*, a chimeric NDV described herein) in a cell line that is susceptible to an NDV infection; and (b) collecting the progeny virus, wherein the virus is grown to sufficient quantities and under sufficient conditions that the virus is free from

contamination, such that the progeny virus is suitable for formulation into a pharmaceutical composition. In another embodiment, a method for producing a pharmaceutical composition comprises: (a) propagating an NDV described herein (*e.g.*, a chimeric NDV described herein) in an embryonated egg; and (b) collecting the progeny virus, wherein the virus is grown to sufficient quantities and under sufficient conditions that the virus is free from contamination, such that the progeny virus is suitable for formulation into a pharmaceutical composition.

[0018] In another aspect, presented herein are methods for treating cancer utilizing a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition comprising such a chimeric NDV. In a specific embodiment, a method for treating cancer comprises infecting a cancer cell in a subject with a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition thereof. In another embodiment, a method for treating cancer comprises administering to a subject in need thereof a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition thereof. In specific embodiments, an effective amount of a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition comprising an effective amount of a chimeric NDV described herein is administered to a subject to treat cancer. In specific embodiments, the chimeric NDV comprises a genome, the genome comprising an agonist of a co-stimulatory signal of an immune cell (*e.g.*, an agonist of a co-stimulatory receptor of an immune cell) and/or an antagonist of an inhibitory signal of an immune cell (*e.g.*, an antagonist of an inhibitory receptor of an immune cell). In certain embodiments, the genome of the NDV also comprises a mutated F protein. In certain embodiments, two or more chimeric NDVs are administered to a subject to treat cancer.

[0019] In another embodiment, a method for treating cancer comprises administering to a subject in need thereof cancer cells infected with a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or composition thereof. In specific embodiments, the cancer cells have been treated with gamma radiation prior to administration to the subject or incorporation into the composition. In another embodiment, a method for treating cancer comprises administering to a subject in need thereof a protein concentrate or plasma membrane fragments from cancer cells infected with a chimeric NDV (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition thereof. In specific embodiments, the chimeric NDV comprises a genome, the genome comprising an agonist of a co-stimulatory signal of an immune

cell (*e.g.*, an agonist of a co-stimulatory receptor of an immune cell) and/or an antagonist of an inhibitory signal of an immune cell (*e.g.*, an antagonist of an inhibitory receptor of an immune cell). In certain embodiments, the genome of the NDV also comprises a mutated F protein.

[0020] In another aspect, presented herein are methods for treating cancer utilizing an NDV described herein (*e.g.*, a chimeric NDV such as described in Section 5.2, *infra*) or a composition comprising such the NDV in combination with one or more other therapies. In one embodiment, presented herein are methods for treating cancer comprising administering to a subject an NDV described herein (*e.g.*, a chimeric NDV, such as described in Section 5.2.1, *infra*) and one or more other therapies. In another embodiment, presented herein are methods for treating cancer comprising administering to a subject an effective amount of an NDV described herein or a composition comprising an effective amount of an NDV described herein, and one or more other therapies. The NDV and one or more other therapies can be administered concurrently or sequentially to the subject. In certain embodiments, the NDV and one or more other therapies are administered in the same composition. In other embodiments, the NDV and one or more other therapies are administered in different compositions. The NDV and one or more other therapies can be administered by the same or different routes of administration to the subject.

[0021] Any NDV type or strain may be used in a combination therapy disclosed herein, including, but not limited to, naturally-occurring strains, variants or mutants, mutagenized viruses, reassortants and/or genetically engineered viruses. In a specific embodiment, the NDV used in a combination with one or more other therapies is a naturally-occurring strain. In another embodiment, the NDV used in combination with one or more other therapies is a chimeric NDV. In a specific embodiment, the chimeric NDV comprises a packaged genome, the genome comprising a cytokine (*e.g.*, IL-2, IL-7, IL-15, IL-17, or IL-21). In specific embodiments, the cytokine is expressed by cells infected with the chimeric NDV. In a specific embodiment, the chimeric NDV comprises a packaged genome, the genome comprising a tumor antigen. In specific embodiments, the tumor antigen is expressed by cells infected with the chimeric NDV. In a specific embodiment, the chimeric NDV comprises a packaged genome, the genome comprising a pro-apoptotic molecule or an anti-apoptotic molecule. In specific embodiments, the pro-apoptotic molecule or anti-apoptotic molecule is expressed by cells infected with the chimeric NDV.

[0022] In another specific embodiment, the chimeric NDV comprises a packaged genome, the genome comprising an agonist of a co-stimulatory signal of an immune cell (*e.g.*, an agonist of a co-stimulatory receptor of an immune cell) and/or an antagonist of an inhibitory signal of an immune cell (*e.g.*, an antagonist of an inhibitory receptor of an immune cell). In specific embodiments, the agonist and/or antagonist are expressed by cells infected with the chimeric NDV. In certain embodiments, the genome of the NDV also comprises a mutated F protein. In certain embodiments, the one or more therapies used in combination with an NDV described herein is one or more other therapies described in Section 5.6.4, *infra*. In particular embodiments, the one or more therapies used in combination with an NDV described herein is an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell (*see, e.g.*, Section 5.6.4.1, *infra*). *See, e.g.*, Section 5.2.1, *infra*, for examples of agonists of a co-stimulatory signal of an immune cell and antagonists of an inhibitory signal of an immune cell. In a specific embodiment, the antagonist of an inhibitory signal of an immune cell is the anti-CTLA-4 antibody described in Sections 6 and 7, *infra*. In another specific embodiment, the antagonist of an inhibitory signal of an immune cell is anti-PD-1 antibody or an anti-PD-L1 antibody described in Section 7, *infra*. In another specific embodiment, the agonist of a co-stimulatory signal of an immune cell is the ICOS ligand described in Sections 6 and 7, *infra*.

3.1 **TERMINOLOGY**

[0023] As used herein, the term “about” or “approximately” when used in conjunction with a number refers to any number within 1, 5 or 10% of the referenced number.

[0024] As used herein, the term “agonist(s)” refers to a molecule(s) that binds to another molecule and induces a biological reaction. In a specific embodiment, an agonist is a molecule that binds to a receptor on a cell and triggers one or more signal transduction pathways. For example, an agonist includes an antibody or ligand that binds to a receptor on a cell and induces one or more signal transduction pathways. In certain embodiments, the antibody or ligand binds to a receptor on a cell and induces one or more signal transduction pathways. In other embodiments, the agonist facilitates the interaction of the native ligand with the native receptor.

[0025] As used herein, the term “antagonist(s)” refers to a molecule(s) that inhibits the action of another molecule without provoking a biological response itself. In a specific embodiment, an antagonist is a molecule that binds to a receptor on a cell and blocks or dampens the biological

activity of an agonist. For example, an antagonist includes an antibody or ligand that binds to a receptor on a cell and blocks or dampens binding of the native ligand to the cell without inducing one or more signal transduction pathways. Another example of an antagonist includes an antibody or soluble receptor that competes with the native receptor on cells for binding to the native ligand, and thus, blocks or dampens one or more signal transduction pathways induced when the native receptor binds to the native ligand.

[0026] As used herein, the terms “antibody” and “antibodies” refer to molecules that contain an antigen binding site, *e.g.*, immunoglobulins. Antibodies include, but are not limited to, monoclonal antibodies, bispecific antibodies, multispecific antibodies, human antibodies, humanized antibodies, synthetic antibodies, chimeric antibodies, polyclonal antibodies, single domain antibodies, camelized antibodies, single-chain Fvs (scFv), single chain antibodies, Fab fragments, F(ab') fragments, disulfide-linked bispecific Fvs (sdFv), intrabodies, and anti-idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id and anti-anti-Id antibodies to antibodies), and epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules. Immunoglobulin molecules can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass. In a specific embodiment, an antibody is a human or humanized antibody. In another specific embodiment, an antibody is a monoclonal antibody or scFv. In certain embodiments, an antibody is a human or humanized monoclonal antibody or scFv. In other specific embodiments, the antibody is a bispecific antibody. In certain embodiments, the bispecific antibody specifically binds to a co-stimulatory receptor of an immune cell or an inhibitory receptor of an immune, and a receptor on a cancer cell. In some embodiments, the bispecific antibody specifically binds to two receptors immune cells, *e.g.*, two co-stimulatory receptors on immune cells, two inhibitory receptors on immune cells, or one co-stimulatory receptor on immune cells and one inhibitory receptor on immune cells.

[0027] As used herein, the term “derivative” in the context of proteins or polypeptides refers to: (a) a polypeptide that is at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% or is 40% to 65%, 50% to 90%, 65% to 90%, 70% to 90%, 75% to 95%, 80% to 95%, or 85% to 99% identical to a native polypeptide; (b) a polypeptide encoded by a nucleic acid sequence that is at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%,

85%, 90%, 95%, 98%, or 99% or is 40% to 65%, 50% to 90%, 65% to 90%, 70% to 90%, 75% to 95%, 80% to 95%, or 85% to 99% identical a nucleic acid sequence encoding a native polypeptide; (c) a polypeptide that contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more, or 2 to 5, 2 to 10, 5 to 10, 5 to 15, 5 to 20, 10 to 15, or 15 to 20 amino acid mutations (*i.e.*, additions, deletions and/or substitutions) relative to a native polypeptide; (d) a polypeptide encoded by nucleic acid sequence that can hybridize under high, moderate or typical stringency hybridization conditions to a nucleic acid sequence encoding a native polypeptide; (e) a polypeptide encoded by a nucleic acid sequence that can hybridize under high, moderate or typical stringency hybridization conditions to a nucleic acid sequence encoding a fragment of a native polypeptide of at least 10 contiguous amino acids, at least 12 contiguous amino acids, at least 15 contiguous amino acids, at least 20 contiguous amino acids, at least 30 contiguous amino acids, at least 40 contiguous amino acids, at least 50 contiguous amino acids, at least 75 contiguous amino acids, at least 100 contiguous amino acids, at least 125 contiguous amino acids, at least 150 contiguous amino acids, or 10 to 20, 20 to 50, 25 to 75, 25 to 100, 25 to 150, 50 to 75, 50 to 100, 75 to 100, 50 to 150, 75 to 150, 100 to 150, or 100 to 200 contiguous amino acids; or (f) a fragment of a native polypeptide. Derivatives also include a polypeptide that comprises the amino acid sequence of a naturally occurring mature form of a mammalian polypeptide and a heterologous signal peptide amino acid sequence. In addition, derivatives include polypeptides that have been chemically modified by, *e.g.*, glycosylation, acetylation, pegylation, phosphorylation, amidation, derivitization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein moiety, etc. Further, derivatives include polypeptides comprising one or more non-classical amino acids. In one embodiment, a derivative is isolated. In specific embodiments, a derivative retains one or more functions of the native polypeptide from which it was derived.

[0028] Percent identity can be determined using any method known to one of skill in the art. In a specific embodiment, the percent identity is determined using the “Best Fit” or “Gap” program of the Sequence Analysis Software Package (Version 10; Genetics Computer Group, Inc., University of Wisconsin Biotechnology Center, Madison, Wisconsin). Information regarding hybridization conditions (*e.g.*, high, moderate, and typical stringency conditions) have been described, see, *e.g.*, U.S. Patent Application Publication No. US 2005/0048549 (*e.g.*, paragraphs 72-73).

[0029] As used herein, the term “fragment” is the context of a fragment of a proteinaceous agent (*e.g.*, a protein) refers to a fragment that is 8 or more contiguous amino acids, 10 or more contiguous amino acids, 15 or more contiguous amino acids, 20 or more contiguous amino acids, 25 or more contiguous amino acids, 50 or more contiguous amino acids, 75 or more contiguous amino acids, 100 or more contiguous amino acids, 150 or more contiguous amino acids, 200 or more contiguous amino acids, or in the range of between 10 to 300 contiguous amino acids, 10 to 200 contiguous amino acids, 10 to 250 contiguous amino acids, 10 to 150 contiguous amino acids, 10 to 100 contiguous amino acids, 10 to 50 contiguous amino acids, 50 to 100 contiguous amino acids, 50 to 150 contiguous amino acids, 50 to 200 contiguous amino acids, 50 to 250 contiguous amino acids, 50 to 300 contiguous amino acids, 25 to 50 contiguous amino acids, 25 to 75 contiguous amino acids, 25 to 100 contiguous amino acids, or 75 to 100 contiguous amino acids of a proteinaceous agent. In a specific embodiment, a fragment of a proteinaceous agent retains one or more functions of the proteinaceous agent – in other words, it is a functional fragment. For example, a fragment of a proteinaceous agent retains the ability to interact with another protein and/or to induce, enhance or activate one or more signal transduction pathways.

[0030] As used herein, the term “functional fragment,” in the context of a proteinaceous agent, refers to a portion of a proteinaceous agent that retains one or more activities or functions of the proteinaceous agent. For example, a functional fragment of an inhibitory receptor may retain the ability to bind one or more of its ligands. A functional fragment of a ligand of a co-stimulatory receptor may retain the ability to bind to the receptor and/or induce, enhance or activate one or more signal transduction pathways mediated by the ligand binding to its co-stimulatory receptor.

[0031] As used herein, the term “heterologous” refers an entity not found in nature to be associated with (*e.g.*, encoded by and/or expressed by the genome of) a naturally occurring NDV.

[0032] As used herein, the term “elderly human” refers to a human 65 years or older.

[0033] As used herein, the term “human adult” refers to a human that is 18 years or older.

[0034] As used herein, the term “human child” refers to a human that is 1 year to 18 years old.

[0035] As used herein, the term “human toddler” refers to a human that is 1 year to 3 years old.

[0036] As used herein, the term “human infant” refers to a newborn to 1 year old year human.

[0037] In certain embodiments, the terms “highly fusogenic” and “increased fusogenic activity”, and the like, as used herein, refers to an increase in the ability of the NDV to form syncytia involving a large number of cells. In a specific embodiment, cells infected with an NDV described herein that is engineered to express a mutated F protein have an increased ability to form syncytia relative to cells infected with the parental virus from which the virus is derived, which parental virus has an unmutated F protein. In another specific embodiment, about 10% to about 25%, about 25% to about 50%, about 25% to about 75%, about 50% to about 75%, about 50% to about 95%, or about 75% to about 99% or about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% more cells infected with an NDV described herein that is engineered to express a mutated F protein form syncytia relative to the number of cells forming syncytia that are infected with the parental virus from the chimeric virus is derived which has an unmutated F protein. In certain embodiments, the syncytia are quantitated microscopically by counting the number of nuclei per syncytium after a certain period of time (*e.g.*, about 8 hours to about 12 hours, about 12 hours to about 24 hours, about 24 hours to about 36 hours, or about 36 hours to about 48 hours).

[0038] As used herein, the term “interferon antagonist” refers to an agent that reduces or inhibits the cellular interferon immune response. In one embodiment, an interferon antagonist is a proteinaceous agent that reduces or inhibits the cellular interferon immune response. In a specific embodiment, an interferon antagonist is a viral protein or polypeptide that reduces or inhibits the cellular interferon response.

[0039] In a specific embodiment, an interferon antagonist is an agent that reduces or inhibits interferon expression and/or activity. In one embodiment, the interferon antagonist reduces or inhibits the expression and/or activity of type I IFN. In another embodiment, the interferon antagonist reduces or inhibits the expression and/or activity of type II IFN. In another embodiment, the interferon antagonist reduces or inhibits the expression and/or activity of type III IFN. In a specific embodiment, the interferon antagonist reduces or inhibits the expression and/or activity of either IFN- α , IFN- β or both. In another specific embodiment, the interferon antagonist reduces or inhibits the expression and/or activity of IFN- γ . In another embodiment,

the interferon antagonist reduces or inhibits the expression and/or activity of one, two or all of IFN- α , IFN- β , and IFN- γ .

[0040] In certain embodiments, the expression and/or activity of IFN- α , IFN- β and/ or IFN- γ in an embryonated egg or cell is reduced approximately 1 to approximately 100 fold, approximately 5 to approximately 80 fold, approximately 20 to approximately 80 fold, approximately 1 to approximately 10 fold, approximately 1 to approximately 5 fold, approximately 40 to approximately 80 fold, or 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 fold by an interferon antagonist relative to the expression and/or activity of IFN- α , IFN- β , and/or IFN- γ in a control embryonated egg or a cell not expressing or not contacted with such an interferon antagonist as measured by the techniques described herein or known to one skilled in the art. In other embodiments, the expression and/or activity of IFN- α , IFN- β and/ or IFN- γ in an embryonated egg or cell is reduced by at least 20% to 25%, at least 25% to 30%, at least 30% to 35%, at least 35% to 40%, at least 40% to 45%, at least 45% to 50%, at least 50% to 55%, at least 55% to 60%, at least 60% to 65%, at least 65% to 70%, at least 70% to 75%, at least 75% to 80%, at least 80% to 85%, at least 85% to 90%, at least 90% to 95%, at least 95% to 99% or by 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% by an interferon antagonist relative to the expression and/or activity of IFN- α , IFN- β , and/or IFN- γ in a control embryonated egg or a cell not expressing or not contacted with such an interferon antagonist as measured by the techniques described herein or known to one skilled in the art.

[0041] As used herein, the phrases “IFN deficient systems” or “IFN-deficient substrates” refer to systems, *e.g.*, cells, cell lines and animals, such as mice, chickens, turkeys, rabbits, rats, horses etc., which do not produce one, two or more types of IFN, or do not produce any type of IFN, or produce low levels of one, two or more types of IFN, or produce low levels of any IFN (*i.e.*, a reduction in any IFN expression of 5-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90% or more when compared to IFN-competent systems under the same conditions), do not respond or respond less efficiently to one, two or more types of IFN, or do not respond to any type of IFN, have a delayed response to one, two or more types of IFN, and/or are deficient in the activity of antiviral genes induced by one, two or more types of IFN, or induced by any type of IFN.

[0042] As used herein, the terms “immunospecifically binds,” “immunospecifically recognizes,” “specifically binds,” and “specifically recognizes” are analogous terms in the context of antibodies and refer to molecules that specifically bind to an antigen (e.g., epitope or immune complex) as understood by one skilled in the art. A molecule that specifically binds to an antigen may bind to other peptides or polypeptides with lower affinity as determined by, e.g., immunoassays (e.g., ELISA), surface plasmon resonance (e.g., BIAcore®), a KinEx assay (using, e.g., a KinExA 3000 instrument (Sapidyne Instruments, Boise, ID)), or other assays known in the art. In a specific embodiment, molecules that specifically bind to an antigen bind to the antigen with a dissociation constant (*i.e.*, K_a) that is at least 2 logs, 2.5 logs, 3 logs, 3.5 logs, 4 logs or greater than the K_a when the molecules bind to another antigen. In another specific embodiment, molecules that specifically bind to an antigen do not cross react with other proteins.

[0043] As used herein, the term “monoclonal antibody” is a term of the art and generally refers to an antibody obtained from a population of homogenous or substantially homogeneous antibodies, and each monoclonal antibody will typically recognize a single epitope (*e.g.*, single conformation epitope) on the antigen.

[0044] As used herein, the phrase “multiplicity of infection” or “MOI” is the average number of virus per infected cell. The MOI is determined by dividing the number of virus added (ml added x Pfu) by the number of cells added (ml added x cells/ml).

[0045] As used herein, the term “native ligand” refers to any naturally occurring ligand that binds to a naturally occurring receptor. In a specific embodiment, the ligand is a mammalian ligand. In another specific embodiment, the ligand is a human ligand.

[0046] As used herein, the term “native polypeptide(s)” in the context of proteins or polypeptides refers to any naturally occurring amino acid sequence, including immature or precursor and mature forms of a protein. In a specific embodiment, the native polypeptide is a human protein or polypeptide.

[0047] As used herein, the term “native receptor” refers to any naturally occurring receptor that binds to a naturally occurring ligand. In a specific embodiment, the receptor is a mammalian receptor. In another specific embodiment, the receptor is a human receptor.

[0048] As used herein, the terms “subject” or “patient” are used interchangeably. As used herein, the terms “subject” and “subjects” refers to an animal. In some embodiments, the subject

is a mammal including a non-primate (*e.g.*, a camel, donkey, zebra, cow, horse, horse, cat, dog, rat, and mouse) and a primate (*e.g.*, a monkey, chimpanzee, and a human). In some embodiments, the subject is a non-human mammal. In certain embodiments, the subject is a pet (*e.g.*, dog or cat) or farm animal (*e.g.*, a horse, pig or cow). In other embodiments, the subject is a human. In certain embodiments, the mammal (*e.g.*, human) is 0 to 6 months old, 6 to 12 months old, 1 to 5 years old, 5 to 10 years old, 10 to 15 years old, 15 to 20 years old, 20 to 25 years old, 25 to 30 years old, 30 to 35 years old, 35 to 40 years old, 40 to 45 years old, 45 to 50 years old, 50 to 55 years old, 55 to 60 years old, 60 to 65 years old, 65 to 70 years old, 70 to 75 years old, 75 to 80 years old, 80 to 85 years old, 85 to 90 years old, 90 to 95 years old or 95 to 100 years old. In specific embodiments, the subject is an animal that is not avian.

[0049] As used herein, the terms “treat” and “treating” in the context of the administration of a therapy refers to a treatment/therapy from which a subject receives a beneficial effect, such as the reduction, decrease, attenuation, diminishment, stabilization, remission, suppression, inhibition or arrest of the development or progression of cancer, or a symptom thereof. In certain embodiments, the treatment/therapy that a subject receives results in at least one or more of the following effects: (i) the reduction or amelioration of the severity of cancer and/or a symptom associated therewith; (ii) the reduction in the duration of a symptom associated with cancer; (iii) the prevention in the recurrence of a symptom associated with cancer; (iv) the regression of cancer and/or a symptom associated therewith; (v) the reduction in hospitalization of a subject; (vi) the reduction in hospitalization length; (vii) the increase in the survival of a subject; (viii) the inhibition of the progression of cancer and/or a symptom associated therewith; (ix) the enhancement or improvement the therapeutic effect of another therapy; (x) a reduction or elimination in the cancer cell population; (xi) a reduction in the growth of a tumor or neoplasm; (xii) a decrease in tumor size; (xiii) a reduction in the formation of a tumor; (xiv) eradication, removal, or control of primary, regional and/or metastatic cancer; (xv) a decrease in the number or size of metastases; (xvi) a reduction in mortality; (xvii) an increase in cancer-free survival rate of patients; (xviii) an increase in relapse-free survival; (xix) an increase in the number of patients in remission; (xx) a decrease in hospitalization rate; (xxi) the size of the tumor is maintained and does not increase in size or increases the size of the tumor by less 5% or 10% after administration of a therapy as measured by conventional methods available to one of skill in the art, such as MRI, X-ray, and CAT Scan; (xxii) the prevention of the development or onset of

cancer and/or a symptom associated therewith; (xxiii) an increase in the length of remission in patients; (xxiv) the reduction in the number of symptoms associated with cancer; (xxv) an increase in symptom-free survival of cancer patients; and/or (xxvi) limitation of or reduction in metastasis. In some embodiments, the treatment/therapy that a subject receives does not cure cancer, but prevents the progression or worsening of the disease. In certain embodiments, the treatment/therapy that a subject receives does not prevent the onset/development of cancer, but may prevent the onset of cancer symptoms.

[0050] As used herein, the term “in combination” in the context of the administration of (a) therapy(ies) to a subject, refers to the use of more than one therapy. The use of the term “in combination” does not restrict the order in which therapies are administered to a subject. A first therapy can be administered prior to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapy to a subject.

[0051] As used herein, the terms “therapies” and “therapy” can refer to any protocol(s), method(s), and/or agent(s) that can be used in the treatment of cancer. In certain embodiments, the terms “therapies” and “therapy” refer to biological therapy, supportive therapy, hormonal therapy, chemotherapy, immunotherapy and/or other therapies useful in the treatment of cancer. In a specific embodiment, a therapy includes adjuvant therapy. For example, using a therapy in conjunction with a drug therapy, biological therapy, surgery, and/or supportive therapy. In certain embodiments, the term “therapy” refers to a chimeric NDV described herein. In other embodiments, the term “therapy” refers to an agent that is not a chimeric NDV.

4. BRIEF DESCRIPTION OF THE FIGURES

[0052] **Figure 1.** NDV infection upregulates the expression of MHC I, MHC II, and ICAM-1 on the surface of *in vitro* infected B16-F10 cells (24 hours post-infection).

[0053] **Figures 2A-2E.** Intratumoral NDV treatment leads to infiltration with macrophages, NK cells, CD8 and CD4 effector cells and decreases the frequency of Tregs. A) Overall study scheme. B) Total CD45⁺ infiltrates. C) Total immune cell infiltrates. D) Representative flow

cytometry dot plots of relative CD4 FoxP3⁺ and FoxP3⁻ subsets. E) Teff/Treg and CD8/Treg ratios.

[0054] Figures 3A-3C. Therapy with NDV exhibits favorable effects on tumor microenvironment of distant tumors. A) Representative flow cytometry dot plots of relative CD4 FoxP3⁺ and FoxP3⁻ subsets. B) Absolute numbers of CD4 effector, Treg, and CD8 cells per gram of tumor. C) Teff/Treg and CD8/Treg ratios.

[0055] Figures 4A-4C. Lymphocytes infiltrating distant tumors upregulate activation, lytic, and proliferation markers. Representative expression plots on CD4 effector cells (left) and the corresponding percentages in the CD4 effector, CD8, Tregs (right) are shown for A) CD44, B) Granzyme B, and C) Ki-67.

[0056] Figures 5A-5D. NDV Monotherapy delays the growth of distant tumors and provides some protection against tumor rechallenge. Bilateral flank tumors were established as described in Figure 2A and the animals were treated and followed for survival. A) Growth of right flank (treated) tumors. B) Growth of left flank (non-treated) tumors. C) Overall survival. Numbers in boxes indicate percent of animals free of tumors. D) Survival in animals cured of B16-F10 melanoma by NDV re-challenged on day 75 with B16-F10 melanoma cells. Representative results of two different experiments with 10 mice per group.

[0057] Figures 6A-6B. Tumor-infiltrating lymphocytes from both treated and non-treated tumors upregulate CTLA-4 in response to NDV therapy. A) Representative dot plots of CTLA-4 expression in CD8, CD4 effector, and Tregs in right (treated) tumors. B) Representative dot plots of CTLA-4 expression in CD8, CD4 effector, and Tregs in left (non-treated) tumors.

[0058] Figure 7A-7C. Combination therapy with NDV and CTLA-4 blockade enhances anti-tumor effect in the injected and distant tumors. Bilateral B16 flank tumors were established and the animals were treated as described in Figure 2A with or without anti-CTLA-4 antibody 9H10. A) Growth of treated tumors. B) Growth of distant tumors. Numbers in boxes represent percentage of mice free of tumors. C) Long-term survival. Representative results of 2 different experiments with 10 mice per group.

[0059] Figure 8. Combination therapy with NDV and anti-CTLA-4 is effective systemically against non-virus-permissive prostate TRAMP tumors. Right (day 12) and left (day 3) flank TRAMP tumors were established and the animals were treated with NDV as described in Figure

2A with or without systemic anti-CTLA-4 antibody. Growth of left flank (non-injected) tumors is shown. Numbers in boxes indicate percent of animals free of tumors.

[0060] Figure 9A-9C. NDV infection upregulates expression of PD-L1 in B16-F10 tumors. A) Surface PD-L1 expression on B16-F10 cells infected with NDV for 24 hours. B) Surface PD-L1 expression on B16-F10 cells treated with UV-inactivated supernatant from infected B16-F10 cells. C) Upregulation of PD-L1 on the surface of tumor cells isolated from injected and distant tumors from the animals treated as in Figure 2A (2 left panels-representative flow cytometry plots, right panel- calculated averages of 5 mice per group).

[0061] Figures 10A-10F. Combination therapy with NDV and anti-PD-1 is effective systemically against B16 melanoma and results in increased T cell infiltration with upregulation of activation markers. A) Overall survival. Animals were treated as described in Figure 2A with or without anti-PD-1 antibody. B) Absolute numbers of CD45, CD3, CD8, and CD4 effector cells in tumors. C) Relative percentage of regulatory T cells in tumor-infiltrating lymphocytes. D-E) Tumor-infiltrating lymphocytes from distant tumors were isolated and stained for expression of ICOS (D) and Granzyme B (E). F) Tumor infiltrating lymphocytes were re-stimulated with dendritic cells loaded with tumor lysates and assessed for expression of IFN gamma by intracellular cytokine staining.

[0062] Figure 11. Combination therapy with NDV and CTLA-4 induces upregulation of ICOS and CD4 effector cells in distant tumors and tumor-draining lymph nodes (TDLN).

[0063] Figures 12A-12D. Generation and in vitro evaluation of NDV-ICOSL virus. A) Viral genomic construct scheme. B) Expression of ICOSL on the surface of B16-F10 cells infected for 24 hours (representative histogram, left and average of 3 samples per group, right). C) Cytolytic activity of NDV in the infected B16-F10 cells determined by LDH assay. D) Replication of recombinant NDV in the B16-F10 cells.

[0064] Figures 13A-13C. Combination therapy with NDV-mICOSL and anti-CTLA-4 protects mice from contralateral tumor challenge and results in long-term animal survival. Animals were challenged with a larger tumor dose and treated with NDV as described in Figure 2A with or without systemic anti-CTLA-4 antibody. Growth of left flank (non-injected) tumors is shown. B) Long-term survival. Numbers in boxes indicate percent of animals protected from tumors. Pooled data of 3 different experiments of 5-10 mice per group. C) Mice treated with combination therapy develop vitiligo at the former tumor sites, but not systemically.

[0065] Figure 14A-14B. Combination therapy with NDV-mICOSL and anti-CTLA-4 protects mice from contralateral tumor challenge and results in long-term animal survival in the CT26 colon carcinoma model. Animals were challenged with a larger tumor dose and treated with NDV as described in Figure 2A with or without systemic anti-CTLA-4 antibody. Growth of left flank (non-injected) tumors is shown. Numbers in boxes indicate percent of animals protected from tumors. B) Long-term survival. Representative experiment with 5-10 mice per group (A) and pooled data of 2 different experiments of 5-10 mice per group (B).

[0066] Figures 15A-15C. NDV treatment leads to distant B16 tumor infiltration with macrophages, NK cells, CD8 and CD4 effector cells and decreases the frequency of Tregs. A) Total CD45+, CD3+, CD8+, CD4+ FoxP3- (Teff), and CD4+ FoxP3+ (Treg) infiltrates. B) Teff/Treg and CD8/Treg ratios. C) Total macrophage, NK, and NKT cell infiltrates.

[0067] Figure 16A-16B. Lymphocytes infiltrating distant B16 tumors upregulate activation, lytic, and proliferation markers. Representative Ki-67, Granzyme B (GrB) and ICOS expression plots (A) and the corresponding percentages in the CD4 effector and CD8 cells (B).

[0068] Figure 17. Tumor infiltrating lymphocytes from treated animals secrete IFN-gamma in response to stimulation with DC's loaded with B16-F10 lysates. Representative dot plots are shown.

[0069] Figures 18A-18B. Animals cured by combination therapy are protected from further tumor challenge. A) B16-F10 melanoma, day 120 re-challenge with 1×10^5 cells. B) CT26 colon carcinoma, day 90 re-challenge with 1×10^6 cells. Representative results of two different experiments with 10 mice per group.

[0070] Figure 19A-19B. Recombinant ICOSL-F chimeric protein is efficiently expressed on surface. A) Schematic diagram of the chimeric protein. B) Expression of the chimeric ICOSL-Ftm fusion protein on the surface of transfected cells.

[0071] Figure 20A-20D. NDV infection is restricted to the injected tumor. A) Recombinant NDV-Fluc was administered intratumorally (IT) or intravenously (IV) into Balb/C animals bearing CT26 tumors and images were acquired over the next 72 hours. B) NDV-Fluc was administered to C57BL/6 mice bearing bilateral B16-F10 melanoma tumors and animals were monitored for 120 hours. Representative luminescence images are shown. C) Quantification of luminescence from the tumor site normalized to background luminescence. D) Area under the

curve (AUC) calculated from the data in panel (C). Data show representative results from 1 of 3 independent experiments with 3-5 mice/group. *** $p < 0.001$.

[0072] Figure 21A-21F. NDV infection increases tumor leukocyte infiltration in the virus-injected tumors. Animals were treated according to the scheme described in Figure 22A. Tumors were excised on day 15, and TILs were labeled and analyzed by flow cytometry. A) Representative flow cytometry plots of percentages of tumor-infiltrating CD45⁺ and CD3⁺ cells. B) Absolute numbers of CD45⁺ cells/g tumor. C) Absolute numbers of innate immune cells/g tumor. D) Representative plots of percentages of CD4⁺FoxP3⁺ (Treg) and CD4⁺FoxP3⁻ (Tconv) cells. E) Absolute numbers of conventional and regulatory CD4⁺ cells and CD8⁺ cells/g tumor. F) Calculated Tconv/Treg and CD8⁺/Treg ratios from the tumors. Data represent cumulative results from 3 independent experiments with 3-5 mice/group. Mean $\pm$ SEM is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

[0073] Figure 22A-22M. NDV increases distant tumor lymphocyte infiltration and delays tumor growth. A) Treatment scheme. B) Representative flow cytometry plots of percentages of tumor-infiltrating CD45⁺ and CD3⁺ cells. C) Absolute numbers of CD45⁺ cells/g tumor. D) Absolute numbers of innate immune cells/g tumor. E) Tumor sections from distant tumors were stained with H&E (upper panels) or labeled for CD3 and FoxP3 (bottom panels) and analyzed by microscopy. Areas denoted by arrows indicate areas of necrosis and inflammatory infiltrates. Scale bars represent 200 μ m. F) Representative flow cytometry plots of percentages of CD4⁺FoxP3⁺ (Treg) and CD4⁺FoxP3⁻ (Tconv) cells. G) Absolute numbers of conventional and regulatory CD4⁺ cells and CD8⁺ cells/g tumor calculated from flow cytometry. H) Relative percentages of Tregs out of CD45⁺ cells. I) Calculated Tconv/Treg and CD8⁺/Treg ratios. (J, K) Upregulation of ICOS, Granzyme B, and Ki-67 on tumor-infiltrating Tconv (J) and CD8⁺ cells (K). L) Growth of NDV-injected and distant tumors. M) Overall animal survival. Data represent cumulative results from 3 (B-K) or 2 (L-M) independent experiments with $n=3-5$ per group. Mean $\pm$ SEM is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

[0074] Figure 23A-23E. NDV therapy increases distant tumor lymphocyte infiltration in bilateral footpad melanoma model. Animals bearing bilateral footpad melanoma tumors were treated according to the schedule described in Figure 22A. Distant tumors were excised on day 15 and TILs were labeled and analyzed by flow cytometry. A) Representative flow cytometry plots of percentages of tumor-infiltrating CD45⁺ and CD3⁺ cells. B) Representative flow

cytometry plots of percentages of CD4⁺FoxP3⁺ and CD4⁺FoxP3⁻ cells. C) Absolute numbers of conventional and regulatory CD4⁺ cells and CD8⁺ cells/g tumor. D, E) Upregulation of ICOS, Granzyme B, and Ki-67 on tumor-infiltrating CD8⁺ (D) and Tconv (E) lymphocytes. Data show representative results from 1 of 2 independent experiments with 5 mice/group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

[0075] Figure 24A-24I. NDV induces infiltration of adoptively-transferred tumor-specific lymphocytes and facilitates tumor inflammation. A) Treatment scheme. B) Representative luminescence images from animals treated with NDV and adoptively-transferred Trp1-Fluc lymphocytes. C) Quantification of average luminescence from the tumor sites. D) The area under the curve (AUC) calculated from the data in panel (C). E) Absolute number of Pmel lymphocytes from distant tumors calculated from flow cytometry. F) Representative flow cytometry plots of percentages of CD45⁺ and CD3⁺ cells infiltrating distant tumors of animals treated per treatment scheme in panel (A). G) Experimental scheme for serum transfer from animals treated intratumorally with single injection of NDV or PBS. H) Representative flow cytometry plots of percentages of CD45⁺ and CD3⁺ cells infiltrating serum-injected tumors. I) Absolute numbers of the indicated cell subsets in serum-injected tumors calculated from flow cytometry. Data for panels B-E represent 1 of 3 experiments with n=4-5 per group. Data for panels G-I represent pooled data from 2 independent experiments with n=5 per group. Mean +/- SEM is shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

[0076] Figure 25. Intratumoral NDV provides protection from tumor rechallenge. Animals cured of B16-F10 melanoma by NDV were injected on day 75 with 1x10⁵ B16-F10 melanoma cells, monitored for tumor growth, and euthanized when the tumors reached 1000mm³. Overall animal survival is shown. Data show cumulative results from 1 of 2 independent experiments with 10 mice/group. ****p<0.0001.

[0077] Figure 26A-26B. Tumor-infiltrating CD8⁺ lymphocytes upregulate CTLA-4 in response to NDV therapy. Representative dot plots (left) and cumulative results (right) of CTLA-4 expression in CD8⁺ cells in NDV-treated (A), and distant (B) tumors. Representative results from 1 of 3 experiments with 3 mice per group. *p<0.05.

[0078] Figure 27A-27K. NDV and CTLA-4 blockade synergize to reject local and distant tumors. A) Treatment scheme. B) Growth of virus-treated (right flank) B16-F10 tumors. C) Growth of distant (left flank) B16-F10 tumors. D) Long-term survival in the B16-F10 model. E)

Surviving animals were injected with 1×10^5 B16-F10 cells in right flank on day 90 and followed for survival. Data represent cumulative results from 3 experiments with n=6-11 per group. F) Growth of virus-treated (right flank) and distant (left flank) TRAMP C2 tumors. G) Long-term survival in the TRAMP C2 model. H) *In vitro* sensitivity of B16-F10 and TRAMP C2 cells to NDV-mediated lysis at different multiplicities of infection (MOI's). I-K) Upregulation of MHC I, MHC II, CD80, and CD86 in B16-F10 and TRAMP C2 cells infected with NDV.

Representative flow cytometry plots from B16-F10 cells (I) and calculated average median fluorescent intensities (MFI) for B16-F10 (J) and TRAMP C2 (K) cells are shown. Mean $\pm$ SEM is shown. Data represent results from 1 of 3 (B-E), or 1 of 2 (F, G) independent experiments with n=5-10 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

[0079] Figure 28A-28E. Systemic anti-tumor effect is restricted to the injected tumor type.

A) Animals were injected i.d. in right flank with B16-F10 melanoma, MC38 colon carcinoma, or PBS, and in the left flank with B16-F10 cells and treated as outlined in the scheme. B, C) Growth of distant tumors (B) and overall survival (C) of animals that received right B16-F10 or no right flank tumors. Data show representative results from 1 out of 2 independent experiments with 5-10 mice/group. D, E) Growth of distant tumors (D) and overall survival (E) of animals that received right B16-F10 or MC38 tumors. Data represent results from 1 out of 2 independent experiments with n=10 per group. ** $p < 0.01$, **** $p < 0.0001$.

[0080] Figure 29A-29E. Combination therapy with NDV and anti-CTLA-4 enhances tumor infiltration with innate and adaptive immune cells. Animals were treated with combination therapy as described in Figure 27A. Tumors were harvested on day 15 and analyzed for infiltrating immune cells by flow cytometry. A) Absolute numbers of CD45+ cells/g tumor. B) Absolute numbers of CD11b+ and NK 1.1+ cells/g tumor. C) Absolute numbers of conventional and regulatory CD4+ cells and CD8+ cells/g tumor. D) Relative percentages of Tregs out of CD45+ cells. E) Calculated Tconv/Treg and CD8+/Treg ratios. Data represent cumulative results from 4 independent experiments with 3-5 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

[0081] Figure 30A-30J. Combination therapy with NDV and CTLA-4 blockade induces inflammatory changes in distant tumors. Animals were treated per schema in Figure 27A. Tumors were harvested on day 15 and analyzed for infiltrating immune cells. A) Tumor sections from distant tumors were stained with H&E (upper panels) or for CD3 and FoxP3 (lower panels)

and analyzed by light and fluorescence microscopy, respectively. Areas denoted by arrows indicate necrosis and inflammatory infiltrates. Scale bars represent 200 μ m. B) Absolute number of tumor-infiltrating CD45+ and CD3+ cells/g tumor calculated from flow cytometry. C) Representative flow cytometry plots of percent of tumor-infiltrating CD4+ and CD8+ cells gated on CD45+ population. D) Absolute numbers of Tconv, Treg, and CD8+ cells per gram of tumor. E) Relative percentages of tumor-infiltrating Tregs out of CD45+ cells. F) Calculated Tconv/Treg and CD8+/Treg ratios. G-I) Upregulation of ICOS, Granzyme B, and Ki-67 on tumor-infiltrating CD8+ and Tconv lymphocytes. Representative flow cytometry plots (upper panels) and cumulative results (bottom panels) are shown. J) TILs were restimulated with DC's pulsed with B16-F10 tumor lysates, and IFN γ production was determined by intracellular cytokine staining. Representative flow cytometry plots (left panel) and cumulative results (right panel) are shown. Data represent cumulative results from 5 (A-I) or 2 (J) independent experiments with n=3-5 per group. Mean $\pm$ SEM is shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

[0082] Figure 31. Antibodies to CD8, CD4, and NK1.1 deplete the cells of interest *in vivo*. Depleting antibodies were injected as discussed in Materials and Methods in Section 7.1, *infra*. Blood samples were collected on day 5 and processed by flow cytometry for CD4+, CD8+, and NK cells with non-crossreactive antibodies. Positive staining is represented by the horizontal bars. Representative plots from 1 of 2 independent experiments with 5 mice per group are shown.

[0083] Figure 32A-32F. Anti-tumor activity of NDV combination therapy depends on CD8+ and NK cells and type I and type II interferons. A-C) Animals were treated as described in Fig. 27A with or without depleting antibodies for CD4+, CD8+, NK cells, or IFN γ . A) Growth of injected tumors. B) Growth of distant tumors. C) Long-term survival. D-F) IFNAR $^{-/-}$ or age-matched C57BL/6 mice (BL/6) were treated as described in Fig. 3A and monitored for tumor growth. D) Growth of injected tumors. E) Growth of distant tumors. F) Long-term survival. Data for all panels represent cumulative results from 2 independent experiments with n=3-10 per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

[0084] Figure 33A-33B. NDV therapy leads to upregulation of PD-L1 on tumors and tumor-infiltrating leukocytes. A). PD-L1 expression on B16-F10 cells infected *in vitro* (left panel), and *in vivo* in virus injected and distant tumors. Left, representative flow cytometry histograms,

right, average median fluorescence intensity (MFI) of PD-L1 expression on B16-F10 cells from tumors. B) PD-L1 expression on the surface of tumor-infiltrating leukocytes isolated from distant tumors. Left: representative flow cytometry histograms, right: calculated average MFI for each cell subset.

[0085] **Figure 34A-34D.** Combination therapy of NDV with antibodies blocking PD-1 leads to enhanced anti-tumor efficacy in bilateral flank B16 melanoma model. A) Treatment scheme. B) Right flank (NDV-injected) tumor growth. C) Left flank (distant) tumor growth. D) Overall survival.

[0086] **Figure 35A-35D.** Combination therapy of NDV with antibodies blocking PD-L1 leads to enhanced anti-tumor efficacy in bilateral flank B16 melanoma model. A) Treatment scheme. B) Right flank (NDV-injected) tumor growth. C) Left flank (distant) tumor growth. D) Overall survival.

[0087] **Figure 36A-36E.** Combination therapy with NDV and anti-PD-1 therapy results in increased distant tumor infiltration with effector but not regulatory T cells. A) Representative flow cytometry plots of percentages of CD4⁺ and CD8⁺ cells in tumors. B) Representative flow cytometry plots of percentages of Tconv (CD4⁺FoxP3⁻) and Treg (CD4⁺FoxP3⁺) cells. C) Absolute numbers of T cell subsets per gram of tumor, calculated from flow cytometry. D) Relative percentages of Tregs from CD4⁺ T cells. E) Calculated Tconv/Treg and CD8/Treg ratios.

[0088] **Figure 37A-37B.** TILs from distant tumors in animals treated with combination NDV and anti-PD-1 therapy upregulate lytic and proliferation markers. A) Representative flow cytometry plots of percentages of Tconv and CD8 lymphocytes positive for Granzyme B and Ki67. B) Percentages of Tconv and CD8⁺ T cells positive for Granzyme B and Ki67.

[0089] **Figure 38A-38C.** NDV induces tumor immune infiltration and upregulation of ICOS on CD4 and CD8 cells in the virus-injected and distant tumors. A) Treatment scheme. B) Expression of ICOS on tumor-infiltrating CD4⁺FoxP3⁻ and CD8⁺ cells isolated from NDV-injected (right flank) tumors. Representative flow cytometry plots (top) and median fluorescence intensities (MFI) (bottom) are shown. C) Expression of ICOS on tumor-infiltrating CD4⁺FoxP3⁻ and CD8⁺ cells isolated from distant (left flank) tumors. Representative flow cytometry plots (top) and median fluorescence intensities (MFI) (bottom) are shown.

[0090] Figure 39A-39D. Generation and in vitro evaluation of NDV-ICOSL virus. A) Viral genomic construct scheme. B) Expression of ICOSL on the surface of B16-F10 cells infected for 24 hours (representative histogram, left and average of 3 samples per group, right). C) Cytolytic activity of NDV in the infected B16-F10 cells determined by LDH assay. D) Replication of recombinant NDV in the B16-F10 cells.

[0091] Figure 40A-40F. NDV-ICOSL causes growth delay of distant tumors and induces enhanced tumor lymphocyte infiltration. Bilateral flank B16-F10 tumors were established as previously and the animals were treated with 4 intratumoral injections of the indicated virus to the right tumor. A) Growth of virus-injected tumors. B) Growth of distant tumors. C) Overall survival. D) Absolute numbers of tumor-infiltrating leukocytes in the right (virus-injected tumors). E) Absolute numbers of tumor-infiltrating leukocytes in the left (distant tumors). F) Relative percentage of Tregs in the distant tumors.

[0092] Figure 41A-41E. Combination therapy of NDV-ICOSL and CTLA-4 blockade results in rejection of the injected and distant tumors in the B16-F10 model and protects against tumor rechallenge. A) Treatment schema. B) Growth of virus-injected (right) tumors. C) Growth of distant (left) tumors. D) Overall survival. E) Surviving animals on day 90 were re-challenged in right flank with 2×10^5 B16-F10 cells and followed for survival.

[0093] Figure 42A-42E. Combination therapy of NDV-ICOSL and CTLA-4 blockade results in rejection of the injected and distant tumors in the CT26 model. A) Treatment schema. B) Growth of virus-injected (right) tumors. C) Growth of distant (left) tumors. D) Overall survival. E) Surviving animals on day 90 were re-challenged in right flank with 1×10^6 CT26 cells and followed for survival.

[0094] Figure 43A-43J. Combination therapy of NDV-ICOSL and anti-CTLA-4 leads to enhanced tumor infiltration with innate and adaptive immune cells. Animals bearing bilateral flank B16-F10 tumors were treated according to the schedule described in figure 41A. On day 15 the animals were sacrificed and distant tumors were processed for analysis of TIL's. A) Representative flow cytometry plots of CD45+ and CD3+ cells gating on the entire tumor cell population. Absolute number of tumor-infiltrating CD45+ (B), CD11b+ (C), and NK1.1+ cells (D) per gram of tumor was calculated from flow cytometry. E) Absolute numbers of tumor-infiltrating, CD3+, CD8+, CD4+FoxP3- (CD4eff), and CD4+FoxP3+ (Treg) per gram of tumor. F) Relative percentage of Tregs of all CD45+ cells. G) Calculated effector/Treg ratios. H, I, J)

relative percentages of tumor-infiltrating CD8⁺ and CD4⁺ effector cells positive for ICOS, granzyme B, and Ki67, respectively.

[0095] Figure 44A-44C. Schematic diagram for additional generated recombinant NDV viruses expressing chimeric and native immunostimulatory proteins. A) Diagram of chimeric proteins of TNF receptor superfamily (GITRL, OX40L, 4-1BBL, CD40L) fused to the NDV HN intracellular and transmembrane region of HN at the N terminus (upper panel). Lower panel demonstrates the diagram of chimeric proteins of immunoglobulin receptor superfamily, with anti-CD28scfv and ICOSL extracellular domains fused to the intracellular and transmembrane region of F at the C terminus. B) Length of intracellular-transmembrane (HN and F) and extracellular domains of each of the described chimeric proteins. C) Schematic diagram of the site of insertion of transgene and list of all recombinant NDVs expressing immunostimulatory ligands generated by this strategy.

[0096] Figure 45A-45C. Confirmation of rescue of recombinant NDV's. A) Hemagglutination assay demonstrating positive hemagglutinating activity in the wells for NDV-HN-GITRL, NDV-aCD28scfv-F, NDV-HN-OX40L, NDV-HN-CD40L, NDV-IL15, and NDV-IL21. B) Primer locations for confirmation of gene insert in the rescued viruses by RT-PCR. C) RT-PCR on RNA isolated from rescued viruses.

[0097] Figure 46. B16-F10 cells infected with recombinant NDVs express the ligands on the surface. B16-F10 cells were infected with the indicated recombinant NDV's at MOI of 2 and were analyzed for surface ligand expression by flow cytometry 18 hours later. Representative flow cytometry plots are shown.

[0098] Figure 47. NDV-HN-4-1BBL induces increased distant tumor immune infiltration. Animals bearing bilateral flank B16 melanoma tumors were treated intratumorally into single flank with the indicated virus as previously. After 3 treatments, animals were euthanized and tumor-infiltrating lymphocytes from distant tumors were analyzed by flow cytometry. Total number of tumor-infiltrating CD3, CD4⁺FoxP3⁺ (Treg), CD4⁺FoxP3⁻(Tconv), CD8, NK, and CD11b⁺ cells per gram of tumor is shown.

5. DETAILED DESCRIPTION

[0099] In one aspect, presented herein are chimeric Newcastle disease viruses (NDVs) engineered to express an agonist of a co-stimulatory signal of an immune cell and/or an

antagonist of an inhibitory signal of an immune cell. In a specific embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an agonist of a co-stimulatory signal of an immune cell, wherein the agonist is expressed. In a specific embodiment, presented herein are chimeric NDVs, comprising a packaged genome which encodes an antagonist of an inhibitory signal of an immune cell, wherein the antagonist is expressed.

[00100] In another aspect, presented herein are methods for propagating the NDVs described herein (*e.g.*, chimeric NDVs described herein). The NDVs described herein (*e.g.*, chimeric NDVs described herein) can be propagated in any cell, subject, tissue, organ or animal susceptible to a NDV infection.

[00101] In another aspect, presented herein are compositions comprising an NDV described herein (*e.g.*, a chimeric NDV described herein). In a specific embodiment, presented herein are pharmaceutical compositions comprising an NDV described herein (*e.g.*, a chimeric NDV described herein) and a pharmaceutically acceptable carrier. In another embodiment, presented herein are pharmaceutical compositions comprising cancer cells infected with an NDV described herein (*e.g.*, a chimeric NDV described herein), and a pharmaceutically acceptable carrier. In another embodiment, presented herein are pharmaceutical compositions comprising protein concentrate from lysed NDV-infected cancer cells (*e.g.*, chimeric-NDV infected cancer cells), and a pharmaceutically acceptable carrier.

[00102] In another aspect, presented herein are methods for producing pharmaceutical compositions comprising an NDV described herein (*e.g.*, a chimeric NDV described herein). In one embodiment, a method for producing a pharmaceutical composition comprises: (a) propagating an NDV described herein (*e.g.*, a chimeric NDV described herein) in a cell line that is susceptible to an NDV infection; and (b) collecting the progeny virus, wherein the virus is grown to sufficient quantities and under sufficient conditions that the virus is free from contamination, such that the progeny virus is suitable for formulation into a pharmaceutical composition. In another embodiment, a method for producing a pharmaceutical composition comprises: (a) propagating an NDV described herein (*e.g.*, a chimeric NDV described herein) in an embryonated egg; and (b) collecting the progeny virus, wherein the virus is grown to sufficient quantities and under sufficient conditions that the virus is free from contamination, such that the progeny virus is suitable for formulation into a pharmaceutical composition.

[00103] In another aspect, presented herein are methods for treating cancer utilizing a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition comprising such a chimeric NDV. In a specific embodiment, a method for treating cancer comprises infecting a cancer cell in a subject with a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition thereof. In another embodiment, a method for treating cancer comprises administering to a subject in need thereof a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition thereof. In specific embodiments, an effective amount of a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition comprising an effective amount of a chimeric NDV described herein is administered to a subject to treat cancer. In specific embodiments, the chimeric NDV comprises a packaged genome, the genome comprising an agonist of a co-stimulatory signal of an immune cell (*e.g.*, an agonist of a co-stimulatory receptor of an immune cell) and/or an antagonist of an inhibitory signal of an immune cell (*e.g.*, an antagonist of an inhibitory receptor of an immune cell), wherein the agonist and/or antagonist are expressed by the NDV. In certain embodiments, the genome of the NDV also comprises a mutated F protein. In certain embodiments, two or more chimeric NDVs are administered to a subject to treat cancer.

[00104] In another embodiment, a method for treating cancer comprises administering to a subject in need thereof cancer cells infected with a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or composition thereof. In specific embodiments, the cancer cells have been treated with gamma radiation prior to administration to the subject or incorporation into the composition. In another embodiment, a method for treating cancer comprises administering to a subject in need thereof a protein concentrate or plasma membrane fragments from cancer cells infected with a chimeric NDV (*e.g.*, a chimeric NDV described in Section 5.2, *infra*) or a composition thereof. In specific embodiments, the chimeric NDV comprises a packaged genome, the genome comprising an agonist of a co-stimulatory signal of an immune cell (*e.g.*, an agonist of a co-stimulatory receptor of an immune cell) and/or an antagonist of an inhibitory signal of an immune cell (*e.g.*, an antagonist of an inhibitory receptor of an immune cell), wherein the agonist and/or antagonist are expressed by the NDV. In certain embodiments, the genome of the NDV also comprises a mutated F protein, which is expressed by the NDV.

[00105] In another aspect, presented herein are methods for treating cancer utilizing an NDV described herein (*e.g.*, a chimeric NDV such as described in Section 5.2, *infra*) or a composition comprising such the NDV in combination with one or more other therapies. In one embodiment, presented herein are methods for treating cancer comprising administering to a subject an NDV described herein (*e.g.*, a chimeric NDV, such as described in Section 5.2, *infra*) and one or more other therapies. In another embodiment, presented herein are methods for treating cancer comprising administering to a subject an effective amount of an NDV described herein or a composition comprising an effective amount of an NDV described herein, and one or more other therapies. The NDV and one or more other therapies can be administered concurrently or sequentially to the subject. In certain embodiments, the NDV and one or more other therapies are administered in the same composition. In other embodiments, the NDV and one or more other therapies are administered in different compositions. The NDV and one or more other therapies can be administered by the same or different routes of administration to the subject.

[00106] Any NDV type or strain may be used in a combination therapy disclosed herein, including, but not limited to, naturally-occurring strains, variants or mutants, mutagenized viruses, reassortants and/or genetically engineered viruses. In a specific embodiment, the NDV used in a combination with one or more other therapies is a naturally-occurring strain. In another embodiment, the NDV used in combination with one or more other therapies is a chimeric NDV. In a specific embodiment, the chimeric NDV comprises a packaged genome, the genome comprising a cytokine (*e.g.*, IL-2, IL-7, IL-15, IL-17 or IL-21). In specific embodiments, the chimeric NDV comprises a packaged genome, the genome comprising a tumor antigen. In specific embodiments, the tumor antigen is expressed by cells infected with the chimeric NDV. In another specific embodiment, the chimeric NDV comprises a packaged genome, the genome comprising a pro-apoptotic molecule (*e.g.*, Bax, Bak, Bad, BID, Bcl-xS, Bim, Noxa, Puma, AIF, FasL, and TRAIL) or an anti-apoptotic molecule (*e.g.*, Bcl-2, Bcl-xL, Mcl-1, and XIAP). In specific embodiments, the pro-apoptotic molecule or anti-apoptotic molecule is expressed by cells infected with the chimeric NDV. In another specific embodiment, the chimeric NDV comprises a packaged genome, the genome comprising an agonist of a co-stimulatory signal of an immune cell (*e.g.*, an agonist of a co-stimulatory receptor of an immune cell) and/or an antagonist of an inhibitory signal of an immune cell (*e.g.*, an antagonist of an inhibitory receptor of an immune cell). In specific embodiments, the agonist and/or antagonist are expressed by

cells infected with the chimeric NDV. In certain embodiments, the genome of the NDV also comprises a mutated F protein, a tumor antigen, a heterologous interferon antagonist, a pro-apoptotic molecule and/or an anti-apoptotic molecule. In certain embodiments, the one or more therapies used in combination with an NDV described herein is one or more other therapies described in Section 5.6.4, *infra*. In particular embodiments, the one or more therapies used in combination with an NDV described herein are an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell. *See, e.g.*, Section 5.2.1, *infra*, for examples of agonists of a co-stimulatory signal of an immune cell and antagonists of an inhibitory signal of an immune cell. In a specific embodiment, the antagonist of an inhibitory signal of an immune cell is the anti-CTLA-4 antibody described in Section 6, *infra*. In another specific embodiment, the agonist of a co-stimulatory signal of an immune cell is the ICOS ligand described in Section 6, *infra*.

5.1 NEWCASTLE DISEASE VIRUS

[00107] Any NDV type or strain may be used in a combination therapy disclosed herein, including, but not limited to, naturally-occurring strains, variants or mutants, mutagenized viruses, reassortants and/or genetically engineered viruses. In a specific embodiment, the NDV used in a combination therapy disclosed herein is a naturally-occurring strain. In certain embodiments, the NDV is a lytic strain. In other embodiments, the NDV used in a combination therapy disclosed herein is a non-lytic strain. In certain embodiments, the NDV used in a combination therapy disclosed herein is lentogenic strain. In some embodiments, the NDV is a mesogenic strain. In other embodiments, the NDV used in a combination therapy disclosed herein is a velogenic strain. Specific examples of NDV strains include, but are not limited to, the 73-T strain, NDV HUI strain, Ulster strain, MTH-68 strain, Italien strain, Hickman strain, PV701 strain, Hitchner B1 strain (*see, e.g.*, Genbank No. AF309418 or NC_002617), La Sota strain (*see, e.g.*, Genbank No. AY845400), YG97 strain, MET95 strain, Roakin strain, and F48E9 strain. In a specific embodiment, the NDV used in a combination therapy disclosed herein that is the Hitchner B1 strain. In another specific embodiment, the NDV used in a combination therapy disclosed herein is a B1 strain as identified by Genbank No. AF309418 or NC_002617. In another specific embodiment, the NDV used in a combination therapy disclosed herein is the NDV identified by ATCC No. VR2239. In another specific embodiment, the NDV used in a combination therapy disclosed herein is the La Sota strain.

[00108] In specific embodiments, the NDV used in a combination therapy disclosed herein is not pathogenic birds as assessed by a technique known to one of skill. In certain specific embodiments, the NDV used in a combination therapy is not pathogenic as assessed by intracranial injection of 1-day-old chicks with the virus, and disease development and death as scored for 8 days. In some embodiments, the NDV used in a combination therapy disclosed herein has an intracranial pathogenicity index of less than 0.7, less than 0.6, less than 0.5, less than 0.4, less than 0.3, less than 0.2 or less than 0.1. In certain embodiments, the NDV used in a combination therapy disclosed herein has an intracranial pathogenicity index of zero.

[00109] In certain embodiments, the NDV used in a combination therapy disclosed herein is a mesogenic strain that has been genetically engineered so as not be a considered pathogenic in birds as assessed by techniques known to one skilled in the art. In certain embodiments, the NDV used in a combination therapy disclosed herein is a velogenic strain that has been genetically engineered so as not be a considered pathogenic in birds as assessed by techniques known to one skilled in the art.

[00110] In certain embodiments, the NDV used in a combination therapy disclosed herein expresses a mutated F protein. In a specific embodiment, the NDV used in a combination therapy expresses a mutated F protein is highly fusogenic and able to form syncytia. In another specific embodiment, the mutated F protein is incorporated into the virion.

[00111] In one embodiment, a genome of a NDV used in a combination therapy disclosed herein is engineered to express a mutated F protein with a mutated cleavage site. In a specific embodiment, the NDV used in a combination therapy disclosed herein is engineered to express a mutated F protein in which the cleavage site of the F protein is mutated to produce a polybasic amino acid sequence, which allows the protein to be cleaved by intracellular proteases, which makes the virus more effective in entering cells and forming syncytia. In another specific embodiment, the NDV used in a combination therapy disclosed herein is engineered to express a mutated F protein in which the cleavage site of the F protein is replaced with one containing one or two extra arginine residues, allowing the mutant cleavage site to be activated by ubiquitously expressed proteases of the furin family. Specific examples of NDVs that express such a mutated F protein include, but are not limited to, rNDV/F2aa and rNDV/F3aa. For a description of mutations introduced into a NDV F protein to produce a mutated F protein with a mutated cleavage site, *see, e.g., Park et al. (2006) Engineered viral vaccine constructs with dual*

specificity: avian influenza and Newcastle disease. PNAS USA 103: 8203-2808, which is incorporated herein by reference in its entirety. In some embodiments, the NDV used in a combination therapy disclosed herein is engineered to express a mutated F protein with the amino acid mutation L289A. In specific embodiments the L289A mutated F protein possesses one, two or three arginine residues in the cleavage site. In certain embodiments, the mutated F protein is from a different type or strain of NDV than the backbone NDV. In some embodiments, the mutated F protein is in addition to the backbone NDV F protein. In specific embodiments, the mutated F protein replaces the backbone NDV F protein.

[00112] In certain embodiments, the NDV used in a combination therapy disclosed herein is attenuated such that the NDV remains, at least partially, infectious and can replicate *in vivo*, but only generate low titers resulting in subclinical levels of infection that are non-pathogenic (see, *e.g.*, Khattar et al., 2009, J. Virol. 83:7779-7782). In a specific embodiment, the NDV is attenuated by deletion of the V protein. Such attenuated NDVs may be especially suited for embodiments wherein the virus is administered to a subject in order to act as an immunogen, *e.g.*, a live vaccine. The viruses may be attenuated by any method known in the art.

[00113] In certain embodiments, the NDV used in a combination therapy disclosed herein does not comprise an NDV V protein encoding sequence. In other embodiments, the NDV used in a combination therapy disclosed herein expresses a mutated V protein. *See, e.g.*, Elankumaran et al., 2010, J. Virol. 84(8): 3835-3844, which is incorporated herein by reference, for examples of mutated V proteins. In certain embodiments, a mesogenic or velogenic NDV strain used in a combination therapy disclosed herein expresses a mutated V protein, such as disclosed by Elankumaran et al., 2010, J. Virol. 84(8): 3835-3844.

[00114] In certain embodiments, the NDV used in a combination therapy disclosed herein is an NDV disclosed in U.S. Patent No. 7,442,379, U.S. Patent No. 6,451,323, or U.S. Patent No. 6,146,642, which is incorporated herein by reference in its entirety. In specific embodiments, the NDV used in a combination therapy disclosed herein is genetically engineered to encode and express a heterologous peptide or protein. In certain embodiments, the NDV used in a combination therapy disclosed herein is a chimeric NDV known to one of skill in the art, or a chimeric NDV disclosed herein (*see, e.g.*, Section 5.2, *infra*). In some embodiments, the NDV used in a combination therapy disclosed herein is a chimeric NDV comprising a genome engineered to express a tumor antigen (*see below for examples of tumor antigens*). In certain

embodiments, the NDV used in a combination therapy disclosed herein is a chimeric NDV comprising a genome engineered to express a heterologous interferon antagonist (*see* below for examples of heterologous interferon antagonists). In some embodiments, the NDV used in a combination therapy disclosed herein is a chimeric NDV disclosed in U.S. patent application publication No. 2012/0058141, which is incorporated herein by reference in its entirety. In certain embodiments, the NDV used in a combination therapy disclosed herein is a chimeric NDV disclosed in U.S. patent application publication No. 2012/0122185, which is incorporated herein by reference in its entirety. In some embodiments, the NDV used in a combination therapy disclosed herein is a chimeric NDV comprising a genome engineered to express a cytokine, such as, *e.g.*, IL-2, IL-7, IL-9, IL-15, IL-17, IL-21, IL-22, IFN-gamma, GM-CSF, and TNF-alpha. In some embodiments, the NDV used in a combination therapy disclosed herein is a chimeric NDV comprising a genome engineered to express IL-2, IL-15, or IL-21. In a specific embodiment, the NDV used in a combination therapy disclosed herein is a chimeric NDV comprising a genome engineered to express a cytokine as described in Section 7, Example 2, *infra*.

5.2 CHIMERIC NEWCASTLE DISEASE VIRUS

[00115] In one aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or Natural Killer (NK) cell. In some embodiments, the agonist and/or antagonist is incorporated into the virion. In a specific embodiment, a genome of a NDV is engineered to express an agonist of a co-stimulatory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell. In another specific embodiment, a genome of a NDV is engineered to express an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell. In other words, the NDV serves as the “backbone” that is engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or Natural Killer (NK) cell. Specific examples of agonists of co-stimulatory signals as well as specific examples of antagonists of inhibitory signal are provided below.

[00116] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a mutated F protein. In

one embodiment, a genome of a NDV is engineered to express an agonist of a co-stimulatory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a mutated F protein. In another embodiment, a genome of a NDV is engineered to express an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a mutated F protein. In a specific embodiment, the mutated F protein is highly fusogenic and able to form syncytia. In another specific embodiment, the mutated F protein is incorporated into the virion. In certain embodiments, the genome of a NDV engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, comprises an NDV V protein encoding sequence.

[00117] In one embodiment, a genome of a NDV is engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a mutated F protein with a mutated cleavage site. In a specific embodiment, the NDV is engineered to express a mutated F protein in which the cleavage site of the F protein is mutated to produce a polybasic amino acid sequence, which allows the protein to be cleaved by intracellular proteases, which makes the virus more effective in entering cells and forming syncytia. In another specific embodiment, the NDV is engineered to express a mutated F protein in which the cleavage site of the F protein is replaced with one containing one or two extra arginine residues, allowing the mutant cleavage site to be activated by ubiquitously expressed proteases of the furin family. Specific examples of NDVs that express such a mutated F protein include, but are not limited to, rNDV/F2aa and rNDV/F3aa. For a description of mutations introduced into a NDV F protein to produce a mutated F protein with a mutated cleavage site, *see, e.g.*, Park *et al.* (2006) Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. *PNAS USA* 103: 8203-2808, which is incorporated herein by reference in its entirety. In some embodiments, the chimeric NDV is engineered to express a mutated F protein with the amino acid mutation L289A. In certain embodiments, the mutated F protein is from a different type or strain of NDV than the backbone NDV. In specific embodiments the L289A mutated F protein possesses one, two or three arginine residues in the cleavage site. In some embodiments, the mutated F protein is in addition to the backbone NDV F protein. In specific embodiments, the mutated F protein replaces the backbone NDV F protein. In specific embodiments, the mutated F protein is incorporated into the virion.

[00118] In some embodiments, the genome of a NDV engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, comprises a mutated NDV V protein encoding sequence, such as disclosed by Elankumaran *et al.*, 2010, J. Virol. 84(8): 3835-3844. In other embodiments, the genome of a NDV engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell does not comprise an NDV V protein encoding sequence. In certain embodiments, parental backbone of the chimeric NDV is a mesogenic or velogenic NDV strain that is engineered to express a mutated V protein, such as disclosed by Elankumaran *et al.*, 2010, J. Virol. 84(8): 3835-3844.

[00119] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a cytokine. In a specific embodiment, a genome of a NDV is engineered to express an agonist of a co-stimulatory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a cytokine. In a specific embodiment, a genome of a NDV is engineered to express an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a cytokine. Specific examples of cytokines include, but are not limited to, interleukin (IL)-2, IL-7, IL-9, IL-15, IL-17, IL-21, IL-22, interferon (IFN) gamma, GM-CSF, and tumor necrosis factor (TNF)-alpha.

[00120] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, a mutated F protein, and a cytokine (*e.g.*, IL-2, IL-7, IL-9, IL-15, IL-17, IL-21, IL-22, IFN-gamma, GM-CSF, and TNF-alpha). In a specific embodiment, the mutated F protein are highly fusogenic. In a specific embodiment, the mutated F protein has a mutant cleavage site (such as described herein). In some embodiments, the mutated F protein comprises the amino acid mutation L289A. In some embodiments, the chimeric NDV is engineered to express a mutated F protein with the amino acid mutation L289A. In certain embodiments, the mutated F protein is from a different type or strain of NDV than the backbone NDV. In specific embodiments the L289A mutated F protein possesses one, two or three arginine residues in the cleavage site. In some embodiments, the mutated F protein is in addition to the backbone NDV F protein. In specific embodiments, the

mutated F protein replaces the backbone NDV F protein. In specific embodiments, the mutated F protein is incorporated into the virion.

[00121] In certain aspects, provided herein are chimeric NDV comprising a genome engineered to express a cytokine such as, *e.g.*, IL-7, IL-15, IL-21 or another cytokine described herein or known to one of skill in the art. See, *e.g.*, Section 7 for examples of chimeric NDVs engineered to express cytokines as well as methods of producing such chimeric NDVs.

[00122] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express (i) an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, and (ii) a tumor antigen. In a specific embodiment, a genome of a NDV is engineered to express an agonist of a co-stimulatory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a tumor antigen. In a specific embodiment, a genome of a NDV is engineered to express an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a tumor antigen.

[00123] Tumor antigens include tumor-associated antigens and tumor-specific antigens. Specific examples of tumor antigens include, but are not limited to, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, N-acetylglucosaminyltransferase-V, p-15, gp100, MART-1/MelanA, TRP-1 (gp75), Tyrosinase, cyclin-dependent kinase 4, β -catenin, MUM-1, CDK4, HER-2/neu, human papillomavirus-E6, human papillomavirus E7, CD20, carcinoembryonic antigen (CEA), epidermal growth factor receptor, MUC-1, caspase-8, CD5, mucin-1, Lewisx, CA-125, p185HER2, IL-2R, Fap- α , tenascin, antigens associated with a metalloproteinase, and CAMPATH-1. Other examples include, but are not limited to, KS 1/4 pan-carcinoma antigen, ovarian carcinoma antigen (CA125), prostatic acid phosphatase, prostate specific antigen, melanoma-associated antigen p97, melanoma antigen gp75, high molecular weight melanoma antigen (HMW-MAA), prostate specific membrane antigen, CEA, polymorphic epithelial mucin antigen, milk fat globule antigen, colorectal tumor-associated antigens (such as: CEA, TAG-72, CO17-1A, GICA 19-9, CTA-1 and LEA), Burkitt's lymphoma antigen-38.13, CD19, B-lymphoma antigen-CD20, CD33, melanoma specific antigens (such as ganglioside GD2, ganglioside GD3, ganglioside GM2, ganglioside GM3), tumor-specific transplantation type of cell-surface antigen (TSTA) (such as virally-induced tumor antigens including T-antigen DNA tumor viruses and Envelope antigens of RNA tumor viruses), oncofetal antigen-alpha-fetoprotein such as CEA of colon, bladder tumor oncofetal antigen, differentiation antigen (such as human

lung carcinoma antigen L6 and L20), antigens of fibrosarcoma, leukemia T cell antigen-Gp37, neoglycoprotein, sphingolipids, breast cancer antigens (such as EGFR (Epidermal growth factor receptor), HER2 antigen (p185.sup.HER2) and HER2 neu epitope), polymorphic epithelial mucin (PEM), malignant human lymphocyte antigen-APO-1, differentiation antigen (such as I antigen found in fetal erythrocytes, primary endoderm, I antigen found in adult erythrocytes, preimplantation embryos, I(Ma) found in gastric adenocarcinomas, M18, M39 found in breast epithelium, SSEA-1 found in myeloid cells, VEP8, VEP9, Myl, VIM-D5, D.sub.156-22 found in colorectal cancer, TRA-1-85 (blood group H), C14 found in colonic adenocarcinoma, F3 found in lung adenocarcinoma, AH6 found in gastric cancer, Y hapten, Le.sup.y found in embryonal carcinoma cells, TL5 (blood group A), EGF receptor found in A431 cells, E₁ series (blood group B) found in pancreatic cancer, FC10.2 found in embryonal carcinoma cells, gastric adenocarcinoma antigen, CO-514 (blood group Le^a) found in Adenocarcinoma, NS-10 found in adenocarcinomas, CO-43 (blood group Le^b), G49 found in EGF receptor of A431 cells, MH2 (blood group AL^e/Le^y) found in colonic adenocarcinoma, 19.9 found in colon cancer, gastric cancer mucins, T₅A₇ found in myeloid cells, R₂₄ found in melanoma, 4.2, G_{D3}, D1.1, OFA-1, G_{M2}, OFA-2, G_{D2}, and M1:22:25:8 found in embryonal carcinoma cells, and SSEA-3 and SSEA-4 found in 4 to 8-cell stage embryos), T cell receptor derived peptide from a Cutaneous T cell Lymphoma, C-reactive protein (CRP), cancer antigen-50 (CA-50), cancer antigen 15-3 (CA15-3) associated with breast cancer, cancer antigen-19 (CA-19) and cancer antigen-242 associated with gastrointestinal cancers, carcinoma associated antigen (CAA), chromogranin A, epithelial mucin antigen (MC5), human epithelium specific antigen (E1A), Lewis(a)antigen, melanoma antigen, melanoma associated antigens 100, 25, and 150, mucin-like carcinoma-associated antigen, multidrug resistance related protein (MRPm6), multidrug resistance related protein (MRP41), Neu oncogene protein (C-erbB-2), neuron specific enolase (NSE), P-glycoprotein (mdr1 gene product), multidrug-resistance-related antigen, p170, multidrug-resistance-related antigen, prostate specific antigen (PSA), CD56, and NCAM.

[00124] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, a mutated F protein, and a tumor antigen. In a specific embodiment, the mutated F protein are highly fusogenic. In a specific embodiment, the mutated F protein has a mutant cleavage site (such as described

herein). In some embodiments, the mutated F protein comprises the amino acid mutation L289A. In some embodiments, the chimeric NDV is engineered to express a mutated F protein with the amino acid mutation L289A. In certain embodiments, the mutated F protein is from a different type or strain of NDV than the backbone NDV. In specific embodiments the L289A mutated F protein possesses one, two or three arginine residues in the cleavage site. In some embodiments, the mutated F protein is in addition to the backbone NDV F protein. In specific embodiments, the mutated F protein replaces the backbone NDV F protein. In specific embodiments, the mutated F protein is incorporated into the virion.

[00125] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express (i) an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, and (ii) a heterologous interferon antagonist. In a specific embodiment, a genome of a NDV is engineered to express an agonist of a co-stimulatory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a heterologous interferon antagonist. In a specific embodiment, a genome of a NDV is engineered to express an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a heterologous interferon antagonist. *See, e.g.*, U.S. patent application publication No. 2012-0058141, which is incorporated herein by reference, for examples of chimeric NDV engineered to express heterologous interferon antagonists.

[00126] Interferon antagonists may be identified using any technique known to one of skill in the art, including, *e.g.*, the techniques described in U.S. Patent Nos. 6,635,416; 7,060,430; and 7,442,527; which are incorporated herein by reference in their entirety. In a specific embodiment, the heterologous interferon antagonist is a viral protein. Such viral proteins may be obtained or derived from any virus and the virus may infect any species (*e.g.*, the virus may infect humans or non-human mammals). Exemplary heterologous interferon antagonists include, without limitation, Nipah virus W protein, Nipah V protein, Ebola virus VP35 protein, vaccinia virus E3L protein, influenza virus NS1 protein, respiratory syncytial virus (RSV) NS2 protein, herpes simplex virus (HSV) type 1 ICP34.5 protein, Hepatitis C virus NS3-4 protease, dominant-negative cellular proteins that block the induction or response to innate immunity (*e.g.*, STAT1, MyD88, IKK and TBK), and cellular regulators of the innate immune response (*e.g.*, SOCS proteins, PIAS proteins, CYLD proteins, IκB protein, Atg5 protein, Pin1 protein, IRAK-M protein, and UBP43). *See, e.g.*, U.S. patent application publication No. 2012-0058141, which is

incorporated herein by reference in its entirety, for additional information regarding heterologous interferon antagonist.

[00127] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, a mutated F protein, and a heterologous interferon antagonist. In a specific embodiment, the mutated F protein are highly fusogenic. In a specific embodiment, the mutated F protein has a mutant cleavage site (such as described herein). In some embodiments, the mutated F protein comprises the amino acid mutation L289A. In some embodiments, the chimeric NDV is engineered to express a mutated F protein with the amino acid mutation L289A. In certain embodiments, the mutated F protein is from a different type or strain of NDV than the backbone NDV. In specific embodiments the L289A mutated F protein possesses one, two or three arginine residues in the cleavage site. In some embodiments, the mutated F protein is in addition to the backbone NDV F protein. In specific embodiments, the mutated F protein replaces the backbone NDV F protein. In specific embodiments, the mutated F protein is incorporated into the virion.

[00128] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a pro-apoptotic molecule. In a specific embodiment, a genome of a NDV is engineered to express an agonist of a co-stimulatory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a pro-apoptotic molecule. In a specific embodiment, a genome of a NDV is engineered to express an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and a pro-apoptotic molecule. Specific examples of pro-apoptotic molecules include, but are not limited to, Bax, Bak, Bad, BID, Bcl-xS, Bim, Noxa, Puma, AIF, FasL, and TRAIL.

[00129] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, a mutated F protein, and a pro-apoptotic molecule. In a specific embodiment, the mutated F protein are highly fusogenic. In a specific embodiment, the mutated F protein has a mutant cleavage site (such as described herein). In some embodiments, the mutated F protein comprises the amino acid mutation L289A. In some embodiments, the chimeric NDV is engineered to express a mutated F protein

with the amino acid mutation L289A. In certain embodiments, the mutated F protein is from a different type or strain of NDV than the backbone NDV. In specific embodiments the L289A mutated F protein possesses one, two or three arginine residues in the cleavage site. In some embodiments, the mutated F protein is in addition to the backbone NDV F protein. In specific embodiments, the mutated F protein replaces the backbone NDV F protein. In specific embodiments, the mutated F protein is incorporated into the virion.

[00130] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and an anti-apoptotic molecule. In a specific embodiment, a genome of a NDV is engineered to express an agonist of a co-stimulatory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and an anti-apoptotic molecule. In a specific embodiment, a genome of a NDV is engineered to express an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and an anti-apoptotic molecule. Specific examples of anti-apoptotic molecules include, but are not limited to, Bcl-2, Bcl-xL, Mcl-1, and XIAP.

[00131] In another aspect, described herein are chimeric NDVs, comprising a genome engineered to express an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, a mutated F protein, and an anti-apoptotic molecule. In a specific embodiment, the mutated F protein are highly fusogenic. In a specific embodiment, the mutated F protein has a mutant cleavage site (such as described herein). In some embodiments, the mutated F protein comprises the amino acid mutation L289A. In some embodiments, the chimeric NDV is engineered to express a mutated F protein with the amino acid mutation L289A. In certain embodiments, the mutated F protein is from a different type or strain of NDV than the backbone NDV. In specific embodiments the L289A mutated F protein possesses one, two or three arginine residues in the cleavage site. In some embodiments, the mutated F protein is in addition to the backbone NDV F protein. In specific embodiments, the mutated F protein replaces the backbone NDV F protein. In specific embodiments, the mutated F protein is incorporated into the virion.

[00132] In certain aspects, provided herein are chimeric NDVs comprising a genome engineered express a pro-apoptotic molecule. In certain aspects, provided herein are chimeric

NDVs comprising a genome engineered to express an anti-apoptotic molecule. Examples of pro-apoptotic molecules and anti-apoptotic molecules are provided herein.

[00133] Any NDV type or strain may be used as a backbone that is engineered to express an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and in certain embodiments, engineered to express a cytokine, tumor antigen, heterologous interferon antagonist, pro-apoptotic molecule, anti-apoptotic molecule and/or mutated F protein, including, but not limited to, naturally-occurring strains, variants or mutants, mutagenized viruses, reassortants and/or genetically engineered viruses. In a specific embodiment, the NDV used in a combination therapy disclosed herein is a naturally-occurring strain. In certain embodiments, the NDV that serves as the backbone for genetic engineering is a lytic strain. In other embodiments, the NDV that serves as the backbone for genetic engineering is a non-lytic strain. In certain embodiments, the NDV that serves as the backbone for genetic engineering is lentogenic strain. In some embodiments, the NDV that serves as the backbone for genetic engineering is mesogenic strain. In other embodiments, the NDV that serves as the backbone for genetic engineering is a velogenic strain. Specific examples of NDV strains include, but are not limited to, the 73-T strain, NDV HUI strain, Ulster strain, MTH-68 strain, Italien strain, Hickman strain, PV701 strain, Hitchner B1 strain, La Sota strain (*see, e.g.*, Genbank No. AY845400), YG97 strain, MET95 strain, Roakin strain, and F48E9 strain. In a specific embodiment, the NDV that serves as the backbone for genetic engineering is the Hitchner B1 strain. In another specific embodiment, the NDV that serves as the backbone for genetic engineering is a B1 strain as identified by Genbank No. AF309418 or NC_002617. In another specific embodiment, the NDV that serves as the backbone for genetic engineering is the NDV identified by ATCC No. VR2239. In another specific embodiment, the NDV that serves as the backbone for genetic engineering is the La Sota strain.

[00134] In certain embodiments, attenuation, or further attenuation, of the chimeric NDV is desired such that the chimeric NDV remains, at least partially, infectious and can replicate *in vivo*, but only generate low titers resulting in subclinical levels of infection that are non-pathogenic (*see, e.g.*, Khattar et al., 2009, J. Virol. 83:7779-7782). In a specific embodiment, the NDV is attenuated by deletion of the V protein. Such attenuated chimeric NDVs may be especially suited for embodiments wherein the virus is administered to a subject in order to act as

an immunogen, *e.g.*, a live vaccine. The viruses may be attenuated by any method known in the art.

[00135] In certain embodiments, a chimeric NDV described herein expresses one, two, three, or more, or all of the following, and a suicide gene: (1) an agonist of a co-stimulatory signal of an immune cell; (2) an antagonist of an inhibitory signal of an immune cell; (3) a cytokine; (4) a tumor antigen; (5) a heterologous interferon antagonist; (6) a pro-apoptotic molecule; (7) an anti-apoptotic molecule; and/or (8) a mutated F protein. In specific embodiments, in addition to expressing an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and in certain embodiments, a mutated F protein and a cytokine, a chimeric NDV is engineered to express a suicide gene (*e.g.*, thymidine kinase) or another molecule that inhibits NDV replication or function (a gene that makes NDV sensitive to an antibiotic or an anti-viral agent). In some embodiments, in addition to expressing an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte or NK cell, and in certain embodiments, a mutated F protein and a cytokine, a chimeric NDV is engineered to encode tissue-specific microRNA (miRNA) target sites (*e.g.*, sites targeted by miR-21, miR-184, miR-133a/133b, miR-137, and/or miR-193a microRNAs).

[00136] In certain embodiments, the tropism of the chimeric NDV is altered. In a specific embodiment, the tropism of the virus is altered by modification of the F protein cleavage site to be recognized by tissue-specific or tumor-specific proteases such as matrix metalloproteases (MMP) and urokinase. In other embodiments, tropism of the virus is altered by introduction of tissue-specific miRNA target sites. In certain embodiments, NDV HN protein is mutated to recognize tumor-specific receptor.

[00137] In certain embodiments, one or more of the following are expressed by a chimeric NDV as a chimeric protein or fusion protein: (1) an agonist of a co-stimulatory signal of an immune cell; (2) an antagonist of an inhibitory signal of an immune cell; (3) a cytokine; (4) a tumor antigen; (5) a heterologous interferon antagonist; (6) a pro-apoptotic molecule; (7) an anti-apoptotic molecule; and/or (8) a mutated F protein. In specific embodiments, the chimeric protein or fusion protein comprises the transmembrane and cytoplasmic domains or fragments thereof of the NDV F or NDV HN protein and an extracellular domain that comprises one of the molecules referenced in the previous sentence. *See* U.S. Patent Application No. 2012-0122185

for a description of such chimeric proteins or fusion proteins, and International Application Publication No. WO 2007/064802, which are incorporated herein by reference.

[00138] In embodiments herein, the agonist of a co-stimulatory signal and/or the antagonist of an inhibitory signal of an immune cell may be inserted into the genome of the backbone NDV between two transcription units. In a specific embodiment, the agonist of a co-stimulatory signal and/or the antagonist of an inhibitory signal of an immune cell is inserted into the genome of the backbone NDV between the M and P transcription units or between the HN and L transcription units. In accordance with other embodiments herein, the cytokine, tumor antigen, heterologous interferon antagonist, pro-apoptotic molecule, anti-apoptotic molecule and/or mutated F protein are inserted into the genome of the backbone NDV between two or more transcription units (*e.g.*, between the M and P transcription units or between the HN and L transcription units).

5.2.1. IMMUNE CELL STIMULATORY AGENTS

[00139] The chimeric NDVs described herein may be engineered to express any agonist of a co-stimulatory signal and/or any antagonist of an inhibitory signal of an immune cell, such as, *e.g.*, a T-lymphocyte, NK cell or antigen-presenting cell (*e.g.*, a dendritic cell or macrophage), known to one of skill in the art. In specific embodiments, the agonist and/or antagonist is an agonist of a human co-stimulatory signal of an immune cell and/or antagonist of a human inhibitory signal of an immune cell. In certain embodiments, the agonist of a co-stimulatory signal is an agonist of a co-stimulatory molecule (*e.g.*, co-stimulatory receptor) found on immune cells, such as, *e.g.*, T-lymphocytes (*e.g.*, CD4⁺ or CD8⁺ T-lymphocytes), NK cells and/or antigen-presenting cells (*e.g.*, dendritic cells or macrophages). Specific examples of co-stimulatory molecules include glucocorticoid-induced tumor necrosis factor receptor (GITR), Inducible T-cell costimulator (ICOS or CD278), OX40 (CD134), CD27, CD28, 4-1BB (CD137), CD40, lymphotoxin alpha (LT alpha), LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes), CD226, cytotoxic and regulatory T cell molecule (CRTAM), death receptor 3 (DR3), lymphotoxin-beta receptor (LTBR), transmembrane activator and CAML interactor (TACI), B cell-activating factor receptor (BAFFR), and B cell maturation protein (BCMA). In specific embodiments, the agonist is an agonist of a human co-stimulatory receptor of an immune cell. In certain embodiments, the agonist of a co-stimulatory receptor is not an agonist of ICOS. In some embodiments, the antagonist is an antagonist of an inhibitory molecule (*e.g.*,

inhibitory receptor) found on immune cells, such as, *e.g.*, T-lymphocytes (*e.g.*, CD4⁺ or CD8⁺ T-lymphocytes), NK cells and/or antigen-presenting cells (*e.g.*, dendritic cells or macrophages). Specific examples of inhibitory molecules include cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4 or CD52), programmed cell death protein 1 (PD1 or CD279), B and T-lymphocyte attenuator (BTLA), killer cell immunoglobulin-like receptor (KIR), lymphocyte activation gene 3 (LAG3), T-cell membrane protein 3 (TIM3), CD160, adenosine A2a receptor (A2aR), T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), and CD160. In specific embodiments, the antagonist is an antagonist of a human inhibitory receptor of an immune cell.

[00140] In a specific embodiment, the agonist of a co-stimulatory receptor is an antibody or antigen-binding fragment thereof that specifically binds to the co-stimulatory receptor. Specific examples of co-stimulatory receptors include GITR, ICOS, OX40, CD27, CD28, 4-1BB, CD40, , LT alpha, LIGHT, CD226, CRTAM, DR3, LTBR, TACI, BAFFR, and BCMA. In certain specific embodiments, the antibody is a monoclonal antibody. In other specific embodiments, the antibody is an sc-Fv. In a specific embodiment, the antibody is a bispecific antibody that binds to two receptors on an immune cell. In other embodiments, the bispecific antibody binds to a receptor on an immune cell and to another receptor on a cancer cell. In specific embodiments, the antibody is a human or humanized antibody. In some embodiments, the antibody is expressed as a chimeric protein with NDV F protein or fragment thereof, or NDV HN protein or fragment thereof. *See, e.g.*, U.S. patent application Publication No. 2012/0122185, which is incorporated herein by reference for a description regarding generation of chimeric F or chimeric HN proteins. In a specific embodiment, the chimeric protein is the chimeric F protein described in Sections 6 and/or 7, *infra*. The techniques described below for generating the chimeric ICOSL-F protein and the chimeric CD28-F protein can be used to generate other chimeric F proteins or chimeric HN proteins.

[00141] In another embodiment, the agonist of a co-stimulatory receptor is a ligand of the co-stimulatory receptor. In certain embodiments, the ligand is fragment of a native ligand. Specific examples of native ligands include ICOSL, B7RP1, CD137L, OX40L, CD70, herpes virus entry mediator (HVEM), CD80, and CD86. The nucleotide sequences encoding native ligands as well as the amino acid sequences of native ligands are known in the art. For example, the nucleotide and amino acid sequences of B7RP1 (otherwise known as ICOSL; GenBank human:

NM_015259.4, NP_056074.1 murine: NM_015790.3, NP_056605.1), CD137L(GenBank human: NM_003811.3, NP_003802.1, murine: NM_009404.3, NP_033430.1), OX40L(GenBank human: NM_003326.3, NP_003317.1, murine: NM_009452.2, NP_033478.1), CD70(GenBank human: NM_001252.3, NP_001243.1, murine: NM_011617.2, AAD00274.1), CD80(GenBank human: NM_005191.3, NP_005182.1, murine: NM_009855.2, NP_033985.3), and CD86(GenBank human: NM_005191.3, CAG46642.1, murine: NM_019388.3, NP_062261.3) can be found in GenBank. In other embodiments, the ligand is a derivative of a native ligand. In some embodiments, the ligand is a fusion protein comprising at least a portion of the native ligand or a derivative of the native ligand that specifically binds to the co-stimulatory receptor, and a heterologous amino acid sequence. In specific embodiments, the fusion protein comprises at least a portion of the native ligand or a derivative of the native ligand that specifically binds to the co-stimulatory receptor, and the Fc portion of an immunoglobulin or a fragment thereof. An example of a ligand fusion protein is a 4-1BB ligand fused to Fc portion of immunoglobulin (described by Meseck M et al., J Immunother. 2011 34:175-82). In some embodiments, the ligand is expressed as a chimeric protein with the NDV F protein or fragment thereof, or NDV HN protein or fragment thereof. In a specific embodiment, the protein is the chimeric HN protein described in Section 7, *infra*. The techniques described below for generating the chimeric HN-GITRL, chimeric HN-OX40-L, chimeric HN-4-1BBL, and/or chimeric HN-CD40L can be used to generate other chimeric F proteins or chimeric HN proteins.

[00142] In another embodiment, the antagonist of an inhibitory receptor is an antibody (or an antigen-binding fragment) or a soluble receptor that specifically binds to the native ligand for the inhibitory receptor and blocks the native ligand from binding to the inhibitory receptor and transducing an inhibitory signal(s). Specific examples of native ligands for inhibitory receptors include PDL-1, PDL-2, B7-H3, B7-H4, HVEM, Gal9 and adenosine. Specific examples of inhibitory receptors that bind to a native ligand include CTLA-4, PD-1, BTLA, KIR, LAG3, TIM3, and A2aR.

[00143] In specific embodiments, the antagonist of an inhibitory receptor is a soluble receptor that specifically binds to the native ligand for the inhibitory receptor and blocks the native ligand from binding to the inhibitory receptor and transducing an inhibitory signal(s). In certain embodiments, the soluble receptor is a fragment of a native inhibitory receptor or a fragment of a derivative of a native inhibitory receptor that specifically binds to native ligand (*e.g.*, the

extracellular domain of a native inhibitory receptor or a derivative of an inhibitory receptor). In some embodiments, the soluble receptor is a fusion protein comprising at least a portion of the native inhibitory receptor or a derivative of the native inhibitory receptor (*e.g.*, the extracellular domain of the native inhibitory receptor or a derivative of the native inhibitory receptor), and a heterologous amino acid sequence. In specific embodiments, the fusion protein comprises at least a portion of the native inhibitory receptor or a derivative of the native inhibitory receptor, and the Fc portion of an immunoglobulin or a fragment thereof. An example of a soluble receptor fusion protein is a LAG3-Ig fusion protein (described by Huard B et al., Eur J Immunol. 1995 25:2718-21).

[00144] In specific embodiments, the antagonist of an inhibitory receptor is an antibody (or an antigen-binding fragment) that specifically binds to the native ligand for the inhibitory receptor and blocks the native ligand from binding to the inhibitory receptor and transducing an inhibitory signal(s). In certain specific embodiments, the antibody is a monoclonal antibody. In other specific embodiments, the antibody is an scFv. In particular embodiments, the antibody is a human or humanized antibody. A specific example of an antibody to inhibitory ligand is anti-PD-L1 antibody (Iwai Y, et al. PNAS 2002; 99:12293-12297).

[00145] In another embodiment, the antagonist of an inhibitory receptor is an antibody (or an antigen-binding fragment) or ligand that binds to the inhibitory receptor, but does not transduce an inhibitory signal(s). Specific examples of inhibitory receptors include CTLA-4, PD1, BTLA, KIR, LAG3, TIM3, and A2aR. In certain specific embodiments, the antibody is a monoclonal antibody. In other specific embodiments, the antibody is an scFv. In particular embodiments, the antibody is a human or humanized antibody. A specific example of an antibody to inhibitory receptor is anti-CTLA-4 antibody (Leach DR, et al. Science 1996; 271: 1734-1736). Another example of an antibody to inhibitory receptor is anti-PD-1 antibody (Topalian SL, NEJM 2012; 28:3167-75).

[00146] In certain embodiments, a chimeric NDV described herein is engineered to an antagonist of CTLA-4, such as, *e.g.*, Ipilimumab or Tremelimumab. In certain embodiments, a chimeric NDV described herein is engineered to an antagonist of PD1, such as, *e.g.*, MDX-1106 (BMS-936558), MK3475, CT-011, AMP-224, or MDX-1105. In certain embodiments, a chimeric NDV described herein is engineered to express an antagonist of LAG3, such as, *e.g.*, IMP321. In certain embodiments, a chimeric NDV described herein is engineered to express an

antibody (*e.g.*, a monoclonal antibody or an antigen-binding fragment thereof, or scFv) that binds to B7-H3, such as, *e.g.*, MGA271. In specific embodiments, a chimeric NDV described herein is engineered to express an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell described in Section 6 and/or Section 7, *infra*. In specific embodiments, NDV described herein is engineered to express anti-CD28 scvFv, ICOSL, CD40L, OX40L, CD137L, GITRL, and/or CD70.

[00147] In certain embodiments, an agonist of a co-stimulatory signal of an immune cell induces (*e.g.*, selectively) induces one or more of the signal transduction pathways induced by the binding of a co-stimulatory receptor to its ligand. In specific embodiments, an agonist of a co-stimulatory receptor induces one or more of the signal transduction pathways induced by the binding of the co-stimulatory receptor to one or more of its ligands by at least 25%, 30%, 40%, 50%, 60%, 75%, 80%, 85%, 90%, 95%, 98% or 99%, or in the range of between 25% to 50%, 25% to 75%, 50% to 75%, 50% to 95%, 75% to 95%, or 75% to 100% relative to the one or more signal transduction pathways induced by the binding of the co-stimulatory receptor to one or more of its ligands in the absence of the agonist. In specific embodiments, an agonist of a co-stimulatory receptor: (i) induces one or more of the signal transduction pathways induced by the binding of the co-stimulatory receptor to one particular ligand by at least 25%, 30%, 40%, 50%, 60%, 75%, 80%, 85%, 90%, 95%, 98% or 99%, or in the range of between 25% to 50%, 25% to 75%, 50% to 75%, 50% to 95%, 75% to 95%, or 75% to 100% relative to the one or more signal transduction pathways induced by the binding of the co-stimulatory receptor to the particular ligand in the absence of the agonist; and (ii) does not induce, or induces one or more of the signal transduction pathways induced by the binding of the co-stimulatory receptor to one or more other ligands by less than 20%, 15%, 10%, 5%, or 2%, or in the range of between 2% to 5%, 2% to 10%, 5% to 10%, 5% to 15%, 5% to 20%, 10% to 15%, or 15% to 20% relative to the one or more signal transduction pathways induced by the binding of the co-stimulatory receptor to such one or more other ligands in the absence of the agonist.

[00148] In certain embodiments, an agonist of a co-stimulatory signal of an immune cell activates or enhances (*e.g.*, selectively activates or enhances) one or more of the signal transduction pathways induced by the binding of a co-stimulatory receptor to its ligand. In specific embodiments, an agonist of a co-stimulatory receptor activates or enhances one or more of the signal transduction pathways induced by the binding of the co-stimulatory receptor to one

or more of its ligands by at least 25%, 30%, 40%, 50%, 60%, 75%, 80%, 85%, 90%, 95%, 98% or 99%, or in the range of between 25% to 50%, 25% to 75%, 50% to 75%, 50% to 95%, 75% to 95%, or 75% to 100% relative to the one or more signal transduction pathways induced by the binding of co-stimulatory receptor to one or more of its ligands in the absence of the agonist. In specific embodiments, an agonist of a co-stimulatory receptor: (i) an agonist of a co-stimulatory signal activates or enhances one or more of the signal transduction pathways induced by the binding of the co-stimulatory receptor to one particular ligand by at least 25%, 30%, 40%, 50%, 60%, 75%, 80%, 85%, 90%, 95%, 98% or 99%, or in the range of between 25% to 50%, 25% to 75%, 50% to 75%, 50% to 95%, 75% to 95%, or 75% to 100% relative to the one or more signal transduction pathways induced by the binding of the co-stimulatory receptor to the particular ligand in the absence of the agonist; and (ii) does not activate or enhance, or activates or enhances one or more of the signal transduction pathways induced by the binding of the co-stimulatory receptor to one or more other ligands by less than 20%, 15%, 10%, 5%, or 2%, or in the range of between 2% to 5%, 2% to 10%, 5% to 10%, 5% to 15%, 5% to 20%, 10% to 15%, or 15% to 20% relative to the one or more signal transduction pathways induced by the binding of the co-stimulatory receptor to such one or more other ligands in the absence of the agonist.

[00149] In some embodiments, an antagonist of an inhibitory signal of an immune cell (*e.g.*, selectively) inhibits or reduces one or more of the signal transduction pathways induced by the binding of an inhibitory receptor to its ligand. In specific embodiments, an antagonist of an inhibitory receptor inhibits or reduces one or more of the signal transduction pathways induced by the binding of the inhibitory receptor to one or more of its ligands by at least 25%, 30%, 40%, 50%, 60%, 75%, 80%, 85%, 90%, 95%, 98% or 99%, or in the range of between 25% to 50%, 25% to 75%, 50% to 75%, 50% to 95%, 75% to 95%, or 75% to 100% relative to the one or more signal transduction pathways induced by the binding of the inhibitory receptor to one or more of its ligands in the absence of the antagonist. In specific embodiments, an antagonist of an inhibitory receptor: (i) inhibits or reduces one or more of the signal transduction pathways induced by the binding of the inhibitory receptor to one particular ligand by at least 25%, 30%, 40%, 50%, 60%, 75%, 80%, 85%, 90%, 95%, 98% or 99%, or in the range of between 25% to 50%, 25% to 75%, 50% to 75%, 50% to 95%, 75% to 95%, or 75% to 100% relative to the one or more signal transduction pathways induced by the binding of the inhibitory receptor to the one particular ligand in the absence of the antagonist; and (ii) does not inhibit or reduce, or inhibits or

reduces one or more of the signal transduction pathways induced by the binding of the inhibitory receptor to one or more other ligands by less than 20%, 15%, 10%, 5%, or 2%, or in the range of between 2% to 5%, 2% to 10%, 5% to 10%, 5% to 15%, 5% to 20%, 10% to 15%, or 15% to 20% relative to the one or more signal transduction pathways induced by the binding of inhibitory receptor to such one or more other ligands in the absence of the antagonist.

[00150] In specific embodiments, an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell induces, activates and/or enhances one or more immune activities, functions or responses. The one or more immune activities, functions or responses can be in the form of, *e.g.*, an antibody response (humoral response) or a cellular immune response, *e.g.*, cytokine secretion (*e.g.*, interferon-gamma), helper activity or cellular cytotoxicity. In one embodiment, expression of an activation marker on immune cells (*e.g.*, CD44, Granzyme, or Ki-67), expression of a co-stimulatory receptor on immune cells (*e.g.*, ICOS, CD28, OX40, or CD27), expression of a ligand for a co-stimulatory receptor (*e.g.*, B7HRP1, CD80, CD86, OX40L, or CD70), cytokine secretion, infiltration of immune cells (*e.g.*, T-lymphocytes, B lymphocytes and/or NK cells) to a tumor, antibody production, effector function, T cell activation, T cell differentiation, T cell proliferation, B cell differentiation, B cell proliferation, and/or NK cell proliferation is induced, activated and/or enhanced following contact with an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell. In another embodiment, myeloid-derived suppressor cell (MDSC) tumor infiltration and proliferation, Treg tumor infiltration, activation and proliferation, peripheral blood MDSC and Treg counts are inhibited following contact with an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune cell.

5.3 CONSTRUCTION OF NDVS

[00151] The NDVs described herein can be generated using the reverse genetics technique. The reverse genetics technique involves the preparation of synthetic recombinant viral RNAs that contain the non-coding regions of the negative-strand, viral RNA which are essential for the recognition by viral polymerases and for packaging signals necessary to generate a mature virion. The recombinant RNAs are synthesized from a recombinant DNA template and reconstituted *in vitro* with purified viral polymerase complex to form recombinant ribonucleoproteins (RNPs) which can be used to transfect cells. A more efficient transfection is

achieved if the viral polymerase proteins are present during transcription of the synthetic RNAs either *in vitro* or *in vivo*. The synthetic recombinant RNPs can be rescued into infectious virus particles. The foregoing techniques are described in U.S. Patent No. 5,166,057 issued November 24, 1992; in U.S. Patent No. 5,854,037 issued December 29, 1998; in U.S. Patent No. 6,146,642 issued November 14, 2000; in European Patent Publication EP 0702085A1, published February 20, 1996; in U.S. Patent Application Serial No. 09/152,845; in International Patent Publications PCT WO97/12032 published April 3, 1997; WO96/34625 published November 7, 1996; in European Patent Publication EP A780475; WO 99/02657 published January 21, 1999; WO 98/53078 published November 26, 1998; WO 98/02530 published January 22, 1998; WO 99/15672 published April 1, 1999; WO 98/13501 published April 2, 1998; WO 97/06270 published February 20, 1997; and EPO 780 475A1 published June 25, 1997, each of which is incorporated by reference herein in its entirety.

[00152] The helper-free plasmid technology can also be utilized to engineer a NDV described herein. Briefly, a complete cDNA of a NDV (*e.g.*, the Hitchner B1 strain) is constructed, inserted into a plasmid vector and engineered to contain a unique restriction site between two transcription units (*e.g.*, the NDV P and M genes; or the NDV HN and L genes). A nucleotide sequence encoding a heterologous amino acid sequence (*e.g.*, a nucleotide sequence encoding an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell) may be inserted into the viral genome at the unique restriction site. Alternatively, a nucleotide sequence encoding a heterologous amino acid sequence (*e.g.*, a nucleotide sequence encoding an agonist of a co-stimulatory signal and/or an antagonist of an inhibitory signal of an immune cell) may be engineered into a NDV transcription unit so long as the insertion does not affect the ability of the virus to infect and replicate. The single segment is positioned between a T7 promoter and the hepatitis delta virus ribozyme to produce an exact negative transcript from the T7 polymerase. The plasmid vector and expression vectors comprising the necessary viral proteins are transfected into cells leading to production of recombinant viral particles (see, *e.g.*, International Publication No. WO 01/04333; U.S. Patent Nos. 7,442,379, 6,146,642, 6,649,372, 6,544,785 and 7,384,774; Swayne *et al.* (2003). Avian Dis. 47:1047-1050; and Swayne *et al.* (2001). J. Virol. 11868-11873, each of which is incorporated by reference in its entirety).

[00153] Techniques for the production of a chimeric NDV that express an antibody are known in the art. *See, e.g.*, Puhler et al., *Gene Ther.* 15(5): 371-283 (2008) for the generation of a recombinant NDV expressing a full IgG from two transgenes.

[00154] Bicistronic techniques to produce multiple proteins from a single mRNA are known to one of skill in the art. Bicistronic techniques allow the engineering of coding sequences of multiple proteins into a single mRNA through the use of IRES sequences. IRES sequences direct the internal recruitment of ribosomes to the RNA molecule and allow downstream translation in a cap independent manner. Briefly, a coding region of one protein is inserted into the ORF of a second protein. The insertion is flanked by an IRES and any untranslated signal sequences necessary for proper expression and/or function. The insertion must not disrupt the open reading frame, polyadenylation or transcriptional promoters of the second protein (*see e.g.*, García-Sastre *et al.*, 1994, *J. Virol.* 68:6254-6261 and García-Sastre *et al.*, 1994 *Dev. Biol. Stand.* 82:237-246, each of which are incorporated by reference herein in their entirety).

5.4 PROPAGATION OF NDVS

[00155] The NDVs described herein (*e.g.*, the chimeric NDVs) can be propagated in any substrate that allows the virus to grow to titers that permit the uses of the viruses described herein. In one embodiment, the substrate allows the NDVs described herein (*e.g.*, the chimeric NDVs) to grow to titers comparable to those determined for the corresponding wild-type viruses.

[00156] The NDVs described herein (*e.g.*, the chimeric NDVs) may be grown in cells (*e.g.*, avian cells, chicken cells, etc.) that are susceptible to infection by the viruses, embryonated eggs (*e.g.*, chicken eggs or quail eggs) or animals (*e.g.*, birds). Such methods are well-known to those skilled in the art. In a specific embodiment, the NDVs described herein (*e.g.*, the chimeric NDVs) may be propagated in cancer cells, *e.g.*, carcinoma cells (*e.g.*, breast cancer cells and prostate cancer cells), sarcoma cells, leukemia cells, lymphoma cells, and germ cell tumor cells (*e.g.*, testicular cancer cells and ovarian cancer cells). In another specific embodiment, the NDVs described herein (*e.g.*, the chimeric NDVs) may be propagated in cell lines, *e.g.*, cancer cell lines such as HeLa cells, MCF7 cells, THP-1 cells, U87 cells, DU145 cells, Lncap cells, and T47D cells. In certain embodiments, the cells or cell lines (*e.g.*, cancer cells or cancer cell lines) are obtained and/or derived from a human(s). In another embodiment, the NDVs described herein (*e.g.*, the chimeric NDVs) are propagated in chicken cells or embryonated eggs. Representative chicken cells include, but are not limited to, chicken embryo fibroblasts and

chicken embryo kidney cells. In a specific embodiment, the NDVs described herein (*e.g.*, the chimeric NDVs) are propagated in Vero cells. In another specific embodiment, the NDVs described herein (*e.g.*, the chimeric NDVs) are propagated in cancer cells in accordance with the methods described in Section 6 and/or Section 7, *infra*. In another specific embodiment, the NDVs described herein (*e.g.*, the chimeric NDVs) are propagated in chicken eggs or quail eggs. In certain embodiments, a NDV virus described herein (*e.g.*, a chimeric NDV) is first propagated in embryonated eggs and then propagated in cells (*e.g.*, a cell line).

[00157] The NDVs described herein (*e.g.*, the chimeric NDVs) may be propagated in embryonated eggs, *e.g.*, from 6 to 14 days old, 6 to 12 days old, 6 to 10 days old, 6 to 9 days old, 6 to 8 days old, or 10 to 12 days old. Young or immature embryonated eggs can be used to propagate the NDVs described herein (*e.g.*, the chimeric NDVs). Immature embryonated eggs encompass eggs which are less than ten day old eggs, *e.g.*, eggs 6 to 9 days old or 6 to 8 days old that are IFN-deficient. Immature embryonated eggs also encompass eggs which artificially mimic immature eggs up to, but less than ten day old, as a result of alterations to the growth conditions, *e.g.*, changes in incubation temperatures; treating with drugs; or any other alteration which results in an egg with a retarded development, such that the IFN system is not fully developed as compared with ten to twelve day old eggs. The NDVs described herein (*e.g.*, the chimeric NDVs) can be propagated in different locations of the embryonated egg, *e.g.*, the allantoic cavity. For a detailed discussion on the growth and propagation viruses, see, *e.g.*, U.S. Patent No. 6,852,522 and U.S. Patent No. 7,494,808, both of which are hereby incorporated by reference in their entireties.

[00158] For virus isolation, the NDVs described herein (*e.g.*, the chimeric NDVs) can be removed from cell culture and separated from cellular components, typically by well known clarification procedures, *e.g.*, such as gradient centrifugation and column chromatography, and may be further purified as desired using procedures well known to those skilled in the art, *e.g.*, plaque assays.

5.5 COMPOSITIONS & ROUTES OF ADMINISTRATION

[00159] Encompassed herein is the use of a NDV described herein (*e.g.*, the chimeric NDVs) in compositions. Also encompassed herein is the use of plasma membrane fragments from NDV infected cells or whole cancer cells infected with NDV in compositions. In a specific embodiment, the compositions are pharmaceutical compositions, such as immunogenic

formulations (*e.g.*, vaccine formulations). The compositions may be used in methods of treating cancer.

[00160] In one embodiment, a pharmaceutical composition comprises a NDV described herein (*e.g.*, the chimeric NDVs), in an admixture with a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition further comprises one or more additional prophylactic or therapeutic agents, such as described in Section 5.6.4, *infra*. In a specific embodiment, a pharmaceutical composition comprises an effective amount of a NDV described herein (*e.g.*, the chimeric NDVs), and optionally one or more additional prophylactic or therapeutic agents, in a pharmaceutically acceptable carrier. In some embodiments, the NDV (*e.g.*, a chimeric NDV) is the only active ingredient included in the pharmaceutical composition.

[00161] In another embodiment, a pharmaceutical composition (*e.g.*, an oncolysate vaccine) comprises a protein concentrate or a preparation of plasma membrane fragments from NDV infected cancer cells, in an admixture with a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition further comprises one or more additional prophylactic or therapeutic agents, such as described in Section 5.6.4, *infra*.. In another embodiment, a pharmaceutical composition (*e.g.*, a whole cell vaccine) comprises cancer cells infected with NDV, in an admixture with a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition further comprises one or more additional prophylactic or therapeutic agents, such as described in Section 5.6.4, *infra*..

[00162] The pharmaceutical compositions provided herein can be in any form that allows for the composition to be administered to a subject. In a specific embodiment, the pharmaceutical compositions are suitable for veterinary and/or human administration. As used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeiae for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Examples of

suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. The formulation should suit the mode of administration.

[00163] In a specific embodiment, the pharmaceutical compositions are formulated to be suitable for the intended route of administration to a subject. For example, the pharmaceutical composition may be formulated to be suitable for parenteral, intravenous, intraarterial, intrapleural, inhalation, intraperitoneal, oral, intradermal, colorectal, intraperitoneal, intracranial, and intratumoral administration. In a specific embodiment, the pharmaceutical composition may be formulated for intravenous, intraarterial, oral, intraperitoneal, intranasal, intratracheal, intrapleural, intracranial, subcutaneous, intramuscular, topical, pulmonary, or intratumoral administration.

5.6 ANTI-CANCER USES AND OTHER USES

[00164] In one aspect, a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) may be used in the treatment of cancer. In one embodiment, provided herein are methods for treating cancer, comprising administering to a subject in need thereof a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) or a composition thereof. In a specific embodiment, provided herein is a method for treating cancer, comprising administering to a subject in need thereof an effective amount of a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) or a composition thereof.

[00165] In specific embodiments, a chimeric NDV engineered to express an agonist of a co-stimulatory signal of an immune cell, or a composition thereof is administered to a subject to treat cancer. In another specific embodiments, a chimeric NDV engineered to express an antagonist of an inhibitory signal of an immune cell, or a composition thereof is administered to a subject to treat cancer. In certain embodiments, a chimeric NDV engineered to express an agonist of a co-stimulatory signal of an immune cell and a mutated F protein or a composition thereof is administered to a subject to treat cancer. In certain embodiments, a chimeric NDV engineered to express an antagonist of an inhibitory signal of an immune cell and a mutated F protein or a composition thereof is administered to a subject to treat cancer.

[00166] A chimeric NDV (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) described herein or a composition thereof, an oncolysate vaccine, or a whole cell cancer vaccine used in a method for treating cancer may be used as any line of therapy (*e.g.*, a first, second, third, fourth or fifth line therapy).

[00167] In certain embodiments, a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) is the only active ingredient administered to treat cancer. In specific embodiments, a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) is the only active ingredient in a composition administered to treat cancer.

[00168] The chimeric NDV (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) or a composition thereof may be administered locally or systemically to a subject. For example, the chimeric NDV (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) or a composition thereof may be administered parenterally (*e.g.*, intravenously, intraarterially, or subcutaneously), intratumorally, intrapleurally, intranasally, intraperitoneally, intracranially, orally, rectally, by inhalation, intramuscularly, topically or intradermally to a subject. In a specific embodiment, the chimeric NDV is administered via the hepatic artery, by, *e.g.*, hepatic artery injection, which can be performed by interventional radiology or through placement of an arterial infusion pump. In another specific embodiment, the chimeric NDV is administered intraoperatively, laparoscopically, or endoscopically. In a specific embodiment, intraperitoneal administration of the chimeric NDV is performed by direct injection, infusion via catheter, or injection during laparoscopy.

[00169] In certain embodiments, the methods described herein include the treatment of cancer for which no treatment is available. In some embodiments, a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) or a composition thereof is administered to a subject to treat cancer as an alternative to other conventional therapies.

[00170] In one embodiment, provided herein is a method for treating cancer, comprising administering to a subject in need thereof a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) or a composition thereof and one or more additional therapies, such as described in Section 5.6.4, *infra*. In a particular embodiment, one or more therapies are administered to a subject in combination with a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) or a composition thereof to treat cancer. In a specific embodiment, the additional therapies are currently being used, have been used or are known to be useful in treating cancer. In another embodiment, a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) or a composition thereof is administered to a subject in combination with a supportive therapy, a pain relief therapy, or other therapy that does not have a therapeutic effect on cancer. In a specific embodiment, the one or

more additional therapies administered in combination with a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) is one or more of the therapies described in Section 5.6.4.1, *infra*. In certain embodiments, a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) and one or more additional therapies are administered in the same composition. In other embodiments, a chimeric NDV and one or more additional therapies are administered in different compositions.

[00171] In certain embodiments, two, three or multiple NDVs (including one, two or more chimeric NDVs described herein, such as one, two or more of the chimeric NDVs described in Section 5.2, *supra*) are administered to a subject to treat cancer. The second or more chimeric NDVs used in accordance with methods described herein that comprise administration of two, three or multiple NDVs to a subject to treat cancer may be naturally occurring chimeric NDVs or engineered chimeric NDVs that have been engineered to express heterologous amino acid sequence (*e.g.*, a cytokine). The first and second chimeric NDVs may be part of the same pharmaceutical composition or different pharmaceutical compositions. In certain embodiments, the first chimeric NDV and the second chimeric NDV are administered by the same route of administration (*e.g.*, both are administered intratumorally or intravenously). In other embodiments, the first chimeric NDV and the second chimeric NDV are administered by different routes of administration (*e.g.*, one is administered intratumorally and the other is administered intravenously).

[00172] In specific embodiments, a first chimeric NDV engineered to express an agonist of a co-stimulatory signal of an immune cell is administered to a patient to treat cancer in combination with a second chimeric NDV engineered to express an antagonist of an inhibitory signal of an immune cell. In other specific embodiments, a first chimeric NDV engineered to express an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune is administered in combination with a second chimeric NDV engineered to express one, two or more of the following: a cytokine (*e.g.*, IL-2), a heterologous interferon antagonist, a tumor antigen, a pro-apoptotic molecule, and/or anti-apoptotic molecule. In a specific embodiment, the first chimeric NDV, the second chimeric NDV, or both express a mutated F protein that increases the fusogenic activity of the chimeric NDV. In another specific embodiment, the first chimeric NDV, the second chimeric NDV or both express a mutated F protein with a mutation in the cleavage site (such as described herein).

[00173] In specific embodiments, a first composition (*e.g.*, a pharmaceutical composition) comprising a first chimeric NDV engineered to express an agonist of a co-stimulatory signal of an immune cell is administered to a patient to treat cancer in combination with a second composition (*e.g.*, a pharmaceutical composition) comprising a second chimeric NDV engineered to express an antagonist of an inhibitory signal of an immune cell. In other specific embodiments, a first composition (*e.g.*, a pharmaceutical composition) comprising a first chimeric NDV engineered to express an agonist of a co-stimulatory signal of an immune cell and/or an antagonist of an inhibitory signal of an immune is administered in combination with a second composition (*e.g.*, a pharmaceutical composition) comprising a second chimeric NDV engineered to express one, two or more of the following: a cytokine (*e.g.*, IL-2), a heterologous interferon antagonist, a tumor antigen, a pro-apoptotic molecule, and/or anti-apoptotic molecule. In a specific embodiment, the first chimeric NDV, the second chimeric NDV, or both express a mutated F protein that increases the fusogenic activity of the chimeric NDV. In another specific embodiment, the first chimeric NDV, the second chimeric NDV or both express a mutated F protein with a mutation in the cleavage site (such as described herein).

[00174] In another aspect, an NDV described herein (*e.g.*, an NDV described in Section 5.1, *supra*) may be used in combination with one or more additional therapies, such as described herein in Section 5.6.4, *infra* (*e.g.*, Section 5.6.4.1, *infra*), in the treatment of cancer. In one embodiment, provided herein are methods for treating cancer, comprising administering to a subject in need thereof an NDV described herein (*e.g.*, an NDV described in Section 5.1, *supra*) or a composition thereof and one or more additional therapies, such as described herein in Section 5.6.4, *infra*. (*e.g.*, Section 5.6.4.1). In a specific embodiment, provided herein is a method for treating cancer, comprising administering to a subject in need thereof an effective amount of an NDV described herein (*e.g.*, an NDV described in Section 5.1, *supra*) or a composition thereof and an effective amount of one or more additional therapies, such as described in Section 5.6.4, *infra*. (*e.g.*, Section 5.6.4.1). In certain embodiments, an NDV described herein (*e.g.*, an NDV described in Section 5.1, *supra*) and one or more additional therapies, such as described in Section 5.6.4, *infra* (*e.g.*, Section 5.6.4.1), are administered in the same composition. In other embodiments, an NDV (*e.g.*, an NDV described in Section 5.1, *supra*) and one or more additional therapies are administered in different compositions.

[00175] The NDV used in combination with one or more additional therapies can be administered systemically or locally. For example, the NDV or composition thereof may be administered parenterally (*e.g.*, intravenously, intraarterially, or subcutaneously), intratumorally, intrapleurally, intranasally, intraperitoneally, intracranially, orally, rectally, by inhalation, intramuscularly, topically or intradermally to a subject. In a specific embodiment, the NDV is administered via the hepatic artery, by, *e.g.*, hepatic artery injection, which can be performed by interventional radiology or through placement of an arterial infusion pump. In another specific embodiment, the NDV is administered intraoperatively, laparoscopically, or endoscopically. In a specific embodiment, intraperitoneal administration of the NDV is performed by direct injection, infusion via catheter, or injection during laparoscopy.

[00176] An NDV (*e.g.*, an NDV described in Section 5.1, *supra*) described herein or a composition thereof, an oncolysate vaccine, or a whole cell cancer vaccine in combination with one or more additional therapies, such as described herein in Section 5.6.4, *infra*, may be used as any line of therapy (*e.g.*, a first, second, third, fourth or fifth line therapy) for treating cancer in accordance with a method described herein..

[00177] In another aspect, whole cancer cells infected with a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) can be used to treat cancer. In a specific embodiment, a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) may be contacted with a cancer cell or a population of cancer cells and the infected cancer cell or population of cancer cells may be administered to a subject to treat cancer. In one embodiment, the cancer cells are subjected to gamma radiation prior to infection with a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*). In another embodiment, the cancer cells are subjected to gamma radiation after infection with a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*). In a particular embodiment, the cancer cells are treated prior to administration to a subject so that the cancer cells cannot multiply in the subject. In a specific embodiment, the cancer cells cannot multiply in the subject and the virus cannot infect the subject. In one embodiment, the cancer cells are subjected to gamma radiation prior to administration to subject. In another embodiment, the cancer cells are sonicated prior to administration to a subject. In another embodiment, the cancer cells are treated with mitomycin C prior to administration to a subject. In another embodiment, the cancer cells are treated by freezing and thawing prior to administration to a subject. In

another embodiment, the cancer cells are treated with heat treatment prior to administration to a subject. The cancer cells may be administered locally or systemically to a subject. For example, the cancer cells may be administered parenterally (*e.g.*, intravenously or subcutaneously), intratumorally, intranasally, orally, by inhalation, intrapleurally, topically or intradermally to a subject. In a specific embodiment, the cancer cells are administered intratumorally or to the skin (*e.g.*, intradermally) of a subject. The cancer cells used may be autologous or allogeneic. In a specific embodiment, the backbone of the chimeric NDV is a non-lytic strain. The cancer cells may be administered to a subject alone or in combination with an additional therapy. The cancer cells are preferably in a pharmaceutical composition. In certain embodiments, the cancer cells are administered in combination with one or more additional therapies, such as described in Section 5.6.4, *infra*. In certain embodiments, the cancer cells and one or more additional therapies are administered in the same composition. In other embodiments, the cancer cells and one or more additional therapies are administered in different compositions.

[00178] In another aspect, whole cancer cells infected with an NDV described herein (*e.g.*, an NDV described in Section 5.1, *supra*) may be used in combination with one or more additional therapies described herein in Section 5.6.4, *infra*, in the treatment of cancer. In one embodiment, provided herein are methods for treating cancer, comprising administering to a subject in need thereof whole cancer cells infected with an NDV described herein (*e.g.*, an NDV described in Section 5.1, *supra*) in combination with one or more additional therapies described herein in Section 5.6.4, *infra*. In a specific embodiment, provided herein is a method for treating cancer, comprising administering to a subject in need thereof an effective amount of whole cancer cells infected with an NDV described herein (*e.g.*, an NDV described in Section 5.1, *supra*) in combination with an effective amount of one or more additional therapies described in Section 5.6.4, *infra*. In certain embodiments, whole cancer cells infected with an NDV described herein (*e.g.*, an NDV described in Section 5.1, *supra*) and one or more additional therapies described in Section 5.6.4, *infra*, are administered in the same composition. In other embodiments, whole cancer cells infected with an NDV described herein (*e.g.*, an NDV described in Section 5.1, *supra*) and one or more additional therapies are administered in different compositions.

[00179] In another aspect, a protein concentrate or plasma membrane preparation from lysed cancer cells infected with a chimeric NDV (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) can be used to treat cancer. In one embodiment, a plasma membrane preparation

comprising fragments from cancer cells infected with a chimeric NDV described herein can be used to treat cancer. In another embodiment, a protein concentrate from cancer cells infected with a chimeric NDV described herein can be used to treat cancer. Techniques known to one of skill in the art may be used to produce the protein concentrate or plasma membrane preparation. In a specific embodiment, a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) may be contacted with a cancer cell or a population of cancer cells and the infected cancer cell or population of cancer cells may be lysed using techniques known to one of skill in the art to obtain protein concentrate or plasma membrane fragments of the NDV-infected cancer cells, and the protein concentrate or plasma membrane fragments of the NDV-infected cancer cells may be administered to a subject to treat cancer. The protein concentrate or plasma membrane fragments may be administered locally or systemically to a subject. For example, the protein concentrate or plasma membrane fragments may be administered parenterally, intratumorally, intranasally, intrapleurally, orally, by inhalation, topically or intradermally to a subject. In a specific embodiment, such a protein concentrate or plasma membrane preparation is administered intratumorally or to the skin (*e.g.*, intradermally) of a subject. The cancer cells used to produce the protein concentrate or plasma membrane preparation may be autologous or allogeneic. In a specific embodiment, the backbone of the chimeric NDV is a lytic strain. The protein concentrate or plasma membrane preparation may be administered to a subject alone or in combination with an additional therapy. The protein concentrate or plasma membrane preparation is preferably in a pharmaceutical composition. In certain embodiments, the protein concentrate or plasma membrane preparation is administered in combination with one or more additional therapies, such as described in Section 5.6.4, *infra* (*e.g.*, Section 5.6.4.1) In certain embodiments, the protein concentrate or plasma membrane preparation and one or more additional therapies are administered in the same composition. In other embodiments, the protein concentrate or plasma membrane preparation and one or more additional therapies are administered in different compositions.

[00180] In another aspect, a protein concentrate or plasma membrane preparation from lysed cancer cells infected with an NDV (*e.g.*, an NDV described in Section 5.1, *supra*) may be used in combination with one or more additional therapies, such as described herein in Section 5.6.4, *infra* (*e.g.*, Section 5.6.4.1), in the treatment of cancer. In one embodiment, provided herein are methods for treating cancer, comprising administering to a subject in need thereof a protein

concentrate or plasma membrane preparation from lysed cancer cells infected with an NDV (*e.g.*, an NDV described in Section 5.1, *supra*) in combination with one or more additional therapies, such as described herein in Section 5.6.4, *infra*. (*e.g.*, Section 5.6.4.1). In a specific embodiment, provided herein is a method for treating cancer, comprising administering to a subject in need thereof an effective amount of a protein concentrate or plasma membrane preparation from lysed cancer cells infected with an NDV (*e.g.*, an NDV described in Section 5.1, *supra*) in combination with an effective amount of one or more additional therapies, such as described in Section 5.6.4, *infra*. (*e.g.*, Section 5.6.4.1). In certain embodiments, the protein concentrate or plasma membrane preparation and one or more additional therapies, such as described in Section 5.6.4, *infra*, are administered in the same composition. In other embodiments, the protein concentrate or plasma membrane preparation and one or more additional therapies are administered in different compositions.

[00181] In another aspect, the chimeric NDVs described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) can be used to produce antibodies which can be used in diagnostic immunoassays, passive immunotherapy, and the generation of antiidiotypic antibodies. For example, a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*) can be administered to a subject (*e.g.*, a mouse, rat, pig, horse, donkey, bird or human) to generate antibodies which can then be isolated and used, *e.g.*, in diagnostic assays, passive immunotherapy and generation of antiidiotypic antibodies. In certain embodiments, an NDV described herein (*e.g.*, an NDV described in Section 5.1 or 5.2, *supra*) is administered to a subject (*e.g.*, a mouse, rat, pig, horse, donkey, bird, or human) in combination with one or more additional therapies, such as described in Section 5.6.4, *infra*, to generate antibodies which can then be isolated and used, *e.g.*, in diagnostic assays, passive immunotherapy and generation of antiidiotypic antibodies. The generated antibodies may be isolated by standard techniques known in the art (*e.g.*, immunoaffinity chromatography, centrifugation, precipitation, etc.) and used in diagnostic immunoassays, passive immunotherapy and generation of antiidiotypic antibodies.

[00182] In certain embodiments, the antibodies isolated from subjects administered a chimeric NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *supra*), or isolated from subjects administered an NDV described herein (*e.g.*, an NDV described in Section 5.1 or 5.2, *supra*) in combination with one or more additional therapies, such as described in Section 5.6.4,

infra, are used to assess the expression of NDV proteins, a heterologous peptide or protein expressed by a chimeric NDV, or both. Any immunoassay system known in the art may be used for this purpose including but not limited to competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays, to name but a few.

5.6.1. PATIENT POPULATION

[00183] In some embodiments, an NDV (*e.g.*, a chimeric NDV) described herein or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a subject suffering from cancer. In other embodiments, an NDV (*e.g.*, a chimeric NDV) described herein or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a subject predisposed or susceptible to cancer. In some embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a subject diagnosed with cancer. Specific examples of the types of cancer are described herein. In an embodiment, the subject has metastatic cancer. In another embodiment, the subject has stage 1, stage 2, stage 3, or stage 4 cancer. In another embodiment, the subject is in remission. In yet another embodiment, the subject has a recurrence of cancer.

[00184] In certain embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a human that is 0 to 6 months old, 6 to 12 months old, 6 to 18 months old, 18 to 36 months old, 1 to 5 years old, 5 to 10 years old, 10 to 15 years old, 15 to 20 years old, 20 to 25 years old, 25 to 30 years old, 30 to 35 years old, 35 to 40 years old, 40 to 45 years old, 45 to 50 years old, 50 to 55 years old, 55 to 60 years old, 60 to 65 years old, 65 to 70 years old, 70 to 75 years old, 75 to 80 years old, 80 to 85 years old, 85 to 90 years old, 90 to 95 years old or 95 to 100 years old. In some embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine

described herein, or a combination therapy described herein is administered to a human infant. In other embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a human toddler. In other embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a human child. In other embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a human adult. In yet other embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to an elderly human.

[00185] In certain embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a subject in an immunocompromised state or immunosuppressed state or at risk for becoming immunocompromised or immunosuppressed. In certain embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a subject receiving or recovering from immunosuppressive therapy. In certain embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a subject that has or is at risk of getting cancer. In certain embodiments, the subject is, will or has undergone surgery, chemotherapy and/or radiation therapy. In certain embodiments, the patient has undergone surgery to remove the tumor or neoplasm. In specific embodiments, the patient is administered an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein following surgery to remove a tumor or neoplasm. In other embodiment, the patient is administered an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein prior to undergoing surgery to remove a tumor or neoplasm. In certain embodiments, an NDV (*e.g.*, a chimeric NDV) or a

composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a subject that has, will have or had a tissue transplant, organ transplant or transfusion.

[00186] In some embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a patient who has proven refractory to therapies other than the chimeric NDV or composition thereof, oncolysate, whole cell vaccine, or a combination therapy but are no longer on these therapies. In a specific embodiment, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a patient who has proven refractory to chemotherapy. In one embodiment, that a cancer is refractory to a therapy means that at least some significant portion of the cancer cells are not killed or their cell division arrested. The determination of whether the cancer cells are refractory can be made either *in vivo* or *in vitro* by any method known in the art for assaying the effect of a therapy on cancer cells, using the art-accepted meanings of “refractory” in such a context. In a certain embodiment, refractory patient is a patient refractory to a standard therapy. In certain embodiments, a patient with cancer, is refractory to a therapy when the tumor or neoplasm has not significantly been eradicated and/or the symptoms have not been significantly alleviated. The determination of whether a patient is refractory can be made either *in vivo* or *in vitro* by any method known in the art for assaying the effectiveness of a treatment of cancer, using art-accepted meanings of “refractory” in such a context.

[00187] In certain embodiments, the patient to be treated in accordance with the methods described herein is a patient already being treated with antibiotics, anti-virals, anti-fungals, or other biological therapy/immunotherapy or anti-cancer therapy. Among these patients are refractory patients, and patients who are too young for conventional therapies. In some embodiments, the subject being administered an NDV (*e.g.*, a chimeric NDV), an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein has not received therapy prior to the administration of the chimeric NDV or composition, the oncolysate vaccine, or the whole cell vaccine, or the combination therapy.

[00188] In some embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination

therapy described herein is administered to a patient to prevent the onset of cancer in a patient at risk of developing cancer. In some embodiments, compounds are administered to a patient who are susceptible to adverse reactions to conventional therapies.

[00189] In some embodiments, the subject being administered an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein has not received prior therapy. In other embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein is administered to a subject who has received a therapy prior to administration of the NDV (*e.g.*, a chimeric NDV) or composition, the oncolysate vaccine, the whole cell vaccine, or the combination therapy. In some embodiments, the subject administered an NDV (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine described herein, or a whole cell vaccine described herein, or a combination therapy described herein experienced adverse side effects to a prior therapy or a prior therapy was discontinued due to unacceptable levels of toxicity to the subject.

5.6.2. DOSAGE & FREQUENCY

[00190] The amount of an NDV or a composition thereof, an oncolysate vaccine, or a whole cell vaccine which will be effective in the treatment of cancer will depend on the nature of the cancer, the route of administration, the general health of the subject, etc. and should be decided according to the judgment of a medical practitioner. Standard clinical techniques, such as *in vitro* assays, may optionally be employed to help identify optimal dosage ranges. However, suitable dosage ranges of an NDV for administration are generally about 10^2 , 5×10^2 , 10^3 , 5×10^3 , 10^4 , 5×10^4 , 10^5 , 5×10^5 , 10^6 , 5×10^6 , 10^7 , 5×10^7 , 10^8 , 5×10^8 , 1×10^9 , 5×10^9 , 1×10^{10} , 5×10^{10} , 1×10^{11} , 5×10^{11} or 10^{12} pfu, and most preferably about 10^4 to about 10^{12} , 10^6 to 10^{12} , 10^8 to 10^{12} , 10^9 to 10^{12} or 10^9 to 10^{11} , and can be administered to a subject once, twice, three, four or more times with intervals as often as needed. Dosage ranges of oncolysate vaccines for administration may include 0.001 mg, 0.005 mg, 0.01 mg, 0.05 mg, 0.1 mg, 0.5 mg, 1.0 mg, 2.0 mg, 3.0 mg, 4.0 mg, 5.0 mg, 10.0 mg, 0.001 mg to 10.0 mg, 0.01 mg to 1.0 mg, 0.1 mg to 1 mg, and 0.1 mg to 5.0 mg, and can be administered to a subject once, twice, three or more times with intervals as often as needed. Dosage ranges of whole cell vaccines for administration may include 10^2 , 5×10^2 , 10^3 , 5×10^3 , 10^4 , 5×10^4 , 10^5 , 5×10^5 , 10^6 , 5×10^6 , 10^7 , 5×10^7 , 10^8 , $5 \times$

10^8 , 1×10^9 , 5×10^9 , 1×10^{10} , 5×10^{10} , 1×10^{11} , 5×10^{11} or 10^{12} cells, and can be administered to a subject once, twice, three or more times with intervals as often as needed. In certain embodiments, dosages similar to those currently being used in clinical trials for NDV, oncolysate vaccines or whole cell vaccines are administered to a subject. Effective doses may be extrapolated from dose response curves derived from *in vitro* or animal model test systems.

[00191] In certain embodiments, an NDV (*e.g.*, a chimeric NDV) or a composition thereof is administered to a subject as a single dose followed by a second dose 1 to 6 weeks, 1 to 5 weeks, 1 to 4 weeks, 1 to 3 weeks, 1 to 2 weeks later. In accordance with these embodiments, booster inoculations may be administered to the subject at 6 to 12 month intervals following the second inoculation. In certain embodiments, an oncolysate vaccine or a whole cell vaccine is administered to a subject as a single dose followed by a second dose 1 to 6 weeks, 1 to 5 weeks, 1 to 4 weeks, 1 to 3 weeks, 1 to 2 weeks later.

[00192] In certain embodiments, administration of the same NDV (*e.g.*, chimeric NDV) or a composition thereof, oncolysate vaccine, or whole cell vaccine may be repeated and the administrations may be separated by at least 1 day, 2 days, 3 days, 5 days, 6 days, 7 days, 10 days, 14 days, 15 days, 21 days, 28 days, 30 days, 45 days, 2 months, 75 days, 3 months, or at least 6 months. In other embodiments, administration of the same NDV (*e.g.*, a NDV) or a composition thereof, oncolysate vaccine, or whole cell vaccine may be repeated and the administrations may be separated by 1 to 14 days, 1 to 7 days, 7 to 14 days, 1 to 30 days, 15 to 30 days, 15 to 45 days, 15 to 75 days, 15 to 90 days, 1 to 3 months, 3 to 6 months, 3 to 12 months, or 6 to 12 months. In some embodiments, a first NDV (*e.g.*, a first chimeric NDV) or a composition thereof is administered to a subject followed by the administration of a second NDV (*e.g.*, a second chimeric NDV) or a composition thereof. In certain embodiments, the first and second NDVs (*e.g.*, the first and second chimeric NDVs) or compositions thereof may be separated by at least 1 day, 2 days, 3 days, 5 days, 6 days, 7 days, 10 days, 14 days, 15 days, 21 days, 28 days, 30 days, 45 days, 2 months, 75 days, 3 months, or at least 6 months. In other embodiments, the first and second NDVs (*e.g.*, the first and second chimeric NDVs) or compositions thereof may be separated by 1 to 14 days, 1 to 7 days, 7 to 14 days, 1 to 30 days, 15 to 30 days, 15 to 45 days, 15 to 75 days, 15 to 90 days, 1 to 3 months, 3 to 6 months, 3 to 12 months, or 6 to 12 months.

[00193] In certain embodiments, an NDV or composition thereof, or oncolysate vaccine or whole cell vaccine is administered to a subject in combination with one or more additional therapies, such as a therapy described in Section 5.6.4, *infra*. The dosage of the other one or more additional therapies will depend upon various factors including, *e.g.*, the therapy, the nature of the cancer, the route of administration, the general health of the subject, etc. and should be decided according to the judgment of a medical practitioner. In specific embodiments, the dose of the other therapy is the dose and/or frequency of administration of the therapy recommended for the therapy for use as a single agent is used in accordance with the methods disclosed herein. In other embodiments, the dose of the other therapy is a lower dose and/or less frequent administration of the therapy than recommended for the therapy for use as a single agent is used in accordance with the methods disclosed herein. Recommended doses for approved therapies can be found in the Physician's Desk Reference.

[00194] In certain embodiments, an NDV or composition thereof, or oncolysate vaccine or whole cell vaccine is administered to a subject concurrently with the administration of one or more additional therapies. In other embodiments, an NDV or composition thereof, or oncolysate vaccine or whole cell vaccine is administered to a subject every 3 to 7 days, 1 to 6 weeks, 1 to 5 weeks, 1 to 4 weeks, 2 to 4 weeks, 1 to 3 weeks, or 1 to 2 weeks and one or more additional therapies (such as described in Section 5.6.4, *infra*) is administered every 3 to 7 days, 1 to 6 weeks, 1 to 5 weeks, 1 to 4 weeks, 1 to 3 weeks, or 1 to 2 weeks. In certain embodiments, an NDV or composition thereof, or oncolysate vaccine or whole cell vaccine is administered to a subject every 1 to 2 weeks and one or more additional therapies (such as described in Section 5.6.4, *infra*) is administered every 2 to 4 weeks. In some embodiments, an NDV or composition thereof, or oncolysate vaccine or whole cell vaccine is administered to a subject every week and one or more additional therapies (such as described in Section 5.6.4, *infra*) is administered every 2 weeks.

5.6.3. TYPES OF CANCER

[00195] Specific examples of cancers that can be treated in accordance with the methods described herein include, but are not limited to: leukemias, such as but not limited to, acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemias, such as, myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia leukemias and myelodysplastic syndrome; chronic leukemias, such as but not limited to, chronic myelocytic

(granulocytic) leukemia, chronic lymphocytic leukemia, hairy cell leukemia; polycythemia vera; lymphomas such as but not limited to Hodgkin's disease, non-Hodgkin's disease; multiple myelomas such as but not limited to smoldering multiple myeloma, nonsecretory myeloma, osteosclerotic myeloma, placancer cell leukemia, solitary placancercytoma and extramedullary placancercytoma; Waldenström's macroglobulinemia; monoclonal gammopathy of undetermined significance; benign monoclonal gammopathy; heavy chain disease; bone and connective tissue sarcomas such as but not limited to bone sarcoma, osteosarcoma, chondrosarcoma, Ewing's sarcoma, malignant giant cell tumor, fibrosarcoma of bone, chordoma, periosteal sarcoma, soft-tissue sarcomas, angiosarcoma (hemangiosarcoma), fibrosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, neurilemmoma, rhabdomyosarcoma, synovial sarcoma; brain tumors such as but not limited to, glioma, astrocytoma, brain stem glioma, ependymoma, oligodendroglioma, nonglial tumor, glioblastoma multiforme, acoustic neurinoma, craniopharyngioma, medulloblastoma, meningioma, pineocytoma, pineoblastoma, primary brain lymphoma; breast cancer including but not limited to ductal carcinoma, adenocarcinoma, lobular (cancer cell) carcinoma, intraductal carcinoma, medullary breast cancer, mucinous breast cancer, tubular breast cancer, papillary breast cancer, Paget's disease, and inflammatory breast cancer; adrenal cancer such as but not limited to pheochromocytom and adrenocortical carcinoma; thyroid cancer such as but not limited to papillary or follicular thyroid cancer, medullary thyroid cancer and anaplastic thyroid cancer; pancreatic cancer such as but not limited to, insulinoma, gastrinoma, glucagonoma, vipoma, somatostatin-secreting tumor, and carcinoid or islet cell tumor; pituitary cancers such as but limited to Cushing's disease, prolactin-secreting tumor, acromegaly, and diabetes insipidus; eye cancers such as but not limited to ocular melanoma such as iris melanoma, choroidal melanoma, and ciliary body melanoma, and retinoblastoma; vaginal cancers such as squamous cell carcinoma, adenocarcinoma, and melanoma; vulvar cancer such as squamous cell carcinoma, melanoma, adenocarcinoma, basal cell carcinoma, sarcoma, and Paget's disease; cervical cancers such as but not limited to, squamous cell carcinoma, and adenocarcinoma; uterine cancers such as but not limited to endometrial carcinoma and uterine sarcoma; ovarian cancers such as but not limited to, ovarian epithelial carcinoma, borderline tumor, germ cell tumor, and stromal tumor; esophageal cancers such as but not limited to, squamous cancer, adenocarcinoma, adenoid cystic carcinoma, mucoepidermoid carcinoma, adenosquamous carcinoma, sarcoma, melanoma, placancercytoma,

verrucous carcinoma, and oat cell (cancer cell) carcinoma; stomach cancers such as but not limited to, adenocarcinoma, fungating (polypoid), ulcerating, superficial spreading, diffusely spreading, malignant lymphoma, liposarcoma, fibrosarcoma, and carcinosarcoma; colon cancers; rectal cancers; liver cancers such as but not limited to hepatocellular carcinoma and hepatoblastoma; gallbladder cancers such as adenocarcinoma; cholangiocarcinomas such as but not limited to papillary, nodular, and diffuse; lung cancers such as non-cancer cell lung cancer, squamous cell carcinoma (epidermoid carcinoma), adenocarcinoma, large-cell carcinoma and cancer-cell lung cancer; testicular cancers such as but not limited to germinal tumor, seminoma, anaplastic, classic (typical), spermatocytic, nonseminoma, embryonal carcinoma, teratoma carcinoma, choriocarcinoma (yolk-sac tumor), prostate cancers such as but not limited to, prostatic intraepithelial neoplasia, adenocarcinoma, leiomyosarcoma, and rhabdomyosarcoma; penal cancers; oral cancers such as but not limited to squamous cell carcinoma; basal cancers; salivary gland cancers such as but not limited to adenocarcinoma, mucoepidermoid carcinoma, and adenoicystic carcinoma; pharynx cancers such as but not limited to squamous cell cancer, and verrucous; skin cancers such as but not limited to, basal cell carcinoma, squamous cell carcinoma and melanoma, superficial spreading melanoma, nodular melanoma, lentigo malignant melanoma, acral lentiginous melanoma; kidney cancers such as but not limited to renal cell carcinoma, adenocarcinoma, hypernephroma, fibrosarcoma, transitional cell cancer (renal pelvis and/ or uterer); Wilms' tumor; bladder cancers such as but not limited to transitional cell carcinoma, squamous cell cancer, adenocarcinoma, carcinosarcoma. In addition, cancers include myxosarcoma, osteogenic sarcoma, endotheliosarcoma, lymphangioendotheliosarcoma, mesothelioma, synovioma, hemangioblastoma, epithelial carcinoma, cystadenocarcinoma, bronchogenic carcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma and papillary adenocarcinomas (for a review of such disorders, see Fishman et al., 1985, Medicine, 2d Ed., J.B. Lippincott Co., Philadelphia and Murphy et al., 1997, Informed Decisions: The Complete Book of Cancer Diagnosis, Treatment, and Recovery, Viking Penguin, Penguin Books U.S.A., Inc., United States of America).

[00196] In a specific embodiment, the chimeric NDVs described herein or compositions thereof, an oncolysate vaccine described herein, a whole cell vaccine herein, or a combination therapy described herein are useful in the treatment of a variety of cancers and abnormal proliferative diseases, including (but not limited to) the following: carcinoma, including that of

the bladder, breast, colon, kidney, liver, lung, ovary, pancreas, stomach, cervix, thyroid and skin; including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage, including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T cell lymphoma, Burkitt's lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyosarcoma; other tumors, including melanoma, seminoma, teratocarcinoma, neuroblastoma and glioma; tumors of the central and peripheral nervous system, including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin, including fibrosarcoma, rhabdomyosarcoma, and osteosarcoma; and other tumors, including melanoma, xeroderma pigmentosum, keratoactanthoma, seminoma, thyroid follicular cancer and teratocarcinoma.

[00197] In some embodiments, cancers associated with aberrations in apoptosis are treated in accordance with the methods described herein. Such cancers may include, but are not limited to, follicular lymphomas, carcinomas with p53 mutations, hormone dependent tumors of the breast, prostate and ovary, and precancerous lesions such as familial adenomatous polyposis, and myelodysplastic syndromes. In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and dysplasias), or hyperproliferative disorders of the skin, lung, liver, bone, brain, stomach, colon, breast, prostate, bladder, kidney, pancreas, ovary, and/or uterus are treated in accordance with the methods described herein. In other specific embodiments, a sarcoma or melanoma is treated in accordance with the methods described herein.

[00198] In a specific embodiment, the cancer being treated in accordance with the methods described herein is leukemia, lymphoma or myeloma (*e.g.*, multiple myeloma). Specific examples of leukemias and other blood-borne cancers that can be treated in accordance with the methods described herein include, but are not limited to, acute lymphoblastic leukemia "ALL", acute lymphoblastic B-cell leukemia, acute lymphoblastic T-cell leukemia, acute myeloblastic leukemia "AML", acute promyelocytic leukemia "APL", acute monoblastic leukemia, acute erythroleukemic leukemia, acute megakaryoblastic leukemia, acute myelomonocytic leukemia, acute nonlymphocytic leukemia, acute undifferentiated leukemia, chronic myelocytic leukemia "CML", chronic lymphocytic leukemia "CLL", and hairy cell leukemia.

[00199] Specific examples of lymphomas that can be treated in accordance with the methods described herein include, but are not limited to, Hodgkin's disease, non-Hodgkin's Lymphoma,

Multiple myeloma, Waldenström's macroglobulinemia, Heavy chain disease, and Polycythemia vera.

[00200] In another embodiment, the cancer being treated in accordance with the methods described herein is a solid tumor. Examples of solid tumors that can be treated in accordance with the methods described herein include, but are not limited to fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon cancer, colorectal cancer, kidney cancer, pancreatic cancer, bone cancer, breast cancer, ovarian cancer, prostate cancer, esophageal cancer, stomach cancer, oral cancer, nasal cancer, throat cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, uterine cancer, testicular cancer, cancer cell lung carcinoma, bladder carcinoma, lung cancer, epithelial carcinoma, glioma, glioblastoma multiforme, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, skin cancer, melanoma, neuroblastoma, and retinoblastoma. In another embodiment, the cancer being treated in accordance with the methods described herein is a metastatic. In another embodiment, the cancer being treated in accordance with the methods described herein is malignant.

[00201] In a specific embodiment, the cancer being treated in accordance with the methods described herein is a cancer that has a poor prognosis and/or has a poor response to conventional therapies, such as chemotherapy and radiation. In another specific embodiment, the cancer being treated in accordance with the methods described herein is malignant melanoma, malignant glioma, renal cell carcinoma, pancreatic adenocarcinoma, malignant pleural mesothelioma, lung adenocarcinoma, lung small cell carcinoma, lung squamous cell carcinoma, anaplastic thyroid cancer, and head and neck squamous cell carcinoma. In another specific embodiment, the cancer being treated in accordance with the methods described herein is a type of cancer described in Section 6 and/or Section 7, *infra*.

5.6.4. ADDITIONAL THERAPIES

[00202] Additional therapies that can be used in a combination with an NDV described herein or a composition thereof, an oncolysate vaccine, or a whole cell vaccine for the treatment of cancer include, but are not limited to, small molecules, synthetic drugs, peptides (including cyclic peptides), polypeptides, proteins, nucleic acids (*e.g.*, DNA and RNA nucleotides including, but not limited to, antisense nucleotide sequences, triple helices, RNAi, and nucleotide sequences encoding biologically active proteins, polypeptides or peptides), antibodies, synthetic or natural inorganic molecules, mimetic agents, and synthetic or natural organic molecules. In a specific embodiment, the additional therapy is a chemotherapeutic agent.

[00203] In some embodiments, an NDV described herein or a composition thereof, an oncolysate vaccine, or a whole cell vaccine is used in combination with radiation therapy comprising the use of x-rays, gamma rays and other sources of radiation to destroy cancer cells. In specific embodiments, the radiation therapy is administered as external beam radiation or teletherapy, wherein the radiation is directed from a remote source. In other embodiments, the radiation therapy is administered as internal therapy or brachytherapy wherein a radioactive source is placed inside the body close to cancer cells and/or a tumor mass.

[00204] In certain embodiments, an NDV described herein or a composition thereof, an oncolysate vaccine, or a whole cell cancer vaccine is used in combination with adoptive T cell therapy. In a specific embodiment, the T cells utilized in the adoptive T cell therapy are tumor infiltrating lymphocytes that have been isolated from a subject and a particular T cell or clone has been expanded for use thereof. In some embodiments, the T cells utilized in the adoptive T cell therapy are T cells taken from a patient's blood after they have received a cancer vaccine and expanded *in vitro* before use. In another specific embodiment, the T cells utilized in the adoptive T cell therapy are T cells that have been influenced to potently recognize and attack tumors. In another specific embodiment, the T cells utilized in the adoptive T cell therapy have been genetically modified to express tumor-antigen specific T cell receptor or a chimeric antigen receptor (CAR). In a specific embodiment, the adoptive T cell therapy utilized is analogous to that described in Section 7, *infra*.

[00205] In certain embodiments, an NDV described herein or a composition thereof, an oncolysate vaccine, or a whole cell cancer vaccine is used in combination with a cytokine. In a specific embodiment, an NDV described herein or a composition thereof, an oncolysate vaccine, or a whole cell cancer vaccine is used in combination with interferon (*e.g.*, IFN- γ).

[00206] Currently available cancer therapies and their dosages, routes of administration and recommended usage are known in the art and have been described in such literature as the Physician's Desk Reference (67th ed., 2013).

[00207] Specific examples of anti-cancer agents that may be used in combination with an NDV described herein or a composition thereof include: hormonal agents (*e.g.*, aromatase inhibitor, selective estrogen receptor modulator (SERM), and estrogen receptor antagonist), chemotherapeutic agents (*e.g.*, microtubule disassembly blocker, antimetabolite, topoisomerase inhibitor, and DNA crosslinker or damaging agent), anti-angiogenic agents (*e.g.*, VEGF antagonist, receptor antagonist, integrin antagonist, vascular targeting agent (VTA)/vascular disrupting agent (VDA)), radiation therapy, and conventional surgery.

[00208] Non-limiting examples of hormonal agents that may be used in combination with an NDV described herein or a composition thereof include aromatase inhibitors, SERMs, and estrogen receptor antagonists. Hormonal agents that are aromatase inhibitors may be steroidal or nonsteroidal. Non-limiting examples of nonsteroidal hormonal agents include letrozole, anastrozole, aminoglutethimide, fadrozole, and vorozole. Non-limiting examples of steroidal hormonal agents include aromasin (exemestane), formestane, and testolactone. Non-limiting examples of hormonal agents that are SERMs include tamoxifen (branded/marketed as Nolvadex[®]), afimoxifene, arzoxifene, bazedoxifene, clomifene, femarelle, lasofoxifene, ormeloxifene, raloxifene, and toremifene. Non-limiting examples of hormonal agents that are estrogen receptor antagonists include fulvestrant. Other hormonal agents include but are not limited to abiraterone and lonaprisan.

[00209] Non-limiting examples of chemotherapeutic agents that may be used in combination with an NDV described herein or a composition thereof, an oncolysate vaccine, or a whole cell vaccine include microtubule disassembly blocker, antimetabolite, topoisomerase inhibitor, and DNA crosslinker or damaging agent. Chemotherapeutic agents that are microtubule disassembly blockers include, but are not limited to, taxenes (*e.g.*, paclitaxel (branded/marketed as TAXOL[®]), docetaxel, abraxane, larotaxel, ortataxel, and tesetaxel); epothilones (*e.g.*, ixabepilone); and vinca alkaloids (*e.g.*, vinorelbine, vinblastine, vindesine, and vincristine (branded/marketed as ONCOVIN[®])).

[00210] Chemotherapeutic agents that are antimetabolites include, but are not limited to, folate antimetabolites (*e.g.*, methotrexate, aminopterin, pemetrexed, raltitrexed); purine

antimetabolites (*e.g.*, cladribine, clofarabine, fludarabine, mercaptopurine, pentostatin, thioguanine); pyrimidine antimetabolites (*e.g.*, 5-fluorouracil, capecitabine, gemcitabine (GEMZAR[®]), cytarabine, decitabine, floxuridine, tegafur); and deoxyribonucleotide antimetabolites (*e.g.*, hydroxyurea).

[00211] Chemotherapeutic agents that are topoisomerase inhibitors include, but are not limited to, class I (camptotheca) topoisomerase inhibitors (*e.g.*, topotecan (branded/marketed as Hycamtin[®]) irinotecan, rubitecan, and belotecan); class II (podophyllum) topoisomerase inhibitors (*e.g.*, etoposide or VP-16, and teniposide); anthracyclines (*e.g.*, doxorubicin, epirubicin, Doxil, aclarubicin, amrubicin, daunorubicin, idarubicin, pirarubicin, valrubicin, and zorubicin); and anthracenediones (*e.g.*, mitoxantrone, and pixantrone).

[00212] Chemotherapeutic agents that are DNA crosslinkers (or DNA damaging agents) include, but are not limited to, alkylating agents (*e.g.*, cyclophosphamide, mechlorethamine, ifosfamide (branded/marketed as IFEX[®]), trofosfamide, chlorambucil, melphalan, prednimustine, bendamustine, uramustine, estramustine, carmustine (branded/marketed as BiCNU[®]), lomustine, semustine, fotemustine, nimustine, ranimustine, streptozocin, busulfan, mannosulfan, treosulfan, carboquone, N,N'-triethylenethiophosphoramidate, triaziquone, triethylenemelamine); alkylating-like agents (*e.g.*, carboplatin (branded/marketed as PARAPLATIN[®]), cisplatin, oxaliplatin, nedaplatin, triplatin tetranitrate, satraplatin, picoplatin); nonclassical DNA crosslinkers (*e.g.*, procarbazine, dacarbazine, temozolomide (branded/marketed as TEMODAR[®]), altretamine, mitobronitol); and intercalating agents (*e.g.*, actinomycin, bleomycin, mitomycin, and plicamycin).

5.6.4.1 Immune Modulators

[00213] In specific embodiments, an NDV described herein (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine, or a whole cell vaccine are administered to a subject in combination with one or more of the following: any agonist of a co-stimulatory signal of an immune cell (such as, *e.g.*, a T-lymphocyte, NK cell or antigen-presenting cell (*e.g.*, a dendritic cell or macrophage) and/or any antagonist of an inhibitory signal of an immune cell (such as, *e.g.*, a T-lymphocyte, NK cell or antigen-presenting cell (*e.g.*, a dendritic cell or macrophage), known to one of skill in the art. In particular embodiments, an NDV described herein (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine, or a whole cell vaccine are administered to a subject in combination with one or more of the agonists of a co-stimulatory

signal of an immune cell described in Section 5.2.1, *supra*. In some embodiments, an NDV described herein (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine, or a whole cell vaccine are administered to a subject in combination with one or more of the antagonists of an inhibitory signal of an immune cell described in Section 5.2.1, *supra*. In certain embodiments, an NDV described herein (*e.g.*, a chimeric NDV) or a composition thereof, an oncolysate vaccine, or a whole cell vaccine are administered to a subject in combination with one or more of the agonists of a co-stimulatory signal of an immune cell and/or one or more of the antagonists of an inhibitory signal of an immune cell described in Section 6 and/or Section 7, *infra* (*e.g.*, an anti-CTLA-4 antibody, an ICOS-L, an anti-PD-1 antibody, or an anti-PD-L1 antibody)

5.7 BIOLOGICAL ASSAYS

***In Vitro* Viral Assays**

[00214] Viral assays include those that measure altered viral replication (as determined, *e.g.*, by plaque formation) or the production of viral proteins (as determined, *e.g.*, by western blot analysis) or viral RNAs (as determined, *e.g.*, by RT-PCR or northern blot analysis) in cultured cells *in vitro* using methods which are well known in the art.

[00215] Growth of the NDVs described herein can be assessed by any method known in the art or described herein (*e.g.*, in cell culture (*e.g.*, cultures of chicken embryonic kidney cells or cultures of chicken embryonic fibroblasts (CEF)). Viral titer may be determined by inoculating serial dilutions of a NDV described herein into cell cultures (*e.g.*, CEF, MDCK, EFK-2 cells, Vero cells, primary human umbilical vein endothelial cells (HUVEC), H292 human epithelial cell line or HeLa cells), chick embryos, or live animals (*e.g.*, avians). After incubation of the virus for a specified time, the virus is isolated using standard methods. Physical quantitation of the virus titer can be performed using PCR applied to viral supernatants (Quinn & Trevor, 1997; Morgan et al., 1990), hemagglutination assays, tissue culture infectious doses (TCID₅₀) or egg infectious doses (EID₅₀). An exemplary method of assessing viral titer is described in Section 6 and Section 7, below.

[00216] Incorporation of nucleotide sequences encoding a heterologous peptide or protein (*e.g.*, a cytokine, a mutated F protein, a mutated V protein, or miRNA target site into the genome of a chimeric NDV described herein can be assessed by any method known in the art or

described herein (*e.g.*, in cell culture, an animal model or viral culture in embryonated eggs). For example, viral particles from cell culture of the allantoic fluid of embryonated eggs can be purified by centrifugation through a sucrose cushion and subsequently analyzed for fusion protein expression by Western blotting using methods well known in the art.

[00217] Immunofluorescence-based approaches may also be used to detect virus and assess viral growth. Such approaches are well known to those of skill in the art, *e.g.*, fluorescence microscopy and flow cytometry (see Section 6 and Section 7, *infra*).

Antibody Assays

[00218] Antibodies generated by the NDVs described herein may be characterized in a variety of ways well-known to one of skill in the art (*e.g.*, ELISA, Surface Plasmon resonance display (BIAcore), Western blot, immunofluorescence, immunostaining and/or microneutralization assays). In particular, antibodies generated by the chimeric NDVs described herein may be assayed for the ability to specifically bind to an antigen of the virus or a heterologous peptide or protein. Such an assay may be performed in solution (*e.g.*, Houghten, 1992, *Bio/Techniques* 13:412 421), on beads (Lam, 1991, *Nature* 354:82 84), on chips (Fodor, 1993, *Nature* 364:555 556), on bacteria (U.S. Patent No. 5,223,409), on spores (U.S. Patent Nos. 5,571,698; 5,403,484; and 5,223,409), on plasmids (Cull et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:1865 1869) or on phage (Scott and Smith, 1990, *Science* 249:386 390; Cwirla et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:6378 6382; and Felici, 1991, *J. Mol. Biol.* 222:301 310) (each of these references is incorporated herein in its entirety by reference).

[00219] Antibodies generated by the chimeric NDVs described herein that have been identified to specifically bind to an antigen of the virus or a heterologous peptide or protein can be assayed for their specificity to said antigen of the virus or heterologous peptide or protein. The antibodies may be assayed for specific binding to an antigen of the virus or a heterologous peptide or protein and for their cross-reactivity with other antigens by any method known in the art. Immunoassays which can be used to analyze specific binding and cross-reactivity include, but are not limited to, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), “sandwich” immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a

few. Such assays are routine and well known in the art (see, *e.g.*, Ausubel et al., eds., 1994, *Current Protocols in Molecular Biology*, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety).

[00220] The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. Alternatively, a surface plasmon resonance assay (*e.g.*, BIAcore kinetic analysis) or KinExA assay (Blake, et al., *Analytical Biochem.*, 1999, 272:123-134) may be used to determine the binding on and off rates of antibodies to an antigen of the chimeric NDVs described herein.

IFN Assays

[00221] IFN induction and release by an NDV described herein may be determined using techniques known to one of skill in the art or described herein. For example, the amount of IFN induced in cells following infection with an NDV described herein may be determined using an immunoassay (*e.g.*, an ELISA or Western blot assay) to measure IFN expression or to measure the expression of a protein whose expression is induced by IFN. Alternatively, the amount of IFN induced may be measured at the RNA level by assays, such as Northern blots and quantitative RT-PCR, known to one of skill in the art. In specific embodiments, the amount of IFN released may be measured using an ELISPOT assay. (*See, e.g.*, the methods described in Section 6 and Section 7, below.). Further, the induction and release of cytokines may be determined by, *e.g.*, an immunoassay or ELISPOT assay at the protein level and/or quantitative RT-PCR or northern blots at the RNA level. *See* Section 6 and/or Section 7, *infra*, regarding assays to measure cytokine induction and release.

Activation Marker Assays

[00222] Techniques for assessing the expression of activation marker, co-stimulatory molecule, ligand, or inhibitory molecule by immune cells are known to one of skill in the art. For example, the expression of an activation marker, co-stimulatory molecule, ligand, or inhibitory molecule by an immune cell (*e.g.*, T lymphocyte or NK cell) can be assessed by flow cytometry. In a specific embodiment, techniques described in Section 6 and/or Section 7, *infra*, are used to assess the expression of an activation marker, co-stimulatory molecule, ligand, or inhibitory molecule by an immune cell.

Immune Cell Infiltration Assays

[00223] Techniques for assessing immune cell infiltration are known to one of skill in the art. In a specific embodiment, techniques described in Section 6 and/or Section 7, *infra*, are used to assess immune cell infiltration.

Toxicity Studies

[00224] In some embodiments, the NDVs described herein or compositions thereof, oncolysate vaccines described herein, whole cell vaccines described herein, or combination therapies described herein are tested for cytotoxicity in mammalian, preferably human, cell lines (*see, e.g.*, the cytotoxicity assay described in Section 6 and/or Section 7, *infra*). In certain embodiments, cytotoxicity is assessed in one or more of the following non-limiting examples of cell lines: U937, a human monocyte cell line; primary peripheral blood mononuclear cells (PBMC); Huh7, a human hepatoblastoma cell line; HL60 cells, HT1080, HEK 293T and 293H, MLPC cells, human embryonic kidney cell lines; human melanoma cell lines, such as SkMel2, SkMel-119 and SkMel-197; THP-1, monocytic cells; a HeLa cell line; and neuroblastoma cells lines, such as MC-IXC, SK-N-MC, SK-N-MC, SK-N-DZ, SH-SY5Y, and BE(2)-C. In certain embodiments, cytotoxicity is assessed in various cancer cells. In some embodiments, the ToxLite assay is used to assess cytotoxicity.

[00225] Many assays well-known in the art can be used to assess viability of cells or cell lines following infection with an NDV described herein or composition thereof, or treatment with an oncolysate vaccine described herein, a whole cell vaccine described herein, or a combination therapy described herein and, thus, determine the cytotoxicity of the NDV or composition thereof, oncolysate vaccine, whole cell vaccine, or combination therapy. For example, cell proliferation can be assayed by measuring Bromodeoxyuridine (BrdU) incorporation, (³H) thymidine incorporation, by direct cell count, or by detecting changes in transcription, translation or activity of known genes such as proto-oncogenes (*e.g.*, fos, myc) or cell cycle markers (Rb, cdc2, cyclin A, D1, D2, D3, E, etc). The levels of such protein and mRNA and activity can be determined by any method well known in the art. For example, protein can be quantitated by known immunodiagnostic methods such as ELISA, Western blotting or immunoprecipitation using antibodies, including commercially available antibodies. mRNA can be quantitated using methods that are well known and routine in the art, for example, using northern analysis, RNase protection, or polymerase chain reaction in connection with reverse transcription. Cell viability can be assessed by using trypan-blue staining or other cell death or viability markers known in

the art. In a specific embodiment, the level of cellular ATP is measured to determine cell viability. In preferred embodiments, an NDV described herein or composition thereof, oncolysate vaccine, whole cell vaccine, or combination therapy kills cancer cells but does not kill healthy (*i.e.*, non-cancerous) cells. In one embodiment, an NDV described herein or composition thereof, oncolysate vaccine, whole cell vaccine, or combination therapy preferentially kills cancer cells but does not kill healthy (*i.e.*, non-cancerous) cells.

[00226] In specific embodiments, cell viability is measured in three-day and seven-day periods using an assay standard in the art, such as the CellTiter-Glo Assay Kit (Promega) which measures levels of intracellular ATP. A reduction in cellular ATP is indicative of a cytotoxic effect. In another specific embodiment, cell viability can be measured in the neutral red uptake assay. In other embodiments, visual observation for morphological changes may include enlargement, granularity, cells with ragged edges, a filmy appearance, rounding, detachment from the surface of the well, or other changes.

[00227] The NDVs described herein or compositions thereof, oncolysate vaccines, whole cell vaccines or combination therapies can be tested for *in vivo* toxicity in animal models (see, *e.g.*, the animal models described in Section 6 and/or Section 7, below). For example, animal models, described herein and/or others known in the art, used to test the effects of compounds on cancer can also be used to determine the *in vivo* toxicity of the NDVs described herein or compositions thereof, oncolysate vaccines, whole cell vaccines, or combination therapies. For example, animals are administered a range of pfu of an NDV described herein (*e.g.*, a chimeric NDV described in Section 5.2, *infra*). Subsequently, the animals are monitored over time for lethality, weight loss or failure to gain weight, and/or levels of serum markers that may be indicative of tissue damage (*e.g.*, creatine phosphokinase level as an indicator of general tissue damage, level of glutamic oxalic acid transaminase or pyruvic acid transaminase as indicators for possible liver damage). These *in vivo* assays may also be adapted to test the toxicity of various administration mode and/or regimen in addition to dosages.

[00228] The toxicity and/or efficacy of an NDV described herein or a composition thereof, an oncolysate vaccine described herein, a whole cell vaccine described herein, or a combination therapy described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The

dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Therapies that exhibit large therapeutic indices are preferred. While therapies that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such therapies to the site of affected tissue in order to minimize potential damage to noncancerous cells and, thereby, reduce side effects.

[00229] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage of the therapies for use in subjects. The dosage of such agents lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any therapy described herein, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the chimeric NDV that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in subjects. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Anti-Cancer Studies

[00230] The NDVs described herein or compositions thereof, oncolysate vaccines described herein, whole cell vaccines described herein, or combination therapies described herein can be tested for biological activity using animal models for cancer. Such animal model systems include, but are not limited to, rats, mice, chicken, cows, monkeys, pigs, dogs, rabbits, etc. In a specific embodiment, the anti-cancer activity of an NDV described herein or combination therapy is tested in a mouse model system. Such model systems are widely used and well-known to the skilled artisan such as the SCID mouse model or transgenic mice.

[00231] The anti-cancer activity of an NDV described herein or a composition thereof, oncolysate vaccine described herein, whole cell vaccine described herein, or a combination therapy described herein can be determined by administering the NDV or composition thereof, oncolysate vaccine, whole cell vaccine, or combination therapy to an animal model and verifying that the NDV or composition thereof, oncolysate vaccine, whole cell vaccine, or combination therapy is effective in reducing the severity of cancer, reducing the symptoms of cancer, reducing cancer metastasis, and/or reducing the size of a tumor in said animal model (see, e.g.,

Section 6 and/or Section 7, below). Examples of animal models for cancer in general include, include, but are not limited to, spontaneously occurring tumors of companion animals (*see, e.g.,* Vail & MacEwen, 2000, *Cancer Invest* 18(8):781-92). Examples of animal models for lung cancer include, but are not limited to, lung cancer animal models described by Zhang & Roth (1994, *In-vivo* 8(5):755-69) and a transgenic mouse model with disrupted p53 function (*see, e.g.* Morris et al., 1998, *J La State Med Soc* 150(4): 179- 85). An example of an animal model for breast cancer includes, but is not limited to, a transgenic mouse that over expresses cyclin D1 (*see, e.g.,* Hosokawa et al., 2001, *Transgenic Res* 10(5):471-8). An example of an animal model for colon cancer includes, but is not limited to, a TCR b and p53 double knockout mouse (*see, e.g.,* Kado et al., 2001, *Cancer Res.* 61(6):2395-8). Examples of animal models for pancreatic cancer include, but are not limited to, a metastatic model of PancO2 murine pancreatic adenocarcinoma (*see, e.g.,* Wang et al., 2001, *Int. J. Pancreatol.* 29(1):37- 46) and nu-nu mice generated in subcutaneous pancreatic tumors (*see, e.g.,* Ghaneh et al., 2001, *Gene Ther.* 8(3):199-208). Examples of animal models for non-Hodgkin's lymphoma include, but are not limited to, a severe combined immunodeficiency ("SCID") mouse (*see, e.g.,* Bryant et al., 2000, *Lab Invest* 80(4):553-73) and an IgHmu-HOX11 transgenic mouse (*see, e.g.,* Hough et al., 1998, *Proc. Natl. Acad. Sci. USA* 95(23):13853-8). An example of an animal model for esophageal cancer includes, but is not limited to, a mouse transgenic for the human papillomavirus type 16 E7 oncogene (*see, e.g.,* Herber et al., 1996, *J. Virol.* 70(3):1873-81). Examples of animal models for colorectal carcinomas include, but are not limited to, Apc mouse models (*see, e.g.,* Fodde & Smits, 2001, *Trends Mol Med* 7(8):369 73 and Kuraguchi et al., 2000). In a specific embodiment, the animal models for cancer described in Section 6 and/or Section 7, *infra*, are used to assess efficacy of an NDV or composition thereof, an oncolysate, a whole cell vaccine, or a combination therapy.

6. EXAMPLE 1

[00232] This example demonstrates the therapeutic efficacy of NDV therapy in combination with immune checkpoint modulators that are immunostimulatory in the treatment of cancer.

6.1 MATERIALS & METHODS

Mice

[00233] BALB/c mice (6-8 weeks old), and WT C57BL/6 mice were purchased from Jackson Laboratory. All mice were maintained in microisolator cages and treated in accordance with the NIH and American Association of Laboratory Animal Care regulations. All mouse procedures and experiments for this study were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

Cell lines

[00234] The murine cancer cell lines for melanoma (B16-F10), and colon carcinoma (CT26 and MC38) were maintained in RPMI medium supplemented with 10% fetal calf serum and penicillin with streptomycin. The murine prostate cancer cell line TRAMP-C2 was maintained in DMEM medium supplemented with 5% fetal calf serum (FCS; Mediatech, Inc.), 5% Nu Serum IV (BD Biosciences) HEPES, 2-ME, pen/strep, L-glut, 5 µg/mL insulin (Sigma), and 10 nmol/L DHT (Sigma).

Antibodies

[00235] Therapeutic anti-CTLA-4 (clone 9H10), anti-PD-1 (clone RMP1-14), and anti-PD-L1 monoclonal antibodies were produced by BioXcell. Antibodies used for flow cytometry were purchased from eBioscience, Biolegend, Invitrogen, and BD Pharmingen.

Viruses and Cloning

[00236] Recombinant lentogenic NDV LaSota strain was used for all experiments. To generate NDV virus expressing murine ICOSL, a DNA fragment encoding the murine ICOSL flanked by the appropriate NDV-specific RNA transcriptional signals was inserted into the SacII site created between the P and M genes of pT7NDV/LS. Viruses were rescued from cDNA using methods described previously and sequenced by reverse transcription PCR for insert fidelity.

Virus titers were determined by serial dilution and immunofluorescence in Vero cells.

Recombinant ICOSL-F fusion construct was generated by PCR amplification of the ICOSL DNA encoding the extracellular domain (amino acids 1-277) with flanking EcoRI and MluI restriction sites, and the NDV F DNA encoding the F transmembrane and intracellular domains (amino acids 501-554) with flanking MluI and XhoI restriction sites. The resultant DNA fragments were assembled in pCAGGS vector utilizing 3-part ligation.

***In Vitro* Infection Experiments**

[00237] For evaluation of upregulation of surface MHC-I, MHC-II, and ICAM-1 by NDV, and for evaluation of surface expression of the ICOSL transgene from the NDV-ICOSL virus,

B16-F10 cells were infected in 6-well dishes at MOI 2 in triplicate. Twenty-four hours later, the cells were harvested by mechanical scraping and processed for surface labeling and quantification by flow cytometry. For virus growth curve experiments, B16-F10 cells were incubated at room temperature with the virus in 6-well culture dishes at the indicated MOIs in a total volume of 100 μ l. One hour after the incubation, the infection media was aspirated and the cells were incubated at 37° C in 1 ml of DMEM with 10% chick allantoic fluid. After 24, 48, and 72 hours, the supernatants were collected and virus titers were determined as above. For in vitro cytotoxicity experiments, the infections were carried out in a similar fashion. At 24, 48, 72, and 96 hours post infection the cells were washed and incubated with 1% Triton X-100 at 37°C for 30 minutes. LDH activity in the lysates was determined using the Promega CytoTox 96 assay kit, according to the manufacturer's instructions.

Tumor Challenge Survival Experiments.

[00238] Bilateral flank tumor models were established to monitor for therapeutic efficacy in both injected and systemic tumors. Treatment schedules and cell doses were established for each tumor model to achieve 10-20% tumor clearance by NDV or anti-CTLA-4/anti-PD-1 as single agents. For experiments evaluating combination therapy of wild-type NDV (NDV-WT) with immune checkpoint blockade, B16F10 tumors were implanted by injection of 2×10^5 B16F10 cells in the right flank i.d. on day 0 and 5×10^4 cells in the left flank on day 4. On days 7, 10, 13, and 16 the mice were treated with 4 intratumoral injections of 2×10^7 pfu of NDV in PBS in a total volume of 100 μ l. Concurrently, on days 7, 10, 13, and 16 the mice received 4 i.p. injections of anti-CTLA-4 antibody (100 μ g) or anti-PD-1 antibody (250 μ g). Control groups received a corresponding dose of isotype antibody i.p. and intratumoral injection of PBS. Tumor size and incidence were monitored over time by measurement with a caliper.

[00239] For the TRAMP-C2 model, 5×10^5 cells were implanted in right flank on day 0 and 5×10^5 cells were implanted in the left flank on day 8. Treatment was performed on days 11, 14, 17, and 20 in the similar fashion to above.

[00240] For experiments evaluating recombinant NDV expressing ICOSL (NDV-ICOSL), B16F10 tumors were implanted by injection of 2×10^5 B16F10 cells in the right flank i.d. on day 0 and 1×10^5 cells in the left flank on day 4. Treatment was carried out as above.

[00241] For the CT26 model, tumors were implanted by injection of 1×10^6 CT26 cells in the right flank i.d. on day 0 and 1×10^6 cells in the left flank on day 2. Treatment was carried out as above on days 6, 9, and 12.

Isolation of Tumor-Infiltrating Lymphocytes

[00242] B16F10 tumors were implanted by injection of 2×10^5 B16F10 cells in the right flank i.d. on day 0 and 2×10^5 cells in the left flank on day 4. On days 7, 10, and 13 the mice were treated with 3 intratumoral injections of 2×10^7 pfu of NDV, and 100 μ g of i.p. anti-CTLA-4 antibody or 250 μ g of i.p. anti-PD-1 antibody, where specified. On day 15, mice were sacrificed by CO₂ inhalation. Tumors and tumor-draining lymph nodes were removed using forceps and surgical scissors and weighed. Tumors from each group were minced with scissors prior to incubation with 1.67 Wünsch U/mL Liberase and 0.2 mg/mL DNase for 30 minutes at 37° C. Tumors were homogenized by repeated pipetting and filtered through a 70- μ m nylon filter. Cell suspensions were washed once with complete RPMI and purified on a Ficoll gradient to eliminate dead cells. Cells from tumor draining lymph nodes were isolated by grinding the lymph nodes through a 70- μ m nylon filter.

Flow Cytometry

[00243] Cells isolated from tumors or tumor-draining lymph nodes were processed for surface labeling with several antibody panels staining CD45, CD3, CD4, CD8, CD44, PD-1, ICOS, CD11c, CD19, NK1.1, CD11b, F4/80, Ly6C and Ly6G. Fixable viability dye eFluor780 (eBioscience) was used to distinguish the live cells. Cells were further permeabilized using FoxP3 fixation and permeabilization kit (eBioscience) and stained for Ki-67, FoxP3, Granzyme B, CTLA-4, and IFN gamma. Data was acquired using the LSRII Flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

DC Purification and Loading

[00244] Spleens from naïve mice were isolated and digested with 1.67 Wünsch U/mL Liberase and 0.2 mg/mL DNase for 30 minutes at 37° C. The resulting cell suspensions were filtered through 70um nylon filter and washed once with complete RPMI. CD11c+ dendritic cells were purified by positive selection using Miltenyi magnetic beads. Isolated dendritic cells were cultured overnight with recombinant GM-CSF and B16-F10 tumor lysates and were purified on Ficoll gradient.

Analysis of Cytokine Production

Cell suspensions from tumors or tumor-draining lymph nodes were pooled and enriched for T cells using a Miltenyi T-cell purification kit. Isolated T cells were counted and co-cultured for 8 hours with dendritic cells loaded with B16-F10 tumor cell lysates in the presence of 20 U/ml IL-2 (R and D) plus Brefeldin A (BD Bioscience). After restimulation, lymphocytes were processed for flow cytometry as above.

Statistics.

[00245] Data were analyzed by 2-tailed Student's t test, and $P < 0.05$ was considered statistically significant.

6.2 RESULTS

[00246] In order to characterize the anti-tumor immune response induced by Newcastle disease virus (NDV) infection, the expression of MHC I and MHC II molecules as well as ICAM-1 on the surface of *in vitro* infected cells was assessed. . As shown in Figure 1, NDV infection in B16 melanoma cells induces upregulation of MHC class I and II molecules as well as adhesion molecule ICAM-1, all of which are thought to be important for recruitment of tumor-specific lymphocytes and activation of anti-tumor immune response. Next, the anti-tumor immune response induced by NDV infection *in vivo* was assessed in a murine melanoma model and an established 2-flank model that allowed for monitoring of responses both in the virus-injected tumors as well as distant tumors which do not receive the virus. As shown in Figure 2, the virus-infected tumors show dramatic infiltration with immune cells such as NK cells, macrophages, and CD8 and CD4 cells, but not regulatory T cells. Since part of this immune response could be a response to virus, rather than tumor, the immune response with respect to contralateral tumors was assessed (Figure 3). Interestingly, these tumors demonstrated a similar degree of increased CD8 and CD4 effector, but not T reg infiltrate. Analysis of these cells revealed that they upregulate activation, proliferation, and lytic markers (Figure 4). NDV monotherapy was effective in controlling the treated tumors (Figure 5A), but only marginally slowed down the growth of the contralateral tumors (Figure 5B). Mice that cleared the tumors, however, demonstrated some degree of protection against further tumor challenge (Figure 5D), suggesting that NDV therapy can induce a lasting immunity.

[00247] Next, it was assessed whether additional mechanisms could be targeted to enhance the anti-tumor effect generated by NDV. Characterization of tumor-infiltrating lymphocytes from both NDV-injected and non-injected tumors revealed upregulation of the inhibitory

receptor CTLA-4 on lymphocytes (Figure 6). It was then assessed whether inhibition of the CTLA-4 receptor could result in a better therapeutic efficacy of NDV. Strikingly, combination therapy resulted in rejection in bilateral tumors in the majority of the animals, an effect that was not seen with either treatment alone (Figure 7). This effect was present even when the prostate adenocarcinoma TRAMP model was used, which is not susceptible to viral infection (Figure 8), suggesting that the minimal viral replication and the resultant inflammatory response were sufficient for generation of protective anti-tumor immunity.

[00248] To determine whether targeting other immune checkpoints in combination with NDV therapy could be beneficial, the effect on the PD-1 - PD-L1 pathway following NDV infection was assessed. As shown in Figure 9, NDV infected tumor cells both *in vitro* and *in vivo* had upregulated the expression of the inhibitory PD-L1 ligand on the surface of the cells. This effect was not just a result of a direct virus infection, but was also seen when non-infected cells were treated with UV-inactivated supernatants from the virus infected cells (Figure 9B) and in contralateral, noninfected, tumors (Figure 9C). This prompted testing combination therapy with NDV and anti-PD-1 antibody. Similar to CTLA-4 blockade, NDV therapy in combination with anti-PD-1 in the aggressive B16 melanoma model resulted in cures in the majority of animals, an effect that was associated with increased tumor infiltration with activated effector lymphocytes (Figure 10).

[00249] Throughout the studies conducted, the therapeutic efficacy of a combination therapy decreased when larger tumor challenge was used. Next, activation markers that could predict a better response and could be targeted for further improvement in therapeutic efficacy were assessed. Analysis of lymphocytes isolated from the tumors and tumor-draining lymph nodes identified upregulation of the co-stimulatory molecule ICOS as one of the activation markers in the treated animals (Figure 11). ICOS upregulation has been previously been shown to be associated with more durable therapeutic responses and increased survival in patients treated with anti-CTLA-4 therapy for malignant melanoma. It was assessed whether intratumoral expression of the ICOS ligand (ICOSL) could further boost the therapeutic response of combination therapy. Using reverse-genetics system for NDV, NDV expressing murine ICOSL (NDV-ICOSL) were generated. *In vitro* characterization of the virus revealed that it had similar replicative and lytic properties to the parental NDV strain (Figure 12). When tested *in vivo*, however, with a larger B16 tumor challenge, NDV-ICOSL demonstrated significant advantage

over the parental NDV virus when used in combination with CTLA-4 blockade, with long-term survival in the majority of treated animals (Figure 13). This effect was not limited to B16 melanoma and was demonstrated for CT26 colon carcinoma in the Balb/C mouse strain, suggesting that this therapeutic strategy could be translatable to different tumor types (Figure 14). Analysis of B16 tumors from the treated animals demonstrated significant infiltration with different immune cell subtypes with upregulation of the activation markers (Figures 15 and 16). These lymphocytes were tumor-specific and demonstrated secretion of IFN gamma in response to stimulation with dendritic cells loaded with tumor lysates (Figure 17). Finally, animals that were cured of their B16 or CT26 tumors were re-challenged with tumor cells and demonstrated complete protection against tumor re-challenge (Figure 18).

[00250] To further improve the expression of the ICOSL in the tumor and to incorporate the ligand into the virion, a chimeric protein consisting of the extracellular domain of the ICOSL (amino acids 1-277) and the transmembrane and intracellular domains of the NDV F protein (amino acids 501-554) was generated (Figure 19A). Transfection of the resultant construct into B16-F10 cells resulted in increased expression of the chimeric ICOSL-F ligand on the surface of the transfected cells, when compared to the transfected native ICOSL, suggesting that the regulatory mechanisms governing the transport of NDV F protein to the surface can be utilized to increase the surface expression of immune stimulatory ligands (Figure 19B).

[00251] Overall, these studies demonstrate that 1) combination of NDV with immune checkpoint regulatory antibodies can be used as a strategy to circumvent the limitation of both oncolytic virus therapy and antibody therapy; and 2) expression of immunostimulatory ligands by NDV can further improve the therapeutic efficacy of the virus, especially when used in combination with immunoregulatory antibodies. These findings have clinical application.

7. EXAMPLE 2

[00252] This example demonstrates the anti-tumor immune responses induced by oncolytic NDV and the anti-tumor responses induced by NDV in combination with CTLA-4 blockade.

7.1 MATERIALS & METHODS

Mice

[00253] C57BL/6J and Balb/C mice were purchased from Jackson Laboratory. IFNAR^{-/-} mice on C57BL/6J background were a kind gift of Dr. Eric Pamer. Pmel-1 and Trp-1 TCR transgenic mice have been reported (Overwijk et al., 2003, J. Exp. Med, 198:568, Muransky et al., 2008, Blood 112:362) and were provided by N. Restifo (National Cancer Institute, Bethesda, MD). Trp1 mice were crossed to CD2:luciferase mice provided by Patrick Hwu at MD Anderson Cancer Center (Houston, TX) to create Trp1 Luciferase⁺ (Trp1-Fluc) mice. All mice were maintained in microisolator cages and treated in accordance with the NIH and American Association of Laboratory Animal Care regulations. All mouse procedures and experiments for this study were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

Cell lines

[00254] The murine cancer cell lines for melanoma (B16-F10), and colon carcinoma (CT26 and MC38) were maintained in RPMI medium supplemented with 10% fetal calf serum and penicillin with streptomycin. The murine prostate cancer cell line TRAMP-C2 was maintained in DMEM medium supplemented with 5% fetal calf serum (FCS; Mediatech, Inc.), 5% Nu Serum IV (BD Biosciences) HEPES, 2-ME, pen/strep, L-glut, 5 µg/mL insulin (Sigma), and 10 nmol/L DHT (Sigma).

Antibodies

[00255] Therapeutic anti-CTLA-4 (clone 9H10), anti-PD-1 (clone RMP1-14), anti-PD-L1 (clone 9G2), anti-CD8 (clone 2.43), anti-CD4 (clone GK1.5), anti-IFN-gamma (clone XMG1.2), and anti-NK1.1 (clone PK136) monoclonal antibodies were produced by BioXcell. Antibodies used for flow cytometry were purchased from eBioscience, Biolegend, Invitrogen, and BD Pharmingen.

Viruses and cloning

[00256] Recombinant lentogenic NDV LaSota strain was used for all experiments. To generate NDV virus expressing murine ICOSL, a DNA fragment encoding the murine ICOSL flanked by the appropriate NDV-specific RNA transcriptional signals was inserted into the SacII site created between the P and M genes of pT7NDV/LS. Viruses were rescued from cDNA using methods described previously and sequenced by reverse transcription PCR for insert fidelity. Virus titers were determined by serial dilution and immunofluorescence in Vero cells. Recombinant ICOSL-F fusion construct was generated by PCR amplification of the ICOSL

DNA encoding the extracellular domain (amino acids 1-277) with flanking EcoRI and MluI restriction sites, and the NDV F DNA encoding the F transmembrane and intracellular domains (amino acids 501-554) with flanking MluI and XhoI restriction sites. The resultant DNA fragments were assembled in pCAGGS vector utilizing 3-part ligation. Recombinant anti-mouse CD28scfv-F fusion construct was generated by PCR amplification of the cDNA encoding hamster anti-CD28scfv with flanking EcoRI and MluI restriction sites, and the NDV F DNA encoding the F transmembrane and intracellular domains (amino acids 501-554) with flanking MluI and XhoI restriction sites. The resultant DNA fragments were assembled in pCAGGS vector utilizing 3-part ligation and then subcloned into pNDV vector between the P and M genes. To generate recombinant viruses expressing other chimeric proteins (HN-GITRL, HN-4-1BBL, HN-CD40L, HN-OX40L), cDNA encoding extracellular domain of each gene (Figure 44) was amplified with gene-specific primers with flanking EcoRI and MluI restriction sites, and the transmembrane and intracellular domain of HN protein was amplified with specific primers with flanking MluI and XhoI restriction sites. The full chimeric genes were assembled in pCAGGS vector using 3-part ligation and then subcloned into NDV vector between the P and M genes. The details of each chimeric construct are demonstrated in Figure 44. To generate recombinant NDV encoding murine IL-2, IL-15, and IL-21, the cDNA for each gene was amplified with gene-specific primers with flanking SacII restriction sites and then cloned into pNDV between the P and M genes. Viruses were rescued from cDNA using methods described previously and sequenced by reverse transcription PCR for insert fidelity. Virus titers were determined by serial dilution and immunofluorescence in Vero cells.

***In vitro* infection experiments**

[00257] For cell surface labeling, cells were infected in 6-well dishes at MOI 2 (B16-F10) or MOI 5 (TRAMP C2) in triplicate. Twenty-four hours later, the cells were harvested by scraping and processed for surface labeling and quantification by flow cytometry. For *in vitro* cytotoxicity experiments, cells were infected at the indicated MOI's and incubated at 37°C in serum-free media in presence of 250ng/ml TPCK trypsin. At 24, 48, 72, and 96 hours post infection the cells were washed and incubated with 1% Triton X-100 at 37°C for 30 minutes. LDH activity in the lysates was determined using the Promega CytoTox 96 assay kit, according to the manufacturer's instructions.

Tumor challenge survival experiments

[00258] Bilateral flank tumor models were established to monitor for therapeutic efficacy in both injected and systemic tumors. Treatment schedules and cell doses were established for each tumor model to achieve 10-20% tumor clearance by NDV or anti-CTLA-4 as single agents. For experiments evaluating combination therapy of NDV with anti-CTLA-4 antibody, B16-F10 tumors were implanted by injection of 2×10^5 B16-F10 cells in the right flank intradermally (i.d.) on day 0 and 5×10^4 cells in the left flank on day 4. On days 7, 9, 11, and 13 the mice were treated with intratumoral injections of 2×10^7 pfu of NDV in PBS in a total volume of 100 μ l. Concurrently, on days 7, 9, 11, and 13 the mice received intraperitoneal (i.p.) injections of anti-CTLA-4 antibody (100 μ g), anti-PD-1 antibody (250 μ g), or anti-PD-L1 antibody (250 μ g). Control groups received a corresponding dose of isotype antibody i.p. and intratumoral injection of PBS. The animals were euthanized for signs of distress or when the total tumor volume reached 1000 mm³. For depletion of immune cells, mice were injected i.p. with 500 μ g of monoclonal antibodies to CD8+, CD4+, NK1.1 or IFN γ one day before and two days after tumor challenge, followed by injection of 250 μ g every 5 days throughout the experiment. For the TRAMP-C2 model, 1×10^6 cells were implanted in the right flank on day 0 and 5×10^5 cells were implanted in the left flank on day 4. Treatment was performed on days 7, 10, 13, and 16 in the similar fashion to above. For the CT26 model, tumors were implanted by injection of 1×10^6 CT26 cells in the right flank i.d. on day 0 and 1×10^6 cells in the left flank on day 2. Treatment was carried out as above on days 6, 9, and 12. For experiments evaluating recombinant NDV expressing ICOSL, 4-1BBL, OX40L, CD40L, GITRL, anti-CD28scfv, IL-2, IL-15, and IL-21 (NDV-transgene), B16F10 tumors are implanted by injection of 2×10^5 B16F10 cells in the right flank i.d. on day 0 and 1×10^5 cells in the left flank on day 4. On days 7, 9, 11, and 13 the mice are treated with intratumoral injections of 2×10^7 pfu of NDV in PBS in a total volume of 100 μ l. Concurrently, on days 7, 9, 11, and 13 the mice receive intraperitoneal (i.p.) injections of anti-CTLA-4 antibody (100 μ g), anti-PD-1 antibody (250 μ g), or anti-PD-L1 antibody (250 μ g).

Isolation of Trp1 and Pmel lymphocytes and adoptive transfer

[00259] Spleens and lymph nodes from transgenic mice were isolated and grinded through 70- μ m nylon filters. CD4+ and CD8+ cells were purified by positive selection using Miltenyi magnetic beads.

[00260] The isolated Trp1 or Pmel cells were injected into recipient animals via the tail vein at the indicated schedule at 2.5×10^4 cells per mouse and 1×10^6 cells per mouse, respectively.

Serum transfer experiments

[00261] Groups of tumor-bearing mice were treated intratumorally with single injection of NDV or PBS. On day 4, blood was collected by terminal bleeding and serum was isolated by centrifugation. Sera were pooled from each group and UV-treated in Stratalinker 1800 with six pulses of 300mJ/cm² UV light to inactivate any virus that could be potentially present.

Undiluted 100µl of serum was injected intratumorally into naïve B16-F10 tumor-bearing mice for a total of 3 injections given every other day. Tumors were removed 3 days after the last injection and processed for isolation of tumor-infiltrating lymphocytes as described below.

Bioluminescence imaging

[00262] Mice were imaged every 2-3 days starting on day 6. Mice were injected retro-orbitally with 50 µl of 40 mg/ml D-luciferin (Caliper Life Sciences) in PBS and imaged immediately using the IVIS Imaging System (Caliper Life Sciences). Gray-scale photographic images and bioluminescence color images were superimposed using The Living Image, version 4.0 (Caliper Life Sciences) software overlay. A region of interest (ROI) was manually selected over the tumor and the area of the ROI was kept constant.

Isolation of tumor-infiltrating lymphocytes

[00263] B16-F10 tumors were implanted by injection of 2x10⁵ B16-F10 cells in the right flank i.d. on day 0 and 2x10⁵ cells in the left flank on day 4. On days 7, 9, and 11 the mice were treated with intratumoral injections of 2x10⁷ pfu of NDV, and i.p. anti-CTLA-4 or anti-PD-1 antibody where specified. Rare animals that died from tumor burden (always in untreated control groups) or animals that completely cleared the tumors (always in treatment groups) were not used for the analysis. On day 15, mice were sacrificed and tumors and tumor-draining lymph nodes were removed using forceps and surgical scissors and weighed. Tumors from each group were minced with scissors prior to incubation with 1.67 Wünsch U/mL Liberase and 0.2 mg/mL DNase for 30 minutes at 37°C. Tumors were homogenized by repeated pipetting and filtered through a 70-µm nylon filter. Cell suspensions were washed once with complete RPMI and purified on a Ficoll gradient to eliminate dead cells. Cells from tumor draining lymph nodes were isolated by grinding the lymph nodes through a 70- µm nylon filter.

Flow cytometry

[00264] Cells isolated from tumors or tumor-draining lymph nodes were processed for surface labeling with several antibody panels staining for CD45, CD3, CD4, CD8, CD44, ICOS, CD11c,

CD19, NK1.1, CD11b, F4/80, Ly6C and Ly6G. Fixable viability dye eFluor506 (eBioscience) was used to distinguish the live cells. Cells were further permeabilized using FoxP3 fixation and permeabilization kit (eBioscience) and stained for Ki-67, FoxP3, Granzyme B, CTLA-4, and IFN γ . Data was acquired using the LSRII Flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

DC purification and loading

[00265] Spleens from naïve mice were isolated and digested with 1.67 Wunsch U/mL Liberase and 0.2 mg/mL DNase for 30 minutes at 37°C. The resulting cell suspensions were filtered through 70um nylon filter and washed once with complete RPMI. CD11c+ DC's were purified by positive selection using Miltenyi magnetic beads. Isolated DC's were cultured overnight with recombinant GM-CSF and B16-F10 tumor lysates and were purified on Ficoll gradient.

Analysis of cytokine production

[00266] Cell suspensions from tumors or tumor-draining lymph nodes were pooled and enriched for T cells using a Miltenyi T-cell purification kit. Isolated T cells were counted and co-cultured for 8 hours with DC's loaded with B16-F10 tumor cell lysates in the presence of 20 U/ml IL-2 (R and D) plus Brefeldin A (BD Bioscience). After restimulation, lymphocytes were processed for flow cytometry as above.

Immunofluorescence and microscopy

[00267] Tumors were dissected from the mice, washed in PBS, fixed in 4% paraformaldehyde, and processed for paraffin embedding according to protocols described previously. Sections were cut using a microtome, mounted on slides, and processed for staining with hematoxylin and eosin (H&E) or with anti-CD3 and anti-FoxP3 antibody. Slides were analyzed on Zeiss Axio 2 wide-field microscope using 10x and 20x objectives.

Statistics

[00268] Data were analyzed by 2-tailed Student's t test (for comparisons of 2 groups) and ANOVA where appropriate. Data for survival were analyzed by Log-Rank (Mantel-Cox) Test. Two-sided $p < 0.05$ was considered statistically significant ($P \leq 0.05$ (*), $P \leq 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****)).

7.2 RESULTS

NDV replication is restricted to the injected tumor site

[00269] The viral distribution kinetics with intratumoral and systemic administration of NDV were characterized. Intratumoral injection of recombinant NDV expressing firefly luciferase reporter (NDV-Fluc) resulted in sustained luciferase signal in the injected flank tumor, while systemic administration of the virus resulted in no detectable luciferase signal in the tumor (Figure 20A). As limited systemic virus delivery was unlikely to induce sufficient tumor lysis and immune response, the intratumoral NDV injection was explored as a means to elicit an anti-tumor immune response that could potentially overcome the limitations of systemic OV therapy. As such, for further studies modeled metastatic disease was modeled by using the bilateral flank B16-F10 tumor model (Figure 22A). NDV-Fluc administration into the right flank tumor resulted in viral replication within the injected tumor, with the luciferase signal detectable for up to 96 hours (Figure 20B-D). No virus was detected in the contralateral (left flank) tumor by luminescent imaging (Figure 20B-D), by passage in embryonated eggs, or RT-PCR. This system thus allowed for the characterization of the immune responses in both virus-injected and distant tumors, which were not directly affected by NDV.

NDV therapy increases local and distant tumor lymphocyte infiltration and delays tumor growth

[00270] Analysis of the virus-injected tumors revealed an inflammatory response as evidenced by increased infiltration with cells expressing leukocyte common antigen CD45 (Figures 21A-B). The immune infiltrates were characterized by increase in innate immune compartment, including myeloid cells, NK cells, and NKT cells (Figure 21C), and the adaptive compartment, including CD8⁺ and conventional CD4⁺FoxP3⁻ (Tconv) T cells, leading to significant increase of CD8 and Tconv to regulatory (Treg) T cell ratios ($p=0.0131$ and $p=0.0006$, respectively) (Figures 21D-21F). Remarkably, analysis of the contralateral tumors revealed a similar increase in the inflammatory infiltrates (Figure 22B,C), characterized by increased numbers of both innate immune cells (Figure 22D) and effector T cells (Figure 22E,G). Notably, although there were no major changes in the absolute number of Tregs (Figure 22G), there was a substantial decrease in their relative percentages (Figure 22E,F,H), with significant enhancement of the CD8 and Tconv to Treg ratios ($p=0.002$ and $p=0.0021$, respectively) (Figure 22I). Effector T cells isolated from the distal tumors expressed increased activation, proliferation, and lytic markers ICOS, Ki-67, and Granzyme B, respectively (Figure 1J,K). As previously, virus or viral RNA was unable to be isolated from the distant tumors, suggesting that the observed changes in the distant tumor

microenvironment were not due to direct viral infection. In order to further exclude the possibility of undetectable local viral spread, tumors were implanted at other distant sites, such as bilateral posterior footpads, which generated similar findings (Figure 23).

[00271] Consistent with the observed inflammatory effect, intratumoral administration of NDV resulted in growth delay not only of the injected, but also of the contralateral tumors, resulting in prolonged animal survival (Figure 1L,M). To determine whether this effect was transient and whether durable anti-tumor protection was possible, single-flank B16-F10 tumor-bearing mice were intratumorally treated with NDV, and long-term survivors were injected with B16-F10 cells on the opposite flank. The majority of the animals demonstrated tumor growth delay, and 30% of the animals completely rejected rechallenged cells, suggesting that intratumoral therapy with NDV can indeed induce protective anti-tumor memory responses (Figure 25).

NDV induces tumor infiltration and expansion of tumor-specific lymphocytes

[00272] To determine whether the anti-tumor immune response was dependent on the NDV-injected tumor type or a result of nonspecific inflammation generated by NDV infection, the experiment was performed with heterologous tumors (MC38 colon carcinoma and B16-F10 melanoma) implanted at the opposite flanks (Figure 24A). To track tumor-specific lymphocytes, T cell receptor-transgenic congenically-marked CD8⁺ (Pmel) cells or luciferase-marked CD4⁺ (Trp1) cells recognizing the melanoma differentiation antigens gp100 (Pmel) and Trp1 (Trp1) were adoptively transferred (Muranski et al., 2008, Blood, 112: 362; Overwijk et al., 2003, J Exp Med, 198: 569). Bioluminescent imaging was used to measure the distribution and expansion kinetics of the adoptively transferred Trp1 cells. Transfer of Trp1 cells into PBS-treated tumor-bearing animals failed to result in Trp1 accumulation in the tumors, highlighting the highly immunosuppressive nature of the tumor microenvironment in this model (Figure 24B-D). NDV injection into B16-F10 tumors resulted in significant increase in the luciferase signal within the injected tumors (Figure 24B-D), indicating Trp1 T cell expansion (area under the curve (AUC) $p=0.0084$). Remarkably, similar expansion was seen in the contralateral tumor, albeit at a delay ($p=0.0009$) (Figure 24B-D). In contrast, NDV injection into MC38 tumors failed to induce substantial Trp1 infiltration into the injected MC38 tumors or distant B16-F10 tumors (Figure 24B-D), suggesting that the distant tumor-specific lymphocyte infiltration is likely dependent on the antigen identity of the injected tumor. Similarly, intratumoral injection of NDV resulted in

increased infiltration of Pmel cells in distant tumors, which was more pronounced when the injected tumor was B16-F10 rather than MC38 (Figure 24E).

[00273] Interestingly, although infiltration of distant B16-F10 tumors with adoptively-transferred lymphocytes was dependent on the injected tumor identity, distant tumors did demonstrate increased immune infiltration even when the primary injected tumor was MC38 (Figure 24F), suggesting that a nonspecific inflammatory response component may also play a role. Indeed, serum from NDV-treated animals, treated with UV irradiation to inactivate any potential virus, induced tumor leukocyte infiltration when injected intratumorally into naïve B16-F10 tumor-bearing mice (Figure 24G,H), with the majority of the increase seen in the NK and CD8+ compartments ($p=0.0089$ and $p=0.0443$, respectively) (Figure 24I).

NDV and CTLA-4 blockade synergize to reject local and distant tumors

[00274] Despite the prominent inflammatory response and growth delay seen in distant tumors, complete contralateral tumor rejection with long-term survival was only seen in approximately 10% of animals (Figure 22M), suggestive of active immunosuppressive mechanisms in the tumor microenvironment. Characterization of NDV-injected and distant tumors revealed upregulation of CTLA-4 on tumor-infiltrating T cells (Figure 26), suggesting that NDV-induced tumor inflammation would make the tumors sensitive to systemic therapy with CTLA-4 blockade. Remarkably, combination therapy of NDV with anti-CTLA-4 antibody (Figure 27A) resulted in rejection of bilateral tumors and long-term survival in the majority of the animals, an effect that was not seen with either treatment alone (Figure 27B-D). To determine the durability of the observed protection, the surviving animals were injected in the right flank on day 90 with B16-F10 cells without any further therapy. Animals treated with NDV and anti-CTLA-4 combination therapy demonstrated over 80% protection against tumor re-challenge, compared with 40% protection in the animals treated with single agent anti-CTLA-4 antibody (Figure 27E).

Combination therapy with NDV and CTLA-4 blockade is effective against virus non-permissive tumors

[00275] To determine whether this treatment strategy could be extended to other tumor types, the strategy was evaluated in the poorly-immunogenic TRAMP C2 prostate adenocarcinoma model. Similarly to the B16-F10 model, combination therapy caused regression of the injected tumors (Figure 27F), and either delayed the outgrowth of distant tumors or led to complete

distant tumor regression with prolonged long-term survival (Figure 27F,G). Interestingly, whereas B16-F10 cells were susceptible to NDV-mediated lysis in vitro, TRAMP C2 cells were strongly resistant, with low cytotoxicity observed at a multiplicity of infection (MOI) of up to 10 (Figure 27H). In both cell lines, NDV infection in vitro resulted in surface upregulation of MHC and co-stimulatory molecules (Figure 27I-K). MHC class I was upregulated uniformly in all cells, even though not all cells get infected with NDV at the MOI of 1. Previous studies demonstrated that NDV induces type I IFN expression in B16-F10 cells (Zamarin et al., 2009, Mol Ther 17:697). Both type I IFN (Dezfouli et al., 2003, Immunol. Cell. Biol., 81:459, Seliger et al., 2001, Cancer Res., 61:1095) are known to upregulate MHC class I on B16-F10 cells, suggesting that within the context of the infected tumors these mechanisms may play an additional role in enhancement of tumor immunogenicity. These results thus suggest that in vitro sensitivity to virus-mediated lysis is not necessary for sensitivity to NDV therapy in vivo and further highlight the importance of a virus-generated inflammatory response, rather than direct oncolysis, in the observed anti-tumor efficacy.

Systemic anti-tumor effect is antigen-restricted to the injected tumor type

[00276] To determine whether the observed anti-tumor effect in the distant tumor was specific to the injected tumor type, the combination therapy in animals bearing a unilateral distant B16-F10 tumor and in animals with heterologous tumor types (MC38 colon carcinoma and B16-F10 melanoma) implanted at the opposite flanks was evaluated (Figure 28A). Although administration of the virus intradermally into the non-tumor-bearing right flank resulted in delayed left flank tumor outgrowth, it failed to result in long-term protection and tumor rejection seen in the animals bearing bilateral B16-F10 tumors (Figure 28B,C). Similarly, injection of NDV into the right flank MC38 tumors of the animals bearing left flank B16-F10 tumors failed to induce B16-F10 tumor rejection (Figure 28D,E), suggesting that the NDV-induced anti-tumor immune response is likely antigen-restricted to the injected tumor.

Combination therapy with NDV and anti-CTLA-4 induces tumor infiltration with activated lymphocytes

[00277] To examine the B16-F10 tumor microenvironment in the treated animals, bilateral tumors were collected and processed for analysis of infiltrating cells. Analysis of the injected and distant tumors from the treated animals revealed prominent inflammatory infiltrates and large areas of tumor necrosis in the animals treated with combination therapy (Figure 30A,

Figure 29). This correlated with increased numbers of CD45+ cells and T cells in the combination therapy group (Figure 30A-C, Figure 29A-C). As previously, the observed increase in TILs was primarily due to infiltration of CD8+ and Tconv, but not Treg cells, leading to enhanced effector to Treg ratios (Figure 30D-F, Figure 29C-E). Phenotypic characterization of CD4+ and CD8+ TILs from animals receiving the combination treatment demonstrated upregulation of ICOS, Granzyme B, and Ki-67 over the untreated and anti-CTLA-4 treated animals (Figure 30G-I) and a larger percentage of IFN γ -expressing CD8+ cells in response to re-stimulation with dendritic cells (DCs) pulsed with B16-F10 tumor lysates (Figure 30J).

Anti-tumor activity of NDV combination therapy depends on CD8+ cells, NK cells and type I and II interferons

[00278] To determine which components of cellular immunity were responsible for the observed therapeutic effect, the treatment was repeated in the presence of depleting antibodies for CD4+, CD8+, or NK cells. Adequate cell depletion of each cell subset was confirmed by flow cytometry of peripheral blood (Figure 31). Depletion of either CD8+ or NK cells resulted in abrogation of therapeutic effect in both virus-injected and distant tumors (Figure 32A,B), with significant reduction in long-term survival ($p < 0.0001$ for CD8 and $p = 0.0011$ for NK depletion) (Figure 32C). Consistent with these findings, treatment of the animals with an anti-IFN γ neutralizing antibody also decreased therapeutic efficacy. In contrast, depletion of CD4+ cells did not result in appreciable change in anti-tumor effect, though these results must be interpreted with caution since anti-CD4+ depletion also results in concurrent depletion of Tregs.

[00279] Type I IFN has been previously demonstrated to play an important role in priming of CD8+ cells for anti-tumor immune response (Fuentes et al., 2011, J Exp Med, 208: 2005; Diamond et al, 2011, J Exp Med, 208: 1989). To investigate the role of type I IFN in tumor rejection by NDV, the experiments were repeated in the type I IFN receptor knockout (IFNAR $^{-/-}$) mice. The IFNAR $^{-/-}$ mice demonstrated rapid progression of both injected and contralateral tumors and were completely resistant to the combination therapy (Figure 32D-F). Overall, these findings highlight the important role of both innate and adaptive immune responses for the systemic therapeutic efficacy of the virus observed in this study.

NDV therapy leads to upregulation of PD-L1 on tumor cells and on tumor infiltrating leukocytes

[00280] To determine whether targeting other immune checkpoints in combination with NDV therapy could be beneficial, the effect on the PD-1 - PD-L1 pathway following NDV infection was assessed. As shown in Figure 33, NDV infected tumor cells both in vitro and in vivo had upregulated the expression of the inhibitory PD-L1 ligand on the surface of the cells (Figure 33A), which was also seen in the distant, noninfected, tumors. The upregulation of PD-L1 was not just restricted to tumor cells, but was also seen on tumor infiltrating leukocytes of both innate and adaptive immune lineages (Figure 33B).

Combination therapy of NDV with PD-1 and PD-L1-blocking antibodies leads to improved anti-tumor immunity and long-term animal survival

[00281] The combination of NDV with antibody blocking PD-1 and the combination of NDV with antibody blocking PD-L1 were evaluated in the bilateral flank melanoma model described above. Remarkably, similar to CTLA-4 blockade, NDV therapy in combination with either anti-PD-1 or anti-PD-L1 antibody led to improved animal survival (Figures 34 and 35). Distant tumors from animals treated with combination of NDV and anti-PD-1 antibody were characterized. As can be seen from Figure 36, combination of intratumoral NDV with systemic PD-1 blockade led to marked distant tumor infiltration with immune cells, with the increase in tumor-infiltrating CD8 cells being the most pronounced finding. The infiltrating cells upregulated proliferation and lytic markers Ki67 and granzyme B, respectively (Figure 37).

NDV induces tumor immune infiltration upregulation of ICOS on CD4 and CD8 cells in the virus-injected and distant tumors and tumor draining lymph nodes (TDLN)

[00282] The findings above demonstrated that combination of intratumoral NDV with systemic immune checkpoint blockade results in significant synergy between the two therapeutic approaches. To further build on these findings, enhancement of T cell effector function within the tumor microenvironment through a relevant co-stimulatory pathway may drive a better anti-tumor immune response was investigated. Previous studies identified the sustained upregulation of inducible costimulator (ICOS) on T cells as a strong indicator of response to CTLA-4 blockade in patients (Carthon et al., 2010, Clin. Canc. Res., 16:2861). ICOS is a CD28 homologue upregulated on the surface of activated T cells that has been shown to be critical for T cell-dependent B lymphocyte responses and development of all T helper subsets (Simpson et al., 2010 Curr Opin Immunol. 22:326). The role of ICOS in anti-tumor efficacy of CTLA-4 blockade was recently confirmed by mouse studies, where ICOS-deficient mice were severely

compromised in development of anti-tumor response with CTLA-4 blockade (Fu et al., 2011, Cancer Res., 71:5445).

[00283] The expression of ICOS in bilateral flank tumor models treated with NDV were characterized to determine whether the receptor could serve as a target in this therapeutic approach. To characterize the local and abscopal effects of intratumoral NDV therapy, bilateral flank B16-F10 melanoma models were utilized, with the virus administered to a unilateral tumor (Figure 38A). Activation markers that could predict a better response and could be targeted for further improvement in therapeutic efficacy were assessed. The example focused on ICOS, as sustained ICOS upregulation has been previously been shown to be associated with more durable therapeutic responses and increased survival in patients treated with anti-CTLA-4 therapy for malignant melanoma. Analysis of lymphocytes isolated from the tumors and tumor-draining lymph nodes identified upregulation of the co-stimulatory molecule ICOS as one of the activation markers in the treated animals (Figure 38B, C).

Generation and in vitro evaluation of NDV-ICOSL virus.

[00284] Using reverse-genetics system for NDV, NDV expressing murine ICOSL (NDV-ICOSL) was generated (Figure 39A). The expression of ICOSL on the surface of infected B16-F10 cells was confirmed by flow cytometry after 24 hour infection (Figure 39B). *In vitro* characterization of the virus revealed that it had similar replicative (Figure 39D) and lytic (Figure 39C) properties to the parental NDV strain.

NDV-ICOSL growth delay of distant tumors and induces enhanced tumor lymphocyte infiltration

[00285] To evaluate NDV-ICOSL for therapeutic efficacy in the virus-injected and distant tumors, animals bearing bilateral B16-F10 tumors were treated with 4 intratumoral injections of the virus given to a unilateral flank tumor. Both NDV-ICOSL and wild-type NDV were comparable in their ability to cause tumor regressions within the tumors directly injected with the virus (Figure 40A). However, when compared to the wild-type NDV, NDV-ICOSL resulted in significant tumor growth delay of the distant tumors with several animals remaining tumor-free long-term (Figure 40B-C). Analysis of virus-injected tumors revealed enhanced tumor infiltration with CD4 and CD8 effector cells in the animals treated with wild-type NDV and NDV-ICOSL, although the differences between the two viruses were not statistically significant, mirroring the similar activity of the two viruses against the right flank tumors (Figure 40A and

40D). In contrast, analysis of the left flank tumors revealed more prominent increase in tumor-infiltrating CD8 and Tconv cells in the NDV-ICOSL-treated group (Figure 40E). Interestingly, there was also an increase in absolute number of regulatory T cells, with the highest increase seen in the NDV-ICOSL group (Figure 40E), although the relative percentage of regulatory T cells was significantly lower in the NDV-treated animals (Figure 40F).

Combination therapy of NDV-ICOSL and CTLA-4 blockade results in rejection of the injected and distant tumors.

[00286] Overall, the findings above demonstrated that despite the significant inflammatory response seen in distant tumors with intratumoral administration of NDV-ICOSL, the majority of the animals still succumbed to tumors, suggesting that the inhibitory mechanisms active within the tumor microenvironment prevent tumor rejection by the infiltrating immune cells. Thus the efficacy of combination therapy of localized NDV-ICOSL with systemic CTLA-4 blockade was evaluated. For these experiments, the tumor challenge doses were increased to the levels where no significant therapeutic efficacy with NDV or anti-CTLA-4 as single agents was observed. As previously, the animals were treated with 4 doses of NDV administered to a unilateral tumor, concomitantly given with systemic anti-CTLA-4 antibody (Figure 41A). In the B16-F10 model, combination therapy with NDV-ICOSL and anti-CTLA-4 led to regression of the majority of the injected and distant tumors with long-term animal survival, which was significantly superior to the combination of NDV-WT with anti-CTLA-4 (Figure 41B-D). To determine whether these findings could be extended to other tumor models, the same experiment was performed in the bilateral flank CT26 colon carcinoma model. Despite the poor sensitivity of CT26 cells to NDV-mediated lysis *in vitro*, significant therapeutic efficacy of combination therapy of NDV and anti-CTLA-4 against both virus-injected and distant tumors was observed, with superior efficacy again seen in the group utilizing the combination of NDV-ICOSL with anti-CTLA-4 (Figure 42A-D). In both tumor models, animals that completely cleared the tumors were re-challenged with lethal dose of tumor cells on day 90 without further therapy and demonstrated protection against re-challenge in the majority of the animals (Figure 41E and Figure 42E). Interestingly, while in the CT26 model all of the cured animals were protected from re-challenge, in the B16-F10 model the animals treated with combination therapy demonstrated superior protection, when compared to the animals that were cured by anti-CTLA-4 alone (Figure 41E), indicating that the combination approach leads to a more effective protective memory response.

Combination therapy leads to enhanced tumor infiltration with innate and adaptive immune cells

[00287] Analysis of distant B16 tumors from the animals treated with combination of NDV and anti-CTLA-4 therapy demonstrated significant tumor infiltration with different immune cell subtypes (Figure 43A,B). The increased infiltration was evident in both the innate (Figure 43C,D) and the adaptive (Figure 43E) immune compartments, with the highest increase seen in the group treated with combination of NDV-ICOSL and anti-CTLA-4. Interestingly, while this group demonstrated the highest number of infiltrating CD8⁺ lymphocytes, there was also a statistically-significant increase in regulatory T cells seen in this group (Figure 43E), though the overall percentage of Tregs was significantly decreased, when compared to the untreated animals or animals treated with single-agent anti-CTLA-4 (Figure 43F), with resultant increase of the effector to Treg ratios (Figure 43G). A detailed analysis of the TILs demonstrated that the TILs isolated from the animals treated with NDV-ICOSL and anti-CTLA-4 combination expressed the highest levels of activation, lytic, and proliferation makers ICOS, granzyme B, and Ki67 respectively (Figure 43H-J).

Generation of recombinant NDV expressing other co-stimulatory molecules

[00288] This example thus demonstrates that expression of a co-stimulatory ligand by NDV can result in activation of stronger immune responses, which can lead to more effective anti-tumor immunity, especially in the setting of combination therapies with immune checkpoint blockade. To evaluate additional co-stimulatory molecules, ligands targeting the immunoglobulin superfamily of receptors (ICOS and CD28) and the TNF receptor superfamily (GITR, 4-1BB, OX40, and CD40) were studied. For targeting CD28 an artificial ligand composed of a chimeric protein with the cytoplasmic and transmembrane domains of the NDV F glycoprotein and the extracellular domain composed of a single-chain antibody against CD28 (aCD28-scfv) was engineered (Figure 44A,B). For the ligands targeting the TNF receptor superfamily, the extracellular domain of each ligand was fused to the transmembrane and intracellular domains of the NDV HN glycoprotein, in order to ensure enhanced expression of the ligands on the surface of the infected cells (Figure 44A,B). In addition, recombinant viruses expressing cytokines of the common gamma chain receptor family (IL-2, IL-15, and IL-21) were generated. The resultant constructs are illustrated in the diagram in Figure 44C. Recombinant viruses were generated by reverse genetics and presence of the viruses was confirmed by

hemagglutination assays (Figure 45A). To ensure the fidelity of the inserted genes, RNA was isolated from each virus and RT-PCR was performed with primers annealing outside of the cloned gene region (Figure 45B,C). Sequence of each gene was further confirmed by Sanger sequencing. To confirm the expression of co-stimulatory ligands on the surface of infected cells, cultured B16-F10 cells were infected at MOI of 2 and analyzed 24 hours later by flow cytometry with antibodies specific to each gene (Figure 46).

NDV-4-1BBL induces increased tumor infiltration with lymphocytes in the distant tumors

[00289] The ability of the engineered viruses to demonstrate any evidence of enhanced immune response was evaluated, using NDV-4-1BBL as an example. Mice bearing bilateral flank B16-F10 melanomas were treated with intratumoral injection to right tumor of control NDV or NDV-4-1BBL, as described previously and distant tumors were collected on day 15. As can be seen in Figure 47, therapy with NDV-4-1BBL demonstrated enhanced infiltration of both innate and adaptive immune cells into the contralateral tumors, consistent with previous findings demonstrating similar results with NDV expressing ICOSL (Figure 40). Overall, these findings suggest that expression of immunostimulatory molecules by NDV within the context of tumor microenvironment can lead to enhanced anti-tumor immunity.

[00290] The generated viruses NDV-4-1BBL, NDV-GITRL, NDV-OX40L, NDV-CD40L, NDV-IL-2, NDV-IL-15, NDV-IL-21 are evaluated for the ability to induce tumor immune infiltration using similar assays as described in this Section 7. In addition, for therapeutic evaluation, each of the viruses is evaluated in bilateral flank tumor models with the virus being administered to a single-flank tumor in combination with systemic antibodies targeting the inhibitory checkpoints PD-1, PD-L1, and/or CTLA-4.

Conclusion

[00291] To trigger immunogenic tumor cell death and an inflammatory response, nonpathogenic NDV was employed, which, despite its relatively weak lytic activity, has been demonstrated to be a potent inducer of type I IFN and DC maturation (Wilden et al., 2009, Int J Oncol 34: 971; Kato et al., 2005, Immunity 23: 19). A bilateral flank melanoma model with staggered implantation of tumors at a schedule that was previously demonstrated not to be affected by concomitant immunity was utilized (Turk et al., 2004, J Exp Med 200: 771). This example demonstrates that intratumoral injection of NDV results in distant tumor immune infiltration in the absence of distant virus spread. Notably, this effect was associated with

relative reduction in the number of Tregs and marked enhancement of CD4 and CD8 effector to Treg ratios, which has been previously demonstrated to be a marker of a favorable immunological response to immunotherapy (Quezada et al., 2006, J Clin Invest 116: 1935; Curran et al., 2010, Proc Natl Acad Sci U S A 107: 4275).

[00292] The data in this example demonstrates that NDV enhances tumor infiltration with tumor-specific lymphocytes, an effect that was dependent on the identity of the virus-injected tumor. The enhanced tumor infiltration and expansion of adoptively-transferred lymphocytes further suggest the synergy between oncolytic virus therapy and therapeutic approaches utilizing adoptive T cell transfer. It is plausible that the tumor-specific lymphocytes undergo activation and expansion at the site of the initial viral infection, followed by their migration to other tumor sites, which is likely dependent on chemokines and lymphocyte homing receptors (Franciszkiewicz et al., 2012, Cancer Res 72: 6325). The data in this example also demonstrates that distant tumor immune infiltration was in part non-specific and could be induced by NDV infection of a heterologous tumor or by transfer of serum from treated animals to naïve tumor-bearing mice. Increased vascular permeability induced by inflammatory cytokines such as IL-6 may strongly contribute to activation of tumor vasculature and lymphocyte recruitment into the tumors (Fisher et al., 2011, The Journal of clinical investigation 121: 3846).

[00293] Despite the pronounced increase in TILs, therapeutic effect in distant tumors was rather modest with NDV monotherapy, highlighting the immunosuppressive nature of the microenvironment of these tumors (Spranger et al., 2013, Sci Transl Med 5). Remarkably, combination of systemic anti-CTLA-4 antibody with intratumoral NDV led to rejection of distant B16-F10 tumors with long-term animal survival. The animals were also protected against further tumor rechallenge, suggestive of establishment of long-term memory. Interestingly, therapeutic efficacy was also seen with TRAMP C2 and CT26 tumor models, which exhibit poor sensitivity to NDV-mediated cell lysis in vitro. These findings highlight the importance of the NDV-induced anti-tumor immune/inflammatory response, rather than direct lysis, as the primary mechanism driving the anti-tumor efficacy in this model. Indeed, analysis of NDV-injected and distant tumors treated with combination therapy demonstrated prominent infiltration with innate immune cells and activated CD8⁺ and CD4⁺ effector cells, while depletion of CD8⁺ and NK cells abrogated the therapeutic efficacy. Furthermore, the combination strategy was completely ineffective in IFNAR^{-/-} mice, which support the role of the type I IFN pathway in the induction

of anti-tumor immunity in this system (Fuertes et al., 2011, J Exp Med 208, 2005; Diamond et al., 2011, J Exp Med 208: 1989; Swann et al., 2007, J Immunol 178: 7540).

[00294] In summary, this example demonstrates localized intratumoral therapy of B16 melanoma with NDV induces inflammatory responses leading to lymphocytic infiltrates and anti-tumor effect in distant (non-virally injected) tumors without distant virus spread. The inflammatory effect coincided with distant tumor infiltration with tumor-specific CD4+ and CD8+ T cells, which was dependent on the identity of the virus-injected tumor. Combination therapy with localized NDV and systemic CTLA-4 blockade led to rejection of pre-established distant tumors and protection from tumor re-challenge in poorly-immunogenic tumor models, irrespective of tumor cell line sensitivity to NDV-mediated lysis. Therapeutic effect was associated with marked distant tumor infiltration with activated CD8+ and CD4+ effector but not regulatory T cells, and was dependent on CD8+ cells, NK cells and type I interferon. This example demonstrates that localized therapy with oncolytic NDV induces inflammatory immune infiltrates in distant tumors, making them susceptible to systemic therapy with immunomodulatory antibodies.

[00295] The invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described will become apparent to those skilled in the art from the foregoing description and accompanying Figures. Such modifications are intended to fall within the scope of the appended claims.

[00296] All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

WHAT IS CLAIMED:

1. A chimeric Newcastle disease virus (NDV), comprising a packaged genome which encodes an agonist of a co-stimulatory receptor of an immune cell, wherein the agonist is expressed by the virus.
2. A chimeric NDV, comprising a packaged genome which encodes an antagonist of an inhibitory receptor of an immune cell, wherein the antagonist is expressed by the virus.
3. The chimeric NDV of claim 1 or 2, wherein the packaged genome encodes a mutated F protein and the mutated F protein is expressed by the virus.
4. The chimeric NDV of claim 1 or 2, wherein the immune cell is a T lymphocyte or natural killer (NK) cell.
5. The chimeric NDV of claim 1, wherein the co-stimulatory receptor is glucocorticoid-induced tumor necrosis factor receptor (GITR), OX40, CD27, CD28, 4-1BB or CD40.
6. The chimeric NDV of claim 2, wherein the inhibitory receptor is cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD1), B and T-lymphocyte attenuator (BTLA), killer cell immunoglobulin-like receptor (KIR), lymphocyte activation gene 3 (LAG3), or T-cell membrane protein 3 (TIM3).
7. The chimeric NDV of claim 1, wherein the agonist is an antibody that specifically binds to the co-stimulatory receptor.
8. The chimeric NDV of claim 1, wherein the agonist is a ligand that specifically binds to the co-stimulatory receptor.
9. The chimeric NDV of claim 1, wherein the agonist is an antibody specifically binds to GITR, OX40, CD27, CD28, 4-1BB or CD40.

10. The chimeric NDV of claim 7 or 9, wherein the antibody is a monoclonal antibody or single-chain Fv.

11. The chimeric NDV of claim 8, wherein the ligand is GITRL, CD40L, CD137L, OX40L, CD70, or ICOSL.

12. The chimeric NDV of claim 2, wherein the antagonist is an antibody that specifically binds to the inhibitory receptor.

13. The chimeric NDV of claim 2, wherein the antagonist is an antibody that specifically binds to CTLA-4, PD-1, BTLA, KIR, LAG3, or TIM3.

14. The chimeric NDV of claim 2, wherein the antagonist is a soluble receptor of a ligand of the inhibitory receptor.

15. The chimeric NDV of claim 2, wherein the antagonist is an antibody that specifically binds to a ligand of the inhibitory receptor.

16. The chimeric NDV of claim 2, wherein the antagonist is an antibody that specifically binds to PDL1, PDL2, B7-H3, B7-H4, HVEM, or Gal9.

17. The chimeric NDV of claim 14, wherein the soluble receptor is the extracellular domain of PD1, BTLA, KIR, LAG3 or TIM3.

18. The chimeric NDV of claim 12, 13, 15 or 16, wherein the antibody is a monoclonal antibody or sc-Fv.

19. A pharmaceutical composition comprising the chimeric NDV of claim 1, 5, 7, 8, 9 or 11 and a pharmaceutically acceptable carrier.

20. A pharmaceutical composition comprising the chimeric NDV of claim 2, 6, 12, 13, 14, 15, 16 or 17 and a pharmaceutically acceptable carrier.

21. A method for producing a pharmaceutical composition, the method comprising:
- a. propagating the chimeric NDV of any one of claims 1, 2, 5 to 9 or 11 to 17 in a cell line that is susceptible to a NDV infection; and
 - b. collecting the progeny virus,

wherein the virus is grown to sufficient quantities and under sufficient conditions that the virus is free from contamination, such that the progeny virus is suitable for formulation into a pharmaceutical composition.

22. A method for producing a pharmaceutical composition, the method comprising:
- a. propagating the chimeric NDV of any one of claims 1, 2, 5 to 9 or 11 to 17 in an embryonated egg; and
 - b. collecting the progeny virus,

wherein the virus is grown to sufficient quantities and under sufficient conditions that the virus is free from contamination, such that the progeny virus is suitable for formulation into a pharmaceutical composition.

23. A cell line comprising the chimeric NDV of any one of claims 1, 2, 5 to 9 or 11 to 17.

24. An embryonated egg comprising the chimeric NDV of any one of claims 1, 2, 5 to 9 or 11 to 17.

25. A method for treating cancer, comprising administering to a subject in need thereof a pharmaceutical composition comprising the chimeric NDV of any one of claims 1, 5, 7, 8, 9, or 11.

26. A method for treating cancer, comprising administering to a subject in need thereof a pharmaceutical composition comprising the chimeric NDV of any one of claims 2, 6, 12, 13, 14, 15, 16 or 17.

27. The method of claim 25, wherein the packaged genome of the chimeric NDV encodes a mutated F protein with a mutated cleavage site, so that the mutated F protein is expressed by the virus.

28. The method of claim 26, wherein the packaged genome of the chimeric NDV encodes a mutated F protein with a mutated cleavage site, so that the mutated F protein is expressed by the virus.

29. The method of claim 25 further comprising administering to the subject a second agonist of a co-stimulatory receptor of an immune cell.

30. The method of claim 26, further comprising administering to the subject an agonist of a co-stimulatory receptor of an immune cell.

31. The method of claim 26 further comprising administering to the subject a second antagonist of an inhibitory receptor of an immune cell.

32. The method of claim 25 further comprising administering to the subject an antagonist of an inhibitory receptor of an immune cell.

33. A method for treating cancer, comprising administering to a subject in need thereof an NDV and an agonist of a co-stimulatory receptor of an immune cell.

34. A method for treating cancer, comprising administering to a subject in need thereof an NDV and an antagonist of an inhibitory receptor of an immune cell.

35. The method of claim 33, wherein the NDV is a chimeric NDV and wherein the chimeric NDV comprises a packaged genome encoding a cytokine which is expressed by the virus.

36. The method of claim 34, wherein the NDV is a chimeric NDV, which comprises a packaged genome encoding a cytokine, wherein the cytokine is expressed by the virus.

37. The method of claim 33, wherein the NDV is a chimeric NDV, which comprises a packaged genome encoding a second agonist of a co-stimulatory receptor of an immune cell or an antagonist of an inhibitory receptor of an immune cell, wherein the second agonist or antagonist is expressed by the virus.

38. The method of claim 34, wherein the NDV is a chimeric NDV, which comprises a packaged genome encoding an agonist of a co-stimulatory receptor of an immune cell or a second antagonist of an inhibitory receptor of an immune cell, wherein the agonist or second antagonist is expressed by the virus.

39. The method of claim 35 or 36, wherein the cytokine is IL-2, IL-7, IL-15 or IL-21.

40. The method of claim 33, wherein the co-stimulatory receptor is GITR, OX40, CD27, CD28, 4-1BB or CD40.

41. The method of claim 34, wherein the inhibitory receptor is CTLA-4, PD1, BTLA, KIR, LAG3, or TIM3.

42. The method of claim 33, wherein the agonist is an antibody that specifically binds to the co-stimulatory receptor.

43. The method of claim 33, wherein the agonist is a ligand that specifically binds to the co-stimulatory receptor.

44. The method of claim 33, wherein the agonist is an antibody specifically binds to GITR, OX40, CD27, CD28, 4-1BB or CD40.

45. The method of claim 42 or 44, wherein the antibody is a monoclonal antibody or single-chain Fv.

46. The method of claim 43, wherein the ligand is CD137L, OX40L, CD40L, GITRL, CD70, or ICOSL.

47. The method of claim 34, wherein the antagonist is an antibody that specifically binds to the inhibitory receptor.

48. The method of claim 34, wherein the antagonist is an antibody that specifically binds to CTLA-4, PD1, BTLA, KIR, LAG3, or TIM3.

49. The method of claim 34, wherein the antagonist is a soluble receptor of a ligand of the inhibitory receptor.

50. The method of claim 34, wherein the antagonist is an antibody that specifically binds to a ligand of the inhibitory receptor.

51. The method of claim 34, wherein the antagonist is an antibody that specifically binds to PDL1, PDL2, B7-H3, B7-H4, HVEM, or Gal9.

52. The method of claim 51, wherein the soluble receptor is the extracellular domain of PD1, BTLA, KIR, LAG3 or TIM3.

53. The method of claim 47, 48, 50 or 51, wherein the antibody is a monoclonal antibody or scFv.

54. The method of claim 33 or 34 further comprising administering adoptive T lymphocytes.

55. The method of any one of claims 33 to 38, 40 to 44 or 46 to 52, wherein the cancer is melanoma, colorectal cancer, breast cancer, ovarian cancer or renal cell cancer.

56. The method of any one of claims 33 to 38, 40 to 44 or 46 to 52, wherein the cancer is malignant melanoma, malignant glioma, renal cell carcinoma, pancreatic adenocarcinoma, malignant mesothelioma, lung adenocarcinoma, lung small cell carcinoma, lung squamous cell carcinoma, anaplastic thyroid cancer or head and neck squamous cell carcinoma.

57. The method of any one of claims 33 to 38, 40 to 44 or 46 to 52, wherein the subject is a human.