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(54) Title: HIGH AFFINITY ANTIBODIES TO PD-1 AND LAG-3 AND BISPECIFIC BINDING PROTEINS MADE THEREFROM

(57) Abstract: High-affinity antibodies recognizing Programmed Death Ligand-1 (PD-1) and Lymphocyte Activation Gene 3 protein (LAG-3) are disclosed. Binding sites from humanized anti-PD-1 and anti-LAG-3 antibodies are incorporated into a Fabs-in-Tandem Immunoglobulin format without significant loss of binding affinity, and the resultant bispecific, multivalent binding proteins are able to bind to both PD-1 and LAG-3 simultaneously. Such bispecific FIT-Ig binding proteins are useful for treatment of cancer.

HIGH AFFINITY ANTIBODIES TO PD-1 AND LAG-3 AND BISPECIFIC BINDING PROTEINS MADE THEREFROM

Field of the Invention

The present invention relates to new antibodies recognizing Programmed Cell Death Protein 1 (PD-1), new antibodies recognizing Lymphocyte-Activation Gene 3 protein (LAG-3), and bispecific PD-1/LAG-3 binding proteins such as FIT-Ig binding proteins made using those antibodies. The antibodies and bispecific binding proteins are useful for treatment of immunological diseases and hematological cancers.

Background of the Invention

Programmed Cell Death Protein 1 (PD-1)

Programmed Cell Death Protein 1 (PD-1, CD279) is a member of the CD28 family of receptors, which includes CD28, CTLA-4, ICOS, PD-1, and BTLA. Expression of PD-1 is frequently found in immune cells such as T cells, B cells, monocytes and natural killer (NK) cells. PD-1 and like family members are type I transmembrane glycoproteins containing an immunoglobulin-like domain resembling an Ig variable domain that is responsible for ligand binding and a cytoplasmic tail that is responsible for the binding of signaling molecules. The cytoplasmic tail of PD-1 contains two tyrosine-based signaling motifs, an ITIM (immunoreceptor tyrosine-based inhibition motif) and an ITSM (immunoreceptor tyrosine-based switch motif). Vivier et al., *Immunol. Today*, 18:286-291 (1997) and Chemnitz et al., *J. Immunol.*, 173:945-954 (2004).

Two cell surface glycoprotein ligands for PD-1 have been identified, Programmed Death Ligand 1 (PD-L1, CD274, B7-H1) and PD-L2 (CD273, B7-DC), and have been shown to induce intracellular signal transduction that inhibits CD3- and CD28-mediated T cell activation. Riley, *Immunol. Rev.*, 229:114-125 (2009). This downregulation of T cell activation in turn results in reduction of T cell proliferation, IL-2 secretion, IFN-γ secretion, and secretion of other growth factors and cytokines. Freeman et al., *J. Exp. Med.*, 192:1027-1034 (2000); Latchman et al.,

Nat. Immunol., 2:261-8 (2001); Carter et al., *Eur. J. Immunol.*, 32:634-43 (2002); Ohigashi et al., *Clin. Cancer Res.*, 11:2947-53 (2005). Signaling via the PD-1/PD-L1 interaction is believed to serve critical, non-redundant functions within the immune system, by negatively regulating T cell responses. This regulation is involved in T cell development in the thymus, in regulation of chronic inflammatory responses, and in maintenance of both peripheral tolerance and immune privilege. The critical nature of these functions is exemplified in PD-1-deficient mice, which exhibit an autoimmune phenotype. PD-1 deficiency in C57BL/6 mice results in chronic progressive lupus-like glomerulonephritis and arthritis. In Balb/c mice, PD-1 deficiency leads to severe cardiomyopathy due to the presence of heart-tissue-specific self-reacting antibodies.

Following T cell stimulation, PD-1 recruits the tyrosine phosphatase SHP-2 to the ITSM motif within its cytoplasmic tail, leading to the dephosphorylation of effector molecules such as CD3-ζ, PKC-θ and ZAP70 that are involved in the CD3 T cell signaling cascade. The mechanism by which PD-1 down-modulates T cell responses is similar to but distinct from that of CTLA-4, as both molecules regulate an overlapping set of signaling proteins. Parry et al., *Mol. Cell Biol.*, 25:9543-9553 (2005). In general, PD-1-mediated inhibitory signal plays an important role in immune tolerance. Bour-Jordan et al., *Immunol. Rev.*, 241:180-205 (2011).

Increased PD-1 expression is found in tumor-infiltrating lymphocytes (TILs), and expression of PD-1 ligands in tumor cells has been reported in a variety of cancers of different tissues including lung, liver, stomach, kidney, breast, ovary, pancreas, melanocytes, and esophagus. In general, PD-1 ligand expression on tumor cells has been correlated to poor prognosis of cancer patients across multiple tumor types. Okazaki and Honjo, *Int. Immunol.*, 19:813-824 (2007).

Blockade of the PD-1/PD-L1 interaction could lead to enhanced tumor-specific T cell immunity and therefore be helpful in clearance of tumor cells by the immune system. In a murine model of aggressive pancreatic cancer, T. Nomi et al. demonstrated the therapeutic efficacy of PD-1/PD-L1 blockade, showing administration of either anti-PD-1 or anti-PD-L1 antibody significantly inhibited tumor growth. Nomi et al., *Clin. Cancer Res.*, 13:2151-2157 (2007). Antibody blockade effectively promoted tumor-reactive CD8+ T cell infiltration into

the tumor, resulting in the upregulation of anti-tumor effectors including IFN-γ, granzyme B, and perforin. In another study, using a model of squamous cell carcinoma in mice, antibody blockade of PD-1 or PD-L1 significantly inhibited tumor growth. Tsushima et al., *Oral Oncol.*, 42:268-274 (2006).

Recently, it has been shown that PD-1 is highly expressed on T cells from HIV-infected individuals and that receptor expression correlates with impaired T cell function and disease progression. Day et al., *Nature*, 443:350-354 (2006); Trautmann et al., *Nat. Med.*, 12:1198-1202 (2006). In both studies, blockade of the ligand PD-L1 significantly increased the expansion of HIV-specific, IFN-γ-producing cells *in vitro*.

Accordingly, therapeutic modulation of PD-1 signaling by antagonist molecules may revert immune cells from tolerance and reactivate them to eradicate cancer and chronic viral infections.

Lymphocyte Activation Gene 3 (LAG-3)

Lymphocyte Activation Gene 3 protein (LAG-3, CD223) is a negative co-stimulatory receptor that modulates T cell homeostasis, proliferation, and activation. Sierro et al., *Expert Opin. Ther. Targets*, 15: 91-101 (2010). An immunoglobulin superfamily member, LAG-3 is a CD4-like protein which, like CD4, binds to MHC class II molecules, but with two-fold higher affinity and at a distinct site from CD4. Huard et al., *Proc. Natl Acad. Sci. USA*, 94:5744-9 (1997). LAG-3 is expressed on activated CD8+ T cells, γδ T cells, natural killer, B-cells, plasmacytoid dendritic cells, and regulatory T cells (Tregs). The role of LAG-3 as a negative regulator of T cell responses is based on studies with LAG-3 knockout mice and use of blocking anti-LAG-3 antibodies in model *in vitro* and *in vivo* systems. Sierro et al. (2010), *op. cit.*; Hannier et al., *J. Immunol.*, 161:4058-65 (1998); Macon-Lemaitre et al., *Immunology*, 115:170-8 (2005); Workman et al., *Eur. J. Immunol.*, 33:970-9 (2003). Both natural and induced Tregs express increased LAG-3, which is required for their maximal suppressive function. Camisaschi et al., *J. Immunol.*, 184:6545-6551 (2010); Huang, et al., *Immunity*, 21:503-513 (2004). Furthermore, ectopic expression of LAG-3 on CD4+ effector T cells reduces their proliferative capacity and confers on them regulatory potential against third party T cells.

Huang, *ibid.* Recent studies have also shown that high LAG-3 expression on exhausted lymphocytic choriomeningitis virus (LCMV)-specific CD8+ T cells contributes to their unresponsive state and limits CD8+ T cell antitumor responses. Blackburn et al., *Nat. Immunol.*, 10:29-37 (2009) and Grosso et al., *J. Clin. Invest.*, 117:3383-3392 (2007).

The important role LAG-3 plays in antitumor immune response and immune response to infection makes it a target of interest for immunotherapy. Blocking LAG-3 with antagonists, including monoclonal antibodies, has been proposed for treatment of certain cancers and chronic viral infections. Turnis et al., *Eur. J. Immunol.*, 45:1892-1905 (2015).

As the importance of PD-1- and LAG-3-mediated signaling becomes better understood, there is an ongoing need for discovery of new inhibitory anti-PD-1 and anti-LAG-3 antibodies that can effectively alter T cell functionality or increase the reactivity of tumor cells to immune effector cells. Moreover, the design of bispecific molecules that could combine the effects of PD-1 and LAG-3 inhibition would also be a desirable improvement in therapeutic approaches to cancer treatment.

Summary of the Invention

The present invention provides new antibodies that bind to PD-1 with high affinity and new antibodies that bind to LAG-3 with high affinity. The invention also provides PD-1/LAG-3 bispecific Fabs-in-Tandem immunoglobulins (FIT-Igs) that are reactive with both PD-1 and LAG-3. Antibodies and bispecific binding proteins of the present invention can block LAG-3 on TILs to reduce the Tumor-infiltrated Treg cells population or to recover TILs to a cytotoxic phenotype. Additionally, antibodies and bispecific binding proteins of the invention can be used for inhibiting PD-1/PD-L1 signaling, in order to reactivate tumor infiltrated cytotoxic T cells. The bispecifc, multivalent binding proteins described herein will be useful as PD-1/LAG-3 bispecific inhibitors to provide a synergistic combination effect to overcome antitumor immune suppression and thereby improve outcomes even for patients that do not respond or have stopped responding to anti-PD-1 or anti-LAG-3 therapies alone.

The invention also provides methods of making and using the anti-PD-1 and anti-LAG-3 antibodies and PD-1/LAG-3 bispecific binding proteins described herein as well as various compositions that may be used in methods of detecting PD-1 and/or LAG-3 in a sample or in methods of treating or preventing a disorder in an individual that is associated with PD-1 and/or LAG-3 activity.

In a further embodiment, the invention provides a bispecific Fabs-in-Tandem immunoglobulin (FIT-Ig) binding protein comprising first, second, and third polypeptide chains,

wherein said first polypeptide chain comprises, from amino to carboxyl terminus, (i) VL_A-CL-VH_B-CH1-Fc wherein CL is directly fused to VH_B, or (ii) VH_B-CH1-VL_A-CL-Fc wherein CH1 is directly fused to VL_A;

wherein said second polypeptide chain comprises, from amino to carboxyl terminus, VH_A-CH1; and

wherein said third polypeptide chain comprises, from amino to carboxyl terminus, VL_B-CL;

wherein VL is a light chain variable domain, CL is a light chain constant domain, VH is a heavy chain variable domain, CH1 is a heavy chain constant domain, Fc is an immunoglobulin Fc region, A is an epitope of PD-1 or LAG-3 and B is an epitope of PD-1 or LAG-3, with the proviso that A and B are different. In accordance with the present invention, such FIT-Ig binding proteins bind to both PD-1 and LAG-3.

In preferred embodiments, the Fab fragments of such FIT-Ig binding proteins incorporate VL_A-CL and VH_A-CH1 domains from a parental antibody binding to one of the antigen targets PD-1 or LAG-3, and incorporate VL_B-CL and VH_B-CH1 domains from a different parental antibody binding to the other of the antigen targets PD-1 and LAG-3. Thus, VH-CH1/VL-CL pairing will result in tandem Fab moieties recognizing PD-1 and LAG-3.

In accordance with the present invention, a PD-1/LAG-3 FIT-Ig binding protein may advantageously comprise first, second, and third polypeptide chains, wherein said first polypeptide chain comprises, from amino to carboxyl terminus, VL_{PD-1}-CL-VH_{LAG-3}-CH1-Fc wherein CL is directly fused to VH_{LAG-3}, wherein said second polypeptide chain comprises, from

amino to carboxyl terminus, VH_{PD-1}-CH1; and wherein said third polypeptide chain comprises, from amino to carboxyl terminus, VL_{LAG-3}-CL; wherein VL_{PD-1} is a light chain variable domain of an anti-PD-1 antibody, CL is a light chain constant domain, VH_{PD-1} is a heavy chain variable domain of an anti-PD-1 antibody, CH1 is a heavy chain constant domain, VL_{LAG-3} is a light chain variable domain of an anti-LAG-3 antibody, VH_{LAG-3} is a heavy chain variable domain of an anti-LAG-3 antibody, and Fc is an immunoglobulin Fc region. Advantageously, in the first polypeptide chain, the domains VL_{PD-1}-CL are the same as the light chain of an anti-PD-1 parental antibody, the domains VH_{PD-1}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-PD-1 parental antibody, the domains VL_{LAG-3}-CL are the same as the light chain of an anti-LAG-3 parental antibody, and the domains VH_{LAG-3}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-LAG-3 parental antibody.

In alternative embodiments, a PD-1/LAG-3 FIT-Ig binding protein may advantageously comprise first, second, and third polypeptide chains, wherein said first polypeptide chain comprises, from amino to carboxyl terminus, VL_{LAG-3}-CL-VH_{PD-1}-CH1-Fc wherein CL is directly fused to VH_{PD-1}, wherein said second polypeptide chain comprises, from amino to carboxyl terminus, VH_{LAG-3}-CH1; and wherein said third polypeptide chain comprises, from amino to carboxyl terminus, VL_{PD-1}-CL; wherein VL_{PD-1} is a light chain variable domain of an anti-PD-1 antibody, CL is a light chain constant domain, VH_{PD-1} is a heavy chain variable domain of an anti-PD-1 antibody, CH1 is a heavy chain constant domain, VL_{LAG-3} is a light chain variable domain of an anti-LAG-3 antibody, VH_{LAG-3} is a heavy chain variable domain of an anti-LAG-3 antibody, and Fc is an immunoglobulin Fc region. Advantageously, in the first polypeptide chain, the domains VL_{LAG-3}-CL are the same as the light chain of an anti-LAG-3 parental antibody, the domains VH_{LAG-3}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-LAG-3 parental antibody, the domains VL_{PD-1}-CL are the same as the light chain of an anti-PD-1 parental antibody, and the domains VH_{PD-1}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-PD-1 parental antibody.

In alternative embodiments, a PD-1/LAG-3 FIT-Ig binding protein may advantageously comprise first, second, and third polypeptide chains, wherein said first polypeptide chain comprises, from amino to carboxyl terminus, VH_{LAG-3}-CH1-VL_{PD-1}-CL-Fc wherein CH1 is directly fused to VL_{PD-1}, wherein said second polypeptide chain comprises, from amino to carboxyl terminus, VL_{LAG-3}-CL; and wherein said third polypeptide chain comprises, from amino to carboxyl terminus, VH_{PD-1}-CH1; wherein VL_{PD-1} is a light chain variable domain of an anti-PD-1 antibody, CL is a light chain constant domain, VH_{PD-1} is a heavy chain variable domain of an anti-PD-1 antibody, CH1 is a heavy chain constant domain, VL_{LAG-3} is a light chain variable domain of an anti-LAG-3 antibody, VH_{LAG-3} is a heavy chain variable domain of an anti-LAG-3 antibody, and Fc is an immunoglobulin Fc region. Advantageously, in the first polypeptide chain, the domains VL_{LAG-3}-CL are the same as the light chain of an anti-LAG-3 parental antibody, the domains VH_{LAG-3}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-LAG-3 parental antibody, the domains VL_{PD-1}-CL are the same as the light chain of an anti-PD-1 parental antibody, and the domains VH_{PD-1}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-PD-1 parental antibody.

In alternative embodiments, a PD-1/LAG-3 FIT-Ig binding protein may advantageously comprise first, second, and third polypeptide chains, wherein said first polypeptide chain comprises, from amino to carboxyl terminus, VH_{PD-1}-CH1-VL_{LAG-3}-CL-Fc wherein CH1 is directly fused to VL_{LAG-3}, wherein said second polypeptide chain comprises, from amino to carboxyl terminus, VL_{PD-1}-CL; and wherein said third polypeptide chain comprises, from amino to carboxyl terminus, VH_{LAG-3}-CH1; wherein VL_{PD-1} is a light chain variable domain of an anti-PD-1 antibody, CL is a light chain constant domain, VH_{PD-1} is a heavy chain variable domain of an anti-PD-1 antibody, CH1 is a heavy chain constant domain, VL_{LAG-3} is a light chain variable domain of an anti-LAG-3 antibody, VH_{LAG-3} is a heavy chain variable domain of an anti-LAG-3 antibody, and Fc is an immunoglobulin Fc region. Advantageously, in the first polypeptide chain, the domains VL_{LAG-3}-CL are the same as the light chain of an anti-LAG-3 parental antibody, the domains VH_{LAG-3}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-LAG-3 parental antibody, the domains VL_{PD-1}-CL are the same as

the light chain of an anti-PD-1 parental antibody, and the domains VH_{PD-1}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-PD-1 parental antibody.

In the foregoing formulas for the first polypeptide chain of a FIT-Ig binding protein, an Fc region may be a native or a variant Fc region. In particular embodiments, the Fc region is a human Fc region from IgG1, IgG2, IgG3, IgG4, IgA, IgM, IgE, or IgD. In particular embodiments, the Fc is a human Fc from IgG1, or a modified human Fc such as set forth in Table 6, *infra* (SEQ ID NO:28).

In an embodiment of the invention, FIT-Ig binding proteins of the present invention retain one or more properties of parental antibodies from which the sequences of their Fab fragments are utilized and incorporated into the FIT-Ig structure. In preferred embodiments, the FIT-Ig will retain binding affinity for the target antigens (i.e., LAG-3 and PD-1) comparable to that of the parental antibodies, meaning that the binding affinity of the FIT-Ig binding protein for the PD-1 and LAG-3 antigen targets does not vary by greater than 10-fold in comparison to the binding affinity of the parental antibodies for their respective target antigens, as measured by surface plasmon resonance or biolayer interferometry.

In one embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-679 of SEQ ID NO:78; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 20-240 of SEQ ID NO:83; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-236 of SEQ ID NO:86. (See Table 27.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-684 of SEQ ID NO:88; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 20-235 of SEQ ID NO:91; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-236 of SEQ ID NO:93. (See Table 28.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1

and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-679 of SEQ ID NO:95; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 20-242 of SEQ ID NO:98; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-236 of SEQ ID NO:100. (See Table 29.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-684 of SEQ ID NO:102; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 20-235 of SEQ ID NO:105; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-236 of SEQ ID NO:107. (See Table 30.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-679 of SEQ ID NO:140; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:144; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:146. (See FIT107-1-6a-1; Table 41.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-684 of SEQ ID NO:147; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:151; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:153. (See FIT-107-1-6b-1; Table 42.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-679 of SEQ ID NO:154; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ

ID NO:158; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:160. (See FIT-107-1-6a-2; Table 43.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-684 of SEQ ID NO:161; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:165; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:167. (See FIT-107-1-6b-2; Table 44.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-679 of SEQ ID NO:168; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:172; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:174. (See FIT-107-1-6a-3; Table 45.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-684 of SEQ ID NO:175; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:179; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:181. (See FIT-107-1-6b-3; Table 46.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-679 of SEQ ID NO:182; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:186; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:188. (See FIT-107-1-7a-1; Table 47.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or

consisting of the sequence of amino acids 23-687 of SEQ ID NO:189; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:193; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:195. (See FIT-107-1-7b-1; Table 48.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-679 of SEQ ID NO:196; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:200; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:202. (See FIT-107-1-7a-2; Table 49.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-687 of SEQ ID NO:203; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:207; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:209. (See FIT-107-1-7b-2; Table 50.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-679 of SEQ ID NO:210; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:214; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:216. (See FIT-107-1-7a-3; Table 51.)

In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-687 of SEQ ID NO:217; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:221; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids of SEQ ID NO:223. (See FIT-107-1-7b-3; Table 52.)

The invention also provides novel antibodies capable of binding human PD-1, wherein the antigen-binding domain of the antibody comprises a set of six CDRs, i.e., CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2, and CDR-L3, selected from the group of CDR sets defined below:

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
1	CDR-H1	SYMMS	residues 31-35 of SEQ ID NO:4
	CDR-H2	SMSGGGRDTYYPDSVKG	residues 50-66 of SEQ ID NO:4
	CDR-H3	RGTYAMDY	residues 99-106 of SEQ ID NO:4
	CDR-L1	LASQTIGTWLT	residues 24-34 of SEQ ID NO:5
	CDR-L2	AATSLAD	residues 50-56 of SEQ ID NO:5
	CDR-L3	QQLYSTPWT	residues 89-97 of SEQ ID NO:5
2	CDR-H1	TGYYWN	residues 31-36 of SEQ ID NO:6
	CDR-H2	YMSYDGNNNYNPSLKN	residues 51-66 of SEQ ID NO:6
	CDR-H3	DRGTTILGGTMDY	residues 99-111 of SEQ ID NO:6
	CDR-L1	KASQSVSNDVA	residues 24-34 of SEQ ID NO:7
	CDR-L2	YAFYRYT	residues 50-56 of SEQ ID NO:7
	CDR-L3	QQDYSSPWT	residues 89-97 of SEQ ID NO:7
3	CDR-H1	FYTMS	residues 31-35 of SEQ ID NO:8
	CDR-H2	TISGGGRDTYYPDSVKG	residues 50-66 of SEQ ID NO:8
	CDR-H3	QGGNYLFAY	residues 99-107 of SEQ ID NO:8
	CDR-L1	KASQDVNTVVA	residues 24-34 of SEQ ID NO:9
	CDR-L2	WASTRHT	residues 50-56 of SEQ ID NO:9
	CDR-L3	QQHYTTPYT	residues 89-97 of SEQ ID NO:9
4	CDR-H1	DYGMH	residues 31-35 of SEQ ID NO:10
	CDR-H2	YISSGSYTIYYADTVKG	residues 50-66 of SEQ ID NO:10
	CDR-H3	RGGSSHVNVMDY	residues 99-110 of SEQ ID NO:10
	CDR-L1	KASDHINNWLA	residues 24-34 of SEQ ID NO:11

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
	CDR-L2	GATSLET	residues 50-56 of SEQ ID NO:11
	CDR-L3	QQYWSPPYT	residues 89-97 of SEQ ID NO:11
5	CDR-H1	DNNVE	residues 31-35 of SEQ ID NO:12
	CDR-H2	DINPNNGDTLYSQYFKD	residues 50-66 of SEQ ID NO:12
	CDR-H3	GKSDQFDY	residues 99-106 of SEQ ID NO:12
	CDR-L1	LASQTIGTWLA	residues 24-34 of SEQ ID NO:13
	CDR-L2	AATSLAD	residues 50-56 of SEQ ID NO:13
	CDR-L3	QQLYSSPWT	residues 89-97 of SEQ ID NO:13
6	CDR-H1	SYAMS	residues 31-35 of SEQ ID NO:14
	CDR-H2	TISGGGRDTYYPDSVKG	residues 50-66 of SEQ ID NO:14
	CDR-H3	QGGTYLFAS	residues 99-107 of SEQ ID NO:14
	CDR-L1	KASQDVNTAVA	residues 24-34 of SEQ ID NO:15
	CDR-L2	WASTRHT	residues 50-56 of SEQ ID NO:15
	CDR-L3	QQHYTTPYT	residues 89-97 of SEQ ID NO:15
7	CDR-H1	DYEMH	residues 31-35 of SEQ ID NO:16
	CDR-H2	VIEPESGGTVYNQKFKG	residues 51-66 of SEQ ID NO:16
	CDR-H3	EGFNSDHYFDY	residues 99-109 of SEQ ID NO:16
	CDR-L1	RSSQNIVHSNGNTYLE	residues 24-39 of SEQ ID NO:17
	CDR-L2	KVFNRFS	residues 55-61 of SEQ ID NO:17
	CDR-L3	FQGSHVPYT	residues 94-102 of SEQ ID NO:17
8	CDR-H1	SHLMS	residues 31-35 of SEQ ID NO:18
	CDR-H2	AISGGGADTYYPDSVKG	residues 50-66 of SEQ ID NO:18
	CDR-H3	QILAFDS	residues 99-105 of SEQ ID NO:18
	CDR-L1	HASQNIYVWLN	residues 24-34 of SEQ ID NO:19
	CDR-L2	KASNLHT	residues 50-56 of SEQ ID NO:19

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
	CDR-L3	QQGQSYPWT	residues 89-97 of SEQ ID NO:19
9	CDR-H1	SHLMS	residues 31-35 of SEQ ID NO:53
	CDR-H2	AISGGGADTYYPASVKG	residues 50-66 of SEQ ID NO:53
	CDR-H3	QILAFDA	residues 99-105 of SEQ ID NO:53
	CDR-L1	HASQNIYVWLN	residues 24-34 of SEQ ID NO:19
	CDR-L2	KASNLHT	residues 50-56 of SEQ ID NO:19
	CDR-L3	QQGQSYPWT	residues 89-97 of SEQ ID NO:19

The invention also provides novel antibodies capable of binding human LAG-3, wherein the antigen-binding domain of the antibody comprises a set of six CDRs, i.e., CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2, and CDR-L3, selected from the group of CDR sets defined below:

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
10	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:60
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:60
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:60
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:61
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:61
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:61
11	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:60
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:60
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:60
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:62
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:62
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:62

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
12	CDR-H1	DYEMH	residues 31-35 of SEQ ID NO:63
	CDR-H2	AIDPETGGTAYNQKFKG	residues 50-66 of SEQ ID NO:63
	CDR-H3	WGSTVFPY	residues 101-108 of SEQ ID NO:63
	CDR-L1	KSTKSLLNSDGFTYLD	residues 24-39 of SEQ ID NO:64
	CDR-L2	LVSNRFS	residues 55-61 of SEQ ID NO:64
	CDR-L3	FQSNYLPWT	residues 94-102 of SEQ ID NO:64
13	CDR-H1	DYEMH	residues 31-35 of SEQ ID NO:65
	CDR-H2	AIDPATGGTAYNQKFKG	residues 50-66 of SEQ ID NO:65
	CDR-H3	WGTTVFPY	residues 99-106 of SEQ ID NO:65
	CDR-L1	KSTKSLLNSDGFTYLD	residues 24-39 of SEQ ID NO:66
	CDR-L2	LVSNRFS	residues 55-61 of SEQ ID NO:66
	CDR-L3	FQSNYLPWT	residues 94-102 of SEQ ID NO:66
14	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:67
	CDR-H2	WIDPENGDTEYASKFQG	residues 50-66 of SEQ ID NO:67
	CDR-H3	FDY	residues 99-101 of SEQ ID NO:67
	CDR-L1	KSSQSLLDSDGKTYLN	residues 24-39 of SEQ ID NO:68
	CDR-L2	LVSKLDS	residues 55-61 of SEQ ID NO:68
	CDR-L3	WQGSHFPQT	residues 94-102 of SEQ ID NO:68
15	CDR-H1	DDYVH	residues 31-35 of SEQ ID NO:69
	CDR-H2	WIDPENGDTEYASKFQG	residues 50-66 of SEQ ID NO:69
	CDR-H3	WDAEENY	residues 99-105 of SEQ ID NO:69
	CDR-L1	RSSKSLLHSNGNTYLY	residues 24-39 of SEQ ID NO:70
	CDR-L2	RMSNLAS	residues 55-61 of SEQ ID NO:70
	CDR-L3	MQHLEYPFT	residues 94-102 of SEQ ID NO:70
16	CDR-H1	DDYIH	residues 31-35 of SEQ ID NO:71

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
	CDR-H2	WIDPENGDTEYASKFQG	residues 50-66 of SEQ ID NO:71
	CDR-H3	DYRNWY	residues 100-105 of SEQ ID NO:71
	CDR-L1	KSSQSLLDSDGKTYLN	residues 24-39 of SEQ ID NO:68
	CDR-L2	LVSKLDS	residues 55-61 of SEQ ID NO:68
	CDR-L3	WQGSHFPQT	residues 94-102 of SEQ ID NO:68
17	CDR-H1	DFNIKDDYMH	residues 26-35 of SEQ ID NO:114
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:114
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:114
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:117
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:117
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:117
18	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:72
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:72
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:72
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:77
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:77
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:77
19	CDR-H1	DDYMH	residues 30-34 of SEQ ID NO:119
	CDR-H2	WIVPENGNTVYASKFQG	residues 48-64 of SEQ ID NO:119
	CDR-H3	YGDY	residues 95-98 of SEQ ID NO:119
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:120
	CDR-L2	AASALDS	residues 50-56 of SEQ ID NO:120
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:120
20	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:121
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:121

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:121
	CDR-L1	RAMQEISGYLS	residues 24-34 of SEQ ID NO:122
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:122
	CDR-L3	LQYAYYPLT	residues 89-97 of SEQ ID NO:122
21	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:123
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:123
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:123
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:124
	CDR-L2	AASHLDS	residues 50-56 of SEQ ID NO:124
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:124
22	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:125
	CDR-H2	WIVPENGLTEYASKFQG	residues 50-66 of SEQ ID NO:125
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:125
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:126
	CDR-L2	ATSTLDS	residues 50-56 of SEQ ID NO:126
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:126
23	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:127
	CDR-H2	WIVPENGKTEYASKFQG	residues 50-66 of SEQ ID NO:127
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:127
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:128
	CDR-L2	AAMTLDS	residues 50-56 of SEQ ID NO:128
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:128
24	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:129
	CDR-H2	WIVPENGNTHYASKFQG	residues 50-66 of SEQ ID NO:129
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:129

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:130
	CDR-L2	EASTLDS	residues 50-56 of SEQ ID NO:130
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:130
25	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:131
	CDR-H2	WIVPRNGNTMYASKFQG	residues 50-66 of SEQ ID NO:131
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:131
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:132
	CDR-L2	AASTLDL	residues 50-56 of SEQ ID NO:132
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:132
26	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:135
	CDR-H2	WIVPENANTVYASKFQG	SEQ ID NO:224
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:135
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:138
	CDR-L2	AASALDS	residues 50-56 of SEQ ID NO:138
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:138
27	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:136
	CDR-H2	WIVPRNANTVYASKFQG	SEQ ID NO:225
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:136
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:139
	CDR-L2	AASALDL	residues 50-56 of SEQ ID NO:139
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:139
28	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:136
	CDR-H2	WIVPRNANTVYASKFQG	SEQ ID NO:225
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:136
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:117

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:117
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:117

In one embodiment, a binding protein according to the invention is a bispecific, multivalent immunoglobulin binding protein comprising two or more antigen binding sites, wherein at least one antigen binding site comprises a CDR set selected from CDR Sets 1, 2, 3, and 4 above and at least one antigen binding site comprises CDR Set 5 above.

In an embodiment, an anti-PD-1 antibody according to the invention comprises VH and VL domains, wherein the two variable domains comprise amino acid sequences selected from the group consisting of:

SEQ ID NO:4 and SEQ ID NO:5	SEQ ID NO:6 and SEQ ID NO:7
SEQ ID NO:8 and SEQ ID NO:9	SEQ ID NO:10 and SEQ ID NO:11
SEQ ID NO:12 and SEQ ID NO:13	SEQ ID NO:14 and SEQ ID NO:15
SEQ ID NO:16 and SEQ ID NO:17	SEQ ID NO:18 and SEQ ID NO:19
SEQ ID NO:20 and SEQ ID NO:23	SEQ ID NO:21 and SEQ ID NO:23
SEQ ID NO:22 and SEQ ID NO:23	SEQ ID NO:20 and SEQ ID NO:24
SEQ ID NO:21 and SEQ ID NO:24	SEQ ID NO:22 and SEQ ID NO:24
SEQ ID NO:20 and SEQ ID NO:25	SEQ ID NO:21 and SEQ ID NO:25
SEQ ID NO:22 and SEQ ID NO:25	SEQ ID NO:20 and SEQ ID NO:26
SEQ ID NO:21 and SEQ ID NO:26	SEQ ID NO:22 and SEQ ID NO:26
SEQ ID NO:20 and SEQ ID NO:27	SEQ ID NO:21 and SEQ ID NO:27
SEQ ID NO:22 and SEQ ID NO:27	SEQ ID NO:30 and SEQ ID NO:34
SEQ ID NO:31 and SEQ ID NO:34	SEQ ID NO:32 and SEQ ID NO:34
SEQ ID NO:33 and SEQ ID NO:34	SEQ ID NO:30 and SEQ ID NO:35
SEQ ID NO:31 and SEQ ID NO:35	SEQ ID NO:32 and SEQ ID NO:35
SEQ ID NO:33 and SEQ ID NO:35	SEQ ID NO:30 and SEQ ID NO:36
SEQ ID NO:31 and SEQ ID NO:36	SEQ ID NO:32 and SEQ ID NO:36
SEQ ID NO:33 and SEQ ID NO:36	SEQ ID NO:30 and SEQ ID NO:37

SEQ ID NO:31 and SEQ ID NO:37	SEQ ID NO:32 and SEQ ID NO:37
SEQ ID NO:33 and SEQ ID NO:37	SEQ ID NO:38 and SEQ ID NO:43
SEQ ID NO:39 and SEQ ID NO:43	SEQ ID NO:40 and SEQ ID NO:43
SEQ ID NO:41 and SEQ ID NO:43	SEQ ID NO:42 and SEQ ID NO:43
SEQ ID NO:38 and SEQ ID NO:44	SEQ ID NO:39 and SEQ ID NO:44
SEQ ID NO:40 and SEQ ID NO:44	SEQ ID NO:41 and SEQ ID NO:44
SEQ ID NO:42 and SEQ ID NO:44	SEQ ID NO:38 and SEQ ID NO:45
SEQ ID NO:39 and SEQ ID NO:45	SEQ ID NO:40 and SEQ ID NO:45
SEQ ID NO:41 and SEQ ID NO:45	SEQ ID NO:42 and SEQ ID NO:45
SEQ ID NO:38 and SEQ ID NO:46	SEQ ID NO:39 and SEQ ID NO:46
SEQ ID NO:40 and SEQ ID NO:46	SEQ ID NO:41 and SEQ ID NO:46
SEQ ID NO:42 and SEQ ID NO:46	SEQ ID NO:38 and SEQ ID NO:47
SEQ ID NO:39 and SEQ ID NO:47	SEQ ID NO:40 and SEQ ID NO:47
SEQ ID NO:41 and SEQ ID NO:47	SEQ ID NO:42 and SEQ ID NO:47
SEQ ID NO:48 and SEQ ID NO:55	SEQ ID NO:49 and SEQ ID NO:55
SEQ ID NO:50 and SEQ ID NO:55	SEQ ID NO:51 and SEQ ID NO:55
SEQ ID NO:52 and SEQ ID NO:55	SEQ ID NO:53 and SEQ ID NO:55
SEQ ID NO:54 and SEQ ID NO:55	SEQ ID NO:48 and SEQ ID NO:56
SEQ ID NO:49 and SEQ ID NO:56	SEQ ID NO:50 and SEQ ID NO:56
SEQ ID NO:51 and SEQ ID NO:56	SEQ ID NO:52 and SEQ ID NO:56
SEQ ID NO:53 and SEQ ID NO:56	SEQ ID NO:54 and SEQ ID NO:56
SEQ ID NO:48 and SEQ ID NO:57	SEQ ID NO:49 and SEQ ID NO:57
SEQ ID NO:50 and SEQ ID NO:57	SEQ ID NO:51 and SEQ ID NO:57
SEQ ID NO:52 and SEQ ID NO:57	SEQ ID NO:53 and SEQ ID NO:57
SEQ ID NO:54 and SEQ ID NO:57.	

In a further embodiment, an anti-LAG-3 antibody according to the invention comprises VH and VL domains, wherein the two variable domains comprise amino acid sequences selected from the group consisting of:

SEQ ID NO:60 and SEQ ID NO:61	SEQ ID NO:60 and SEQ ID NO:62
SEQ ID NO:63 and SEQ ID NO:64	SEQ ID NO:65 and SEQ ID NO:66
SEQ ID NO:67 and SEQ ID NO:68	SEQ ID NO:69 and SEQ ID NO:70
SEQ ID NO:71 and SEQ ID NO:68	SEQ ID NO:74 and SEQ ID NO:75
SEQ ID NO:74 and SEQ ID NO:76	SEQ ID NO:74 and SEQ ID NO:77
SEQ ID NO:72 and SEQ ID NO:75	SEQ ID NO:72 and SEQ ID NO:76
SEQ ID NO:72 and SEQ ID NO:77	SEQ ID NO:73 and SEQ ID NO:75
SEQ ID NO:73 and SEQ ID NO:76	SEQ ID NO:73 and SEQ ID NO:77
SEQ ID NO:121 and SEQ ID NO:122	SEQ ID NO:123 and SEQ ID NO:124
SEQ ID NO:125 and SEQ ID NO:126	SEQ ID NO:127 and SEQ ID NO:128
SEQ ID NO:129 and SEQ ID NO:130	SEQ ID NO:131 and SEQ ID NO:132
SEQ ID NO:135 and SEQ ID NO:138	SEQ ID NO:136 and SEQ ID NO:139
SEQ ID NO:136 and SEQ ID NO:117	SEQ ID NO:226* and SEQ ID NO:138
SEQ ID NO:227* and SEQ ID NO:139	SEQ ID NO:227* and SEQ ID NO:117

* wherein SEQ ID NO:226 is the same as SEQ ID NO:135 except with an Ala (A) instead of Gly (G) at amino acid 56 (G55A substitution by Kabat numbering); and SEQ ID NO:227 is the same as SEQ ID NO:136 except with an Ala (A) instead of Gly (G) at amino acid 56 (G55A substitution by Kabat numbering).

In another embodiment, an anti-PD-1 antibody or an anti-LAG-3 antibody may be used to make derivative binding proteins recognizing the same target antigen by techniques well established in the field. Such a derivative may be, e.g., a single-chain antibody (scFv), a Fab fragment (Fab), an Fab' fragment, an $F(ab')_2$, an Fv, and a disulfide linked Fv.

In another aspect of the invention, an antibody or bispecific binding protein described herein is capable of modulating a biological function of PD-1, LAG-3, or both. In another aspect, an anti-PD-1 antibody described herein is capable of inhibiting PD-1/PD-L1 signaling. Signal inhibition can be measured in a mixed lymphocyte reaction assay, such as performed in the working examples, *infra*. In another aspect, an anti-LAG-3 antibody described herein is capable of inhibiting MHC Class II/LAG-3 interaction. Such inhibition can be measured in a

PBMC SEB activation assay, such as performed in the working examples, *infra*. In another aspect, bispecific PD-1/LAG-3 FIT-Ig binding protein described herein is capable of inhibiting both PD-1/PD-L1 signaling and MHC Class II/LAG-3 interaction.

In an embodiment, an anti-PD-1 antibody described herein or an antigen-binding fragment thereof has an on rate constant (k_{on}) to human PD-1 of at least 1×10^4 $M^{-1}s^{-1}$, at least 3×10^4 $M^{-1}s^{-1}$, at least 5×10^4 $M^{-1}s^{-1}$, at least 7×10^4 $M^{-1}s^{-1}$, at least 9×10^4 $M^{-1}s^{-1}$, at least 1×10^5 $M^{-1}s^{-1}$, at least 1.1×10^5 $M^{-1}s^{-1}$, at least 1×10^5 $M^{-1}s^{-1}$, at least 1.25×10^5 $M^{-1}s^{-1}$, at least 1.4×10^5 $M^{-1}s^{-1}$, at least 1.5×10^5 $M^{-1}s^{-1}$, at least 3×10^5 $M^{-1}s^{-1}$ or more, as measured by surface plasmon resonance or biolayer interferometry.

In another embodiment, an anti-PD-1 antibody described herein or antigen-binding fragment thereof has an off rate constant (k_{off}) to human PD-1 of less than $1 \times 10^{-3}s^{-1}$, less than $5 \times 10^{-4}s^{-1}$, less than $3 \times 10^{-4}s^{-1}$, less than $1 \times 10^{-4}s^{-1}$, less than $8 \times 10^{-5}s^{-1}$, less than $6 \times 10^{-5}s^{-1}$, less than $4 \times 10^{-5}s^{-1}$, less than $3 \times 10^{-5}s^{-1}$, or less than $1 \times 10^{-5}s^{-1}$, as measured by surface plasmon resonance or biolayer interferometry.

In another embodiment, an anti-PD-1 antibody described herein or antigen-binding fragment thereof has a dissociation constant (K_D) to PD-1 of less than 1×10^{-8} M, less than 5×10^{-9} M, less than 3×10^{-9} M, less than 1×10^{-9} M, less than 8×10^{-10} M, less than 6×10^{-10} M, less than 4×10^{-10} M, less than 2×10^{-10} M, or less than 1×10^{-10} M.

In an embodiment, an anti-LAG-3 antibody described herein or an antigen-binding fragment thereof has an on rate constant (k_{on}) to human LAG-3 of at least 5×10^3 $M^{-1}s^{-1}$, at least 7×10^3 $M^{-1}s^{-1}$, at least 1×10^4 $M^{-1}s^{-1}$, at least 3×10^4 $M^{-1}s^{-1}$, at least 5×10^4 $M^{-1}s^{-1}$, at least 7×10^4 $M^{-1}s^{-1}$, at least 1×10^5 $M^{-1}s^{-1}$, or at least 2×10^5 $M^{-1}s^{-1}$ or more, as measured by surface plasmon resonance or biolayer interferometry.

In another embodiment, an anti-LAG-3 antibody described herein or antigen-binding fragment thereof has an off rate constant (k_{off}) to human LAG-3 of less than $1.5 \times 10^{-3}s^{-1}$, less than $1 \times 10^{-3}s^{-1}$, less than $8 \times 10^{-4}s^{-1}$, less than $6 \times 10^{-4}s^{-1}$, less than $4 \times 10^{-4}s^{-1}$, less than $2 \times 10^{-4}s^{-1}$, less than $1 \times 10^{-4}s^{-1}$, less than $9 \times 10^{-5}s^{-1}$, less than $8 \times 10^{-5}s^{-1}$, less than $7 \times 10^{-5}s^{-1}$, less than $5 \times$

$10^{-5}s^{-1}$, less than $4 \times 10^{-5}s^{-1}$, less than $2 \times 10^{-5}s^{-1}$, or less than $1 \times 10^{-5}s^{-1}$, as measured by surface plasmon resonance or biolayer interferometry.

In another embodiment, an anti-LAG-3 antibody described herein or antigen-binding fragment thereof has a dissociation constant (K_D) to LAG-3 of less than 5×10^{-7} M, less than 2×10^{-7} M, less than 1×10^{-7} M, less than 8×10^{-8} M, less than 6×10^{-8} M, less than 4×10^{-8} M; less than 2×10^{-8} M; less than 1×10^{-8} M; less than 8×10^{-9} M; less than 6×10^{-9} M, less than 4×10^{-9} M; less than 2×10^{-9} M; or less than 1×10^{-9} M.

In an embodiment, a bispecific FIT-Ig binding protein capable of binding PD-1 and LAG-3 according to this invention has an on rate constant (k_{on}) to human PD-1 of at least 5×10^{3} $M^{-1}s^{-1}$, at least 1×10^{4} $M^{-1}s^{-1}$, at least 5×10^{4} $M^{-1}s^{-1}$, at least 1×10^{5} $M^{-1}s^{-1}$, at least 3×10^{5} $M^{-1}s^{-1}$, or at least 5×10^{5} $M^{-1}s^{-1}$, or more, and the same binding protein has an on rate constant (k_{on}) to human LAG-3 of at least 5×10^{3} $M^{-1}s^{-1}$, at least 1×10^{4} $M^{-1}s^{-1}$, at least 5×10^{4} $M^{-1}s^{-1}$, at least 1×10^{5} $M^{-1}s^{-1}$, at least 3×10^{5} $M^{-1}s^{-1}$, or at least 5×10^{5} $M^{-1}s^{-1}$, or more, as measured by surface plasmon resonance or biolayer interferometry. In further embodiments, a bispecific FIT-Ig binding protein capable of binding PD-1 and LAG-3 as described herein will have an on rate constant (k_{on}) to human PD-1 that is no more than a 10-fold decrease from the k_{on} for PD-1 of the parental anti-PD-1 antibody, and is no more than a 10-fold decrease from the k_{on} for LAG-3 of the parental anti-LAG-3 antibody from which the anti-PD-1 and anti-LAG-3 specificities, respectively, of the FIT-Ig binding protein were derived. In other words, the FIT-Ig binding protein will retain an on rate constant for each antigen (PD-1 or LAG-3) that is higher than, the same as, or no more than one order of magnitude less than the on rate constant (k_{on}) exhibited by the parental antibodies reactive with the respective PD-1 or LAG-3 antigens. As disclosed herein, a PD-1/LAG-3 FIT-Ig binding protein for antigen may show improvement in k_{on} for one or both antigens in comparison to the k_{on} for the respective antigens exhibited by the parental antibodies, or the k_{on} for one or both antigens may be essentially the same as exhibited by the parental antibodies, respectively, or, if there is a decrease in k_{on} for one or both antigens shown by the FIT-Ig binding protein in comparison to a parental antibody, then that decrease is no more than a 10-fold decrease. Preferably a decrease in k_{on} for a particular antigen in the FIT-Ig in

comparison to the k_{on} for that antigen of a parental antibody is less than 50%, more preferably less than a 25% decrease. Such high retained k_{on} values in the bispecific FIT-Ig in comparison to the k_{on}s of the parental antibodies is a surprising achievement in the field.

In an embodiment, a bispecific FIT-Ig binding protein capable of binding PD-1 and LAG-3 according to this invention has an off rate constant (k_{off}) to human PD-1 of less than $2 \times 10^{-4}s^{-1}$, less than $1 \times 10^{-4}s^{-1}$, less than $5 \times 10^{-5}s^{-1}$, less than $3 \times 10^{-5}s^{-1}$, less than $2 \times 10^{-5}s^{-1}$, less than $1 \times 10^{-5}s^{-1}$, or less than $8 \times 10^{-6}s^{-1}$, and the same binding protein has an off rate constant (k_{off}) to human LAG-3 of less than $2 \times 10^{-4}s^{-1}$, less than $1 \times 10^{-4}s^{-1}$, less than $5 \times 10^{-5}s^{-1}$, less than $3 \times 10^{-5}s^{-1}$, less than $2 \times 10^{-5}s^{-1}$, less than $1 \times 10^{-5}s^{-1}$, or less than $8 \times 10^{-6}s^{-1}$, as measured by surface plasmon resonance or biolayer interferometry.

In another embodiment, a bispecific FIT-Ig binding protein capable of binding PD-1 and LAG-3 according to this invention has a dissociation constant (K_D) to PD-1 of less than 2×10^{-8} M, less than 1×10^{-8} M, less than 5×10^{-9} M, less than 1×10^{-9} M, less than 6×10^{-10} M, less than 5×10^{-10} M, less than 3×10^{-10} M, less than 2×10^{-10} M, less than 1×10^{-10} M, less than 8×10^{-11} M, less than 6×10^{-11} M, less than 4×10^{-11} M, or less than 1×10^{-11} M, and the same binding protein has a dissociation constant (K_D) for human LAG-3 of less than 2×10^{-8} M, less than 1×10^{-8} M, less than 5×10^{-9} M, less than 1×10^{-9} M, less than 6×10^{-10} M, less than 5×10^{-10} M, less than 3×10^{-10} M, less than 2×10^{-10} M, less than 1×10^{-10} M, less than 8×10^{-11} M, less than 6×10^{-11} M, less than 4×10^{-11} M, or less than 1×10^{-11} M. In further embodiments, a bispecific FIT-Ig binding protein capable of binding PD-1 and LAG-3 as described herein will have a dissociation constant (K_D) to human PD-1 that is no more than 10-fold different from the K_D for PD-1 of the parental anti-PD-1 antibody, and is no more than 10-fold different from the K_D for LAG-3 of the parental anti-LAG-3 antibody from which the anti-PD-1 and anti-LAG-3 specificities, respectively, of the FIT-Ig binding protein were derived. In other words, the FIT-Ig binding protein will retain the binding affinity of the parental antibodies for each antigen (PD-1 or LAG-3) as indicated by a dissociation constant (K_D) that is within one order of magnitude of the K_D exhibited by the parental antibodies reactive with the PD-1 or LAG-3 antigens, respectively. As disclosed herein, a PD-1/LAG-3 FIT-Ig binding protein may show

improvement in K_D (i.e., has a lower K_D value; more tightly binds) for one or both antigens in comparison to the K_D for the respective antigens exhibited by the parental antibodies, or the K_D for one or both antigens may be essentially the same as exhibited by the parental antibodies, respectively, or the K_D for one or both antigens shown by the FIT-Ig binding protein may show a decrease (i.e., have a greater K_D value, binds less tightly) in comparison to the K_D of a parental antibody, but if there is a difference in K_D between FIT-Ig binding protein and parental antibody, then that difference is no more than a 10-fold difference. Preferably, a PD-1/LAG-3 FIT-Ig binding protein shows a lower K_D (binds more tightly) for one or both antigens in comparison to the K_D for the respective antigens exhibited by the one or both parental antibodies. Retention of the binding affinity of the parental anti-PD-1 and anti-LAG-3 antibodies ± 10-fold change in K_D is a surprising achievement in the field.

The invention also provides pharmaceutical compositions comprising at least one anti-PD-1 antibody or antigen-binding fragment thereof as described herein and a pharmaceutically acceptable carrier. The invention also provides pharmaceutical compositions comprising at least one anti-LAG-3 antibody or antigen-binding fragments thereof and a pharmaceutically acceptable carrier. The invention also provides pharmaceutical compositions comprising a combination of anti-PD-1 and anti-LAG-3 antibodies as described herein, or antigen-binding fragment(s) thereof, and a pharmaceutically acceptable carrier. The invention also provides bispecific, multivalent immunoglobulin binding proteins reactive with both PD-1 and LAG-3, which binding proteins incorporate VH/VL binding sites from anti-PD-1 and anti-LAG-3 antibodies described herein. In particular, the invention provides pharmaceutical compositions comprising at least one FIT-Ig binding protein capable of binding PD-1 and LAG-3 and a pharmaceutically acceptable carrier. Pharmaceutical compositions of the invention may further comprise at least one additional active ingredient. In an embodiment, such an additional ingredient includes, but is not limited to, a therapeutic agent, an imaging agent, a cytotoxic agent, an angiogenesis inhibitor, a kinase inhibitor, a co-stimulation molecule blocker, an adhesion molecule blocker, an antibody of different specificity or functional fragment thereof, a detectable label or reporter; an agonist or antagonist for particular cytokine(s), a narcotic, a non-steroid anti-

inflammatory drug (NSAID), an analgesic, an anesthetic, a sedative, a local anesthetic, a neuromuscular blocker, an antimicrobial agent, a corticosteroid, an anabolic steroid, an erythropoietin, an immunogen, an immunosuppressive agent, a growth hormone, a hormone replacement drug, a radiopharmaceutical, an antidepressant, an antipsychotic, a stimulant (e.g., an amphetamine, caffeine, etc.), a beta agonist, an inhaled steroid, an epinephrine or analog, a cytokine.

In another embodiment, a pharmaceutical composition further comprises at least one additional therapeutic agent for treating a disorder in which PD-1-mediated and/or LAG-3-mediated signaling activity is detrimental.

In a further embodiment, the invention provides isolated nucleic acids encoding one or more amino acid sequences of an anti-PD-1 antibody of the invention or an antigen-binding fragment thereof; isolated nucleic acids encoding one or more amino acid sequences of an anti-LAG-3 antibody of the invention or an antigen-binding fragment thereof; and isolated nucleic acids encoding one or more amino acid sequences of a bispecific Fabs-in-Tandem immunoglobulin (FIT-Ig) binding protein capable of binding both PD-1 and LAG-3. Such nucleic acids may be inserted into a vector for carrying out various genetic analyses or for expressing, characterizing, or improving one or more properties of an antibody or binding protein described herein. A vector may comprise a one or more nucleic acid molecules encoding one or more amino acid sequences of an antibody or binding protein described herein in which the one or more nucleic acid molecules is operably linked to appropriate transcriptional and/or translational sequences that permit expression of the antibody or binding protein in a particular host cell carrying the vector. Examples of vectors for cloning or expressing nucleic acids encoding amino acid sequences of binding proteins described herein include, but are not limited to, pcDNA, pTT, pTT3, pEFBOS, pBV, pJV, and pBJ, and derivatives thereof.

The invention also provides a host cell comprising a vector comprising a nucleic acid encoding one or more amino acid sequences of an antibody or binding protein described herein. Host cells useful in the invention may be prokaryotic or eukaryotic. An exemplary prokaryotic host cell is *Escherichia coli*. Eukaryotic cells useful as host cells in the invention include protist

cells, animal cells, plant cells, and fungal cells. An exemplary fungal cell is a yeast cell, including *Saccharomyces cerevisiae*. An exemplary animal cell useful as a host cell according to the invention includes, but is not limited to, a mammalian cell, an avian cell, and an insect cell. Preferred mammalian cells include, but are not limited to, CHO cells, HEK cells, and COS cells. An insect cell useful as a host cell according to the invention is an insect Sf9 cell.

In another aspect, the invention provides a method of producing anti-PD-1 antibody or a functional fragment thereof comprising culturing a host cell comprising an expression vector encoding the antibody or functional fragment in culture medium under conditions sufficient to cause expression by the host cell of the antibody or fragment capable of binding PD-1. In another aspect, the invention provides a method of producing anti-LAG-3 antibody or a functional fragment thereof comprising culturing a host cell comprising an expression vector encoding the antibody or functional fragment in culture medium under conditions sufficient to cause expression by the host cell of the antibody or fragment capable of binding LAG-3. In another aspect, the invention provides a method of producing a bispecific, multivalent binding protein capable of binding PD-1 and LAG-3, specifically a FIT-Ig binding protein binding PD-1 and LAG-3, comprising culturing a host cell comprising an expression vector encoding the FIT-Ig binding protein in culture medium under conditions sufficient to cause expression by the host cell of the binding protein capable of binding PD-1 and LAG-3. The proteins so produced can be isolated and used in various compositions and methods described herein.

In one embodiment, the present invention provides methods for treating cancer in a subject in need thereof, the method comprising administering to the subject an anti-PD-1 antibody or PD-1-binding fragment thereof as described herein, wherein the antibody or binding fragment is capable of binding PD-1 and inhibiting PD-1/PD-L1- or PD-1/PD-L2-mediated signaling in a cell expressing PD-1. In another embodiment, the present invention provides methods for treating cancer in a subject in need thereof, the method comprising administering to the subject an anti-LAG-3 antibody or LAG-3-binding fragment thereof as described herein, wherein the antibody or binding fragment is capable of binding LAG-3 and inhibiting MHC Class II/LAG-3-mediated signaling in a cell expressing LAG-3. In another embodiment, the

present invention provides methods for treating cancer in a subject in need thereof, the method comprising administering to the subject a bispecific FIT-Ig binding protein capable of binding LAG-3 and PD-1 as described herein, wherein the binding protein is capable of binding LAG-3 and PD-1 and of inhibiting MHC Class II/LAG-3-mediated signaling in a cell expressing LAG-3 and of inhibiting PD-1/PD-L1 or PD-1/PD-L2 signaling in a cell expressing PD-1. In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-684 of SEQ ID NO:102; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 20-235 of SEQ ID NO:105; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-236 of SEQ ID NO:107. (See Table 30.) In a further embodiment, a FIT-Ig binding protein of the present invention binds PD-1 and LAG-3 and is comprised of a first polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-687 of SEQ ID NO:189; a second polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 20-235 of SEQ ID NO:192; and a third polypeptide chain comprising, consisting essentially of, or consisting of the sequence of amino acids 23-236 of SEQ ID NO:194. (See Table 48.)

In another embodiment, the present invention provides methods for treating an autoimmune disease or a cancer in a subject in need thereof, wherein the binding protein is capable of binding LAG-3 and PD-1, and wherein the autoimmune disease or cancer is an autoimmune disease or cancer typically responsive to immunotherapy. In another embodiment, the cancer is a cancer that has not been associated with immunotherapy. In another embodiment, the cancer is a cancer that is a refractory or a recurring malignancy. In another embodiment, the binding protein inhibits the growth or survival of tumor cells. In another embodiment, the cancer is selected from the group consisting of melanoma (e.g., metastatic malignant melanoma), renal cancer (e.g. clear cell carcinoma), prostate cancer (e.g. hormone refractory prostate adenocarcinoma), pancreatic adenocarcinoma, breast cancer, colon cancer, lung cancer (e.g. non-small cell lung cancer), esophageal cancer, squamous cell carcinoma of the

head and neck, liver cancer, ovarian cancer, cervical cancer, thyroid cancer, glioblastoma, glioma, leukemia, lymphoma, and other neoplastic malignancies.

Methods of treatment described herein may further comprise administering to a subject in need thereof, of an immunostimulatory adjuvant, such as a CpG oligodeoxynucleotide (CpG ODN) comprising a full or partial phosphodiester or phosphorothioate backbone. For example, in a method of treatment of the invention, an immunostimulatory adjuvant may be incorporated into a composition comprising an antibody or FIT-Ig binding protein of the invention, and the composition administered to a subject in need of treatment. In another embodiment, a method of treatment of the invention may comprise a step of administering to a subject in need of treatment an antibody or FIT-Ig binding protein described herein and a separate step of administering an immunostimulatory adjuvant to the subject before, concurrently, or after the step of administering to the subject an antibody or FIT-Ig binding protein of the invention.

Brief Description of the Drawings

Figure 1A and 1B are bar graphs showing IL-2 production levels in a mixed lymphocyte reaction testing the effect of various anti-PD-1 antibodies disclosed herein, in comparison to two recombinant anti-PD-1 antibodies produced from published sequences (nivolumab and pembrolizumab) and control human and murine antibodies directed against irrelevant antigens ("hIgG4" and "mIgG"). Figure 1A and 1B show separate MLR tests, using responder lymphocytes from different donors.

Figure 2A and 2B are bar graphs showing gamma interferon (IFN-gamma) production levels in a mixed lymphocyte reaction testing the effect of various anti-PD-1 antibodies disclosed herein, in comparison to two recombinant anti-PD-1 antibodies produced from published sequences (nivolumab and pembrolizumab) and control human and murine antibodies directed against irrelevant antigens ("hIgG4" and "mIgG"). Figure 2A and 2B show separate MLR tests, using responder lymphocytes from different donors.

Figure 3 shows bar graphs of IL-2 production levels in a mixed lymphocyte reaction testing the effect of various humanized anti-PD-1 antibodies disclosed herein, in comparison to a

recombinant therapeutic anti-PD-1 antibody produced from published sequences (nivolumab), and a control human antibody directed against irrelevant antigen ("HuF0323-1").

Figure 4 shows bar graphs of IL-2 production levels in a mixed lymphocyte reaction testing the effect of various humanized anti-PD-1 antibodies disclosed herein, in comparison to the parental murine mAb709, a recombinant therapeutic anti-PD-1 antibody produced from published sequences (nivolumab), and a control human antibody directed against irrelevant antigen ("HuF0323-1").

Figure 5 shows bar graphs of IL-2 production levels in a mixed lymphocyte reaction testing the effect of various humanized anti-PD-1 antibodies disclosed herein, in comparison to a chimera with the parental murine mAb713 variable domains (mAb713c), a recombinant therapeutic anti-PD-1 antibody produced from published sequences (nivolumab), and a control human antibody directed against irrelevant antigen ("HuF0323-1"). Figures 3, 4, and 5 show separate MLR tests, using responder lymphocytes from different donors.

Figure 6 is a bar graph showing IL-2 production in a SEB T cell activation assay comparing the reversal of T cell suppression effect at various concentrations of two murine anti-LAG-3 antibodies described herein. The functionality of anti-LAG-3 antibodies of the invention ("3502-mAb746" and 3502-mAb747") is compared against a recombinant anti-LAG-3 mAb produced from a published sequence ("BMS-986016"), a recombinant murine anti-LAG-3 antibody produced from a published sequence ("BAP050"), and control human and murine antibodies directed against irrelevant antigens ("hIgG4" and "mIgG").

Figure 7 is a bar graph showing IL-2 production in a SEB T cell activation assay comparing the reversal of T cell suppression effect at various concentrations of several a FIT-Ig bispecific binding protein, FIT107-1-2a, described herein. The functionality of FIT107-1-2a is compared against a combination of a recombinant anti-LAG-3 mAb of known sequence (BMS-986016) and a recombinant anti-PD-1 mAb of known sequence (nivolumab), and against an anti-PD-1 antibody alone ("PD-1", mAb709 disclosed herein).

Figure 8 presents curves showing relative gamma interferon (IFN-g) production levels in a mixed lymphocyte reaction testing the effect of FIT107-1-2a bispecific FIT-Ig binding protein

at various concentrations, in comparison to a combination of a recombinant anti-LAG-3 mAb of known sequence (BMS-986016) and a recombinant anti-PD-1 mAb of known sequence (nivolumab), and against humanized anti-PD-1 antibody HumAb709-8 disclosed herein).

Figure 9 is a bar graph showing IL-2 production in a SEB T cell activation assay comparing the reversal of T cell suppression effect at various concentrations of a chimeric anti-LAG-3 antibody mAb747c and a humanized anti-LAG-3 antibody HumAb747-60. See, Example 13. The functionality of humanized anti-LAG-3 antibody of the invention (HumAb747-60) is compared against a chimeric anti-LAG-3 mAb produced using murine variable domains described herein and a human antibody directed against an irrelevant antigen ("hIgG4", control).

Figure 10 is a bar graph showing IL-2 production in a SEB T cell activation assay comparing the reversal of T cell suppression effect at various concentrations of a chimeric anti-LAG-3 antibody mAb747c and a high-affinity variants of humanized anti-LAG-3 antibody HumAb747-60 incorporating mutations indicated after affinity maturation experiments. See, Example 14. The functionality of anti-LAG-3 variant antibodies of the invention (HumAb747V-66 to HumAb747V-73) is compared against a chimeric anti-LAG-3 mAb produced using murine variable domains described herein and a human antibody directed against an irrelevant antigen ("hIgG4", control).

Figure 11 is a bar graph showing IL-2 production in a SEB T cell activation assay comparing the reversal of T cell suppression effect at various concentrations of a FIT-Ig binding protein specific for both LAG-3 and PD-1 targets. See, Example 16.2. The functionality of PD-1/LAG-3 FIT-Ig bispecific antibodies of the invention is compared against a combination of recombinant anti-PD-1 and anti-LAG-3 monoclonal antibodies prepared from published sequences ("nivolumab + BMS 986016") and a human antibody directed against an irrelevant antigen ("hIgG", control).

Figure 12 is a bar graph showing the results of a receptor blocking assay showing the ability of an anti-LAG-3 antibody according to the invention (HumAb747V-67) and a PD-1/LAG-3 FIT-Ig binding protein according to the invention (FIT107-1-7b-1) to block interaction

between human LAG-3 and fibrinogen-like protein 1 (FGL1). See, Example 16.5.

Figure 13 is a series of graphs evaluating the cell-surface binding to PD-1 and LAG-3 expressed on T cells. The results show that the bispecific FIT-Ig protein FIT107-1-7b-1 recognizes both PD-1 and LAG-3 surface proteins on T cells.

Detailed Description of the Invention

This invention pertains to novel anti-PD-1 antibodies, novel anti-LAG-3 antibodies, antigen-binding portions thereof, and multivalent, bispecific binding proteins such as Fabs-in-Tandem immunoglobulins (FIT-Igs) that bind both to PD-1 and LAG-3 targets. Various aspects of the invention relate to anti-PD-1 and anti-LAG-3 antibodies and antibody fragments, FIT-Ig binding proteins binding to human PD-1 and human LAG-3, and pharmaceutical compositions thereof, as well as nucleic acids, recombinant expression vectors and host cells for making such antibodies, functional antibody fragments, and binding proteins. Methods of using the antibodies, functional antibody fragments, and bispecific binding proteins of the invention to detect human PD-1, human LAG-3, or both; to inhibit human PD-1 and/or human LAG-3 activity, either *in vitro* or *in vivo*; and to treat diseases, especially cancer, that are mediated by PD-1 and/or LAG-3 binding to their respective ligands, i.e., PD-1 and MHC Class II, are also encompassed by the invention.

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. The meaning and scope of the terms should be clear, however, in the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. In this application, the use of "or" means "and/or" unless stated otherwise. Furthermore, the use of the term "including", as well as other forms, such as "includes" and "included", is not limiting. Also, terms such as "element" or "component" encompass both elements and components comprising one unit and elements and components that comprise more than one subunit unless specifically stated otherwise.

Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art. The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclatures used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

That the present invention may be more readily understood, select terms are defined below.

The term "polypeptide" refers to any polymeric chain of amino acids. The terms "peptide" and "protein" are used interchangeably with the term polypeptide and also refer to a polymeric chain of amino acids. The term "polypeptide" encompasses native or artificial proteins, protein fragments and polypeptide analogs of a protein amino acid sequence. The term "polypeptide" encompasses fragments and variants (including fragments of variants) thereof, unless otherwise contradicted by context. For an antigenic polypeptide, a fragment of polypeptide optionally contains at least one contiguous or nonlinear epitope of polypeptide. The precise boundaries of the at least one epitope fragment can be confirmed using ordinary skill in the art. The fragment comprises at least about 5 contiguous amino acids, such as at least about 10 contiguous amino acids, at least about 15 contiguous amino acids, or at least about 20 contiguous amino acids. A variant of a polypeptide is as described herein.

The term "isolated protein" or "isolated polypeptide" is a protein or polypeptide that by virtue of its origin or source of derivation is not associated with naturally associated components

that accompany it in its native state, is substantially free of other proteins from the same species, is expressed by a cell from a different species, or does not occur in nature. Thus, a polypeptide that is chemically synthesized or synthesized in a cellular system different from the cell from which it naturally originates will be "isolated" from its naturally associated components. A protein may also be rendered substantially free of naturally associated components by isolation, using protein purification techniques well known in the art.

The term "recovering" refers to the process of rendering a chemical species such as a polypeptide substantially free of naturally associated components by isolation, e.g., using protein purification techniques well known in the art.

The term "biological activity" refers to all inherent biological properties of the anti-PD-1 or anti-LAG-3 antibodies described herein. Biological properties of anti-PD-1 antibodies include, but are not limited to, binding to PD-1 protein; biological properties of anti-LAG-3 antibodies include, but are not limited to, binding to MHC Class II proteins.

The term "specific binding" or "specifically binding" in reference to the interaction of an antibody, a binding protein, or a peptide with a second chemical species, means that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the second chemical species. For example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

The term "antibody" broadly refers to any immunoglobulin (Ig) molecule comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains, or any functional fragment, mutant, variant, or derivation thereof, which retains the essential epitope binding features of an Ig molecule. Such mutant, variant, or derivative antibody formats are known in the art. Nonlimiting embodiments of which are discussed below.

In a full-length antibody, each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region

is comprised of three domains: CH1, CH2, and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Each VH and VL is comprised of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. First, second and third CDRs of a VH domain are commonly enumerated as CDR-H1, CDR-H2, and CDR-H3; likewise, first, second and third CDRs of a VL domain are commonly enumerated as CDR-L1, CDR-L2, and CDR-L3. 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.

The term "Fc region" is used to define the C-terminal region of an immunoglobulin heavy chain, which may be generated by papain digestion of an intact antibody. The Fc region may be a native sequence Fc region or a variant Fc region. The Fc region of an immunoglobulin generally comprises two constant domains, i.e., a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain, for example, as in the case of the Fc regions of IgM and IgE antibodies. The Fc region of IgG, IgA, and IgD antibodies comprises a hinge region, a CH2 domain, and a CH3 domain. In contrast, the Fc region of IgM and IgE antibodies lacks a hinge region but comprises a CH2 domain, a CH3 domain and a CH4 domain. Variant Fc regions having replacements of amino acid residues in the Fc portion to alter antibody effector function are known in the art (see, e.g., Winter et al., US Patent Nos. 5,648,260 and 5,624,821). The Fc portion of an antibody mediates several important effector functions, for example, cytokine induction, ADCC, phagocytosis, complement dependent cytotoxicity (CDC), and half-life/clearance rate of antibody and antigen-antibody complexes. In some cases these effector functions are desirable for therapeutic antibody but in other cases might be unnecessary or even deleterious, depending on the therapeutic objectives. Certain human IgG isotypes, particularly IgG1 and IgG3, mediate ADCC and CDC via binding to FcγRs and complement C1q, respectively. In still another embodiment at least one amino acid residue is replaced in the

constant region of the antibody, for example the Fc region of the antibody, such that effector functions of the antibody are altered. The dimerization of two identical heavy chains of an immunoglobulin is mediated by the dimerization of CH3 domains and is stabilized by the disulfide bonds within the hinge region that connects CH1 constant domains to the Fc constant domains (e.g., CH2 and CH3). The anti-inflammatory activity of IgG is completely dependent on sialylation of the N-linked glycan of the IgG Fc fragment. The precise glycan requirements for anti-inflammatory activity have been determined, such that an appropriate IgG1 Fc fragment can be created, thereby generating a fully recombinant, sialylated IgG1 Fc with greatly enhanced potency (see, Anthony et al., *Science*, 320:373-376 (2008)).

The terms "antigen-binding portion" and "antigen-binding fragment" or "functional fragment" of an antibody are used interchangeably and refer to one or more fragments of an antibody that retain the ability to specifically bind to an antigen, i.e., the same antigen (e.g., PD-1, LAG-3) as the full-length antibody from which the portion or fragment is derived. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Such antibody embodiments may also be bispecific, dual specific, or multi-specific formats; specifically binding to two or more different antigens (e.g., PD-1 and a different antigen, such as LAG-3). Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL, and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., *Nature*, 341: 544-546 (1989); PCT Publication No. WO 90/05144), which comprises a single variable domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see, for example, Bird et al., *Science*, 242: 423-426 (1988); and Huston et al., *Proc. Natl.*

Acad. Sci. USA, 85: 5879-5883 (1988)). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody and equivalent terms given above. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see, for example, Holliger et al., *Proc. Natl. Acad. Sci. USA*, 90: 6444-6448 (1993). Such antibody binding portions are known in the art (Kontermann and Dübel eds., Antibody Engineering (Springer-Verlag, New York, 2001), p. 790 (ISBN 3-540-41354-5)). In addition, single chain antibodies also include "linear antibodies" comprising a pair of tandem Fv segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata et al., *Protein Eng.*, 8(10): 1057-1062 (1995); and US Patent No. 5,641,870)).

An immunoglobulin constant (C) domain refers to a heavy (CH) or light (CL) chain constant domain. Murine and human IgG heavy chain and light chain constant domain amino acid sequences are known in the art.

The term "monoclonal antibody" or "mAb" refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic determinant (epitope). Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each mAb is directed against a single determinant on the antigen. The modifier "monoclonal" is not to be construed as requiring production of the antibody by any particular method.

The term "human antibody" includes antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by

somatic mutation *in vivo*), for example in the CDRs and in particular CDR3. However, the term "human antibody" does not include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "recombinant human antibody" includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial human antibody library (Hoogenboom, H.R., *Trends Biotechnol.*, 15: 62-70 (1997); Azzazy and Highsmith, *Clin. Biochem.*, 35: 425-445 (2002); Gavilondo and Larrick, *BioTechniques*, 29: 128-145 (2000); Hoogenboom and Chames, *Immunol. Today*, 21: 371-378 (2000)), antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes (see, e.g., Taylor et al., *Nucl. Acids Res.*, 20: 6287-6295 (1992); Kellermann and Green, *Curr. Opin. Biotechnol.*, 13: 593-597 (2002); Little et al., *Immunol. Today*, 21: 364-370 (2000)); or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

The term "chimeric antibody" refers to antibodies that comprise heavy and light chain variable region sequences from one species and constant region sequences from another species, such as antibodies having murine heavy and light chain variable regions linked to human constant regions.

The term "CDR-grafted antibody" refers to antibodies that comprise heavy and light chain variable region sequences from one species but in which the sequences of one or more of the CDR

regions of VH and/or VL are replaced with CDR sequences of another species, such as antibodies having human heavy and light chain variable regions in which one or more of the human CDRs has been replaced with murine CDR sequences.

The term "humanized antibody" refers to antibodies that comprise heavy and light chain variable region sequences from a non-human species (e.g., a mouse) but in which at least a portion of the VH and/or VL sequence has been altered to be more "human-like", i.e., more similar to human germline variable sequences. One type of humanized antibody is a CDR-grafted antibody, in which CDR sequences from a non-human species (e.g., mouse) are introduced into human VH and VL framework sequences. A humanized antibody is an antibody or a variant, derivative, analog or fragment thereof which immunospecifically binds to an antigen of interest and which comprises framework regions and constant regions having substantially the amino acid sequence of a human antibody but complementarity determining regions (CDRs) having substantially the amino acid sequence of a non-human antibody. As used herein, the term "substantially" in the context of a CDR refers to a CDR having an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the amino acid sequence of a non-human antibody CDR. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (i.e., donor antibody) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. In an embodiment, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. In some embodiments, a humanized antibody contains both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain. In some embodiments, a humanized antibody only contains a humanized light chain. In some embodiments, a humanized antibody only contains a humanized heavy chain. In specific embodiments, a humanized antibody only contains a humanized variable domain of a light chain and/or humanized heavy chain.

A humanized antibody may be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including without limitation IgG1, IgG2, IgG3, and IgG4. The humanized antibody may comprise sequences from more than one class or isotype, and particular constant domains may be selected to optimize desired effector functions using techniques well known in the art.

The framework and CDR regions of a humanized antibody need not correspond precisely to the parental sequences, e.g., the donor antibody CDR or the acceptor framework may be mutagenized by substitution, insertion and/or deletion of at least one amino acid residue so that the CDR or framework residue at that site does not correspond to either the donor antibody or the consensus framework. In an exemplary embodiment, such mutations, however, will not be extensive. Usually, at least 80%, preferably at least 85%, more preferably at least 90%, and most preferably at least 95% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences. Back mutation at a particular framework position to restore the same amino acid that appears at that position in the donor antibody is often utilized to preserve a particular loop structure or to correctly orient the CDR sequences for contact with target antigen.

The term "CDR" refers to the complementarity determining regions within antibody variable domain sequences. There are three CDRs in each of the variable regions of the heavy chain and the light chain, which are designated CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2, and CDR-L3. The term "CDR set" as used herein refers to a group of three CDRs that occur in a single variable region capable of binding the antigen. The exact boundaries of these CDRs have been defined differently according to different systems. The system described by Kabat (Kabat et al., Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Maryland (1987) and (1991)) not only provides an unambiguous residue numbering system applicable to any variable region of an antibody, but also provides precise residue boundaries defining the three CDRs.

The term "Kabat numbering", which is recognized in the art, refers to a system of numbering amino acid residues which are more variable (i.e., hypervariable) than other amino

acid residues in the heavy and light chain variable regions of an antibody or an antigen-binding portion thereof. See, Kabat et al., *Ann. NY Acad. Sci.*, 190: 382-391 (1971); and Kabat et al., Sequences of Proteins of Immunological Interest, *Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242 (1991).

The growth and analysis of extensive public databases of amino acid sequences of variable heavy and light regions over the past twenty years have led to the understanding of the typical boundaries between framework regions (FRs) and CDR sequences within variable region sequences and have enabled persons skilled in the art to accurately determine the CDRs according to Kabat numbering, Chothia numbering, or other systems. See, e.g., Martin, "Protein Sequence and Structure Analysis of Antibody Variable Domains," *In* Kontermann and Dübel, eds., Antibody Engineering (Springer-Verlag, Berlin, 2001), chapter 31, pages 432-433.

The term "multivalent binding protein" denotes a binding protein comprising two or more antigen binding sites. A multivalent binding protein is preferably engineered to have three or more antigen binding sites, and is generally not a naturally occurring antibody. The term "bispecific binding protein" refers to a binding protein capable of binding two targets of different specificity. "Fabs-in-Tandem immunoglobulin" (FIT-Ig) binding proteins of the invention comprise two or more antigen binding sites and are typically tetravalent binding proteins. A FIT-Ig may be monospecific, i.e., capable of binding one antigen, or multispecific, i.e., capable of binding two or more antigens. A preferred FIT-Ig according to this invention binds both PD-1 and LAG-3 and, therefore, is bispecific. A FIT-Ig binding protein comprising two long (heavy) V-C-V-C-Fc chain polypeptides and four short (light) V-C chain polypeptides forms a hexamer exhibiting four Fab antigen binding sites (VH-CH1 paired with VL-CL, sometimes notated VH-CH1::VL-CL). Each half of a FIT-Ig comprises a heavy chain polypeptide and two light chain polypeptides, and complementary immunoglobulin pairing of the VH-CH1 and VL-CL elements of the three chains results in two Fab-structured antigen binding sites, arranged in tandem. In the present invention, it is preferred that the immunoglobulin domains comprising the Fab elements are directly fused in the heavy chain polypeptide, without the use of interdomain linkers. That is, the N-terminal V-C element of the long (heavy) polypeptide chains

is directly fused at its C-terminus to the N-terminus of another V-C element, which in turn is linked to a C-terminal Fc region. In bispecific FIT-Ig binding proteins, the tandem Fab elements will be reactive with different antigens. Each Fab antigen binding site comprises a heavy chain variable domain and a light chain variable domain with a total of six CDRs per antigen binding site.

A description of the design, expression, and characterization of FIT-Ig molecules is provided in PCT Publication WO 2015/103072. A preferred example of such FIT-Ig molecules comprises a heavy chain and two different light chains. The heavy chain comprises the structural formula VL_A-CL-VH_B-CH1-Fc where CL is directly fused to VH_B or VH_B-CH1-VL_A-CL-Fc where CH1 is directly fused to VL_A, wherein VL_A is a variable light domain from a parental antibody that binds antigen A, VH_B is a variable heavy domain from a parental antibody that binds antigen B, CL is a light chain constant domain, CH1 is a heavy chain constant domain, and Fc is an immunoglobulin Fc region (e.g., the C-terminal hinge-CH2-CH3 portion of a heavy chain of an IgG1 antibody). The two light polypeptide chains of the FIT-Ig have the formulas VH_A-CH1 and VL_B-CL, respectively. In bispecific FIT-Ig embodiments, antigen A and antigen B are different antigens, or different epitopes of the same antigen. In the present invention, one of A and B is PD-1 and the other is LAG-3.

The term "activity" includes properties such as the ability to bind a target antigen with specificity, the affinity of an antibody for an antigen, the ability to neutralize the biological activity of a target antigen, the ability to inhibit interaction of a target antigen with its natural receptor(s), and the like. Preferred antibodies and binding proteins of the present invention have the ability to inhibit PD-1 binding to its ligand PD-L1, the ability to inhibit LAG-3 binding to its ligand MHC Class II, or both in the case of bispecific binding proteins described herein.

The term "k_{on}" (also "Kon", "kon"), as used herein, is intended to refer to the on rate constant for association of a binding protein (e.g., an antibody) to an antigen to form an association complex, e.g., antibody/antigen complex, as is known in the art. The "k_{on}" also is known by the terms "association rate constant", or "ka", as used interchangeably herein. This

value indicates the binding rate of an antibody to its target antigen or the rate of complex formation between an antibody and antigen as is shown by the equation below:

Antibody ("Ab") + Antigen ("Ag")→Ab-Ag.

The term "k_{off}" (also "Koff", "koff"), as used herein, is intended to refer to the off rate constant for dissociation, or "dissociation rate constant", of a binding protein (e.g., an antibody) from an association complex (e.g., an antibody/antigen complex) as is known in the art. This value indicates the dissociation rate of an antibody from its target antigen or separation of Ab-Ag complex over time into free antibody and antigen as shown by the equation below:

Ab + Ag←Ab-Ag.

The term "K_D" (also "K_d"), as used herein, is intended to refer to the "equilibrium dissociation constant" and refers to the value obtained in a titration measurement at equilibrium, or by dividing the dissociation rate constant (k_{off}) by the association rate constant (k_{on}). The association rate constant (k_{on}), the dissociation rate constant (k_{off}), and the equilibrium dissociation constant (K_D) are used to represent the binding affinity of an antibody to an antigen. Methods for determining association and dissociation rate constants are well known in the art. Using fluorescence-based techniques offers high sensitivity and the ability to examine samples in physiological buffers at equilibrium. Other experimental approaches and instruments such as a BIAcore® (biomolecular interaction analysis) assay can be used (e.g., instrument available from BIAcore International AB, a GE Healthcare company, Uppsala, Sweden). Biolayer interferometry (BLI) using, e.g., the Octet® RED96 system (Pall FortéBio LLC), is another affinity assay technique. Additionally, a KinExA® (Kinetic Exclusion Assay) assay, available from Sapidyne Instruments (Boise, Idaho) can also be used.

The term "isolated nucleic acid" shall mean a polynucleotide (e.g., of genomic, cDNA, or synthetic origin, or some combination thereof) that, by human intervention, is not associated with all or a portion of the polynucleotides with which it is found in nature; is operably linked to a polynucleotide that it is not linked to in nature; or does not occur in nature as part of a larger sequence.

The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequence. "Operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act *in trans* or at a distance to control the gene of interest. The term "expression control sequence" as used herein refers to polynucleotide sequences that are necessary to effect the expression and processing of coding sequences to which they are ligated. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability;

and when desired, sequences that enhance protein secretion. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader or signal sequences and fusion partner sequences.

"Transformation", as defined herein, refers to any process by which exogenous DNA enters a host cell. Transformation may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the host cell being transformed and may include, but is not limited to, transfection, viral infection, electroporation, lipofection, and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. They also include cells which transiently express the inserted DNA or RNA for limited periods of time.

The term "recombinant host cell" (or simply "host cell"), is intended to refer to a cell into which exogenous DNA has been introduced. In an embodiment, the host cell comprises two or more (e.g., multiple) nucleic acids encoding antibodies, such as the host cells described in US Patent No. 7,262,028, for example. Such terms are intended to refer not only to the particular subject cell, but also to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. In an embodiment, host cells include prokaryotic and eukaryotic cells selected from any of the Kingdoms of life. In another embodiment, eukaryotic cells include protist, fungal, plant and animal cells. In another embodiment, host cells include but are not limited to the prokaryotic cell line *Escherichia coli*; mammalian cell lines CHO, HEK 293, COS,

NS0, SP2 and PER.C6; the insect cell line Sf9; and the fungal cell *Saccharomyces cerevisiae*.

Standard techniques may be used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures may be generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

The term "agonist", as used herein, refers to a modulator that, when contacted with a molecule of interest, causes an increase in the magnitude of a certain activity or function of the molecule compared to the magnitude of the activity or function observed in the absence of the agonist. The terms "antagonist" and "inhibitor", as used herein, refer to a modulator that, when contacted with a molecule of interest causes a decrease in the magnitude of a certain activity or function of the molecule compared to the magnitude of the activity or function observed in the absence of the antagonist. Particular antagonists of interest include those that block or reduce the biological or immunological activity of human PD-1 and human LAG-3.

As used herein, the term "effective amount" refers to the amount of a therapy that is sufficient to reduce or ameliorate the severity and/or duration of a disorder or one or more symptoms thereof; prevent the advancement of a disorder; cause regression of a disorder; prevent the recurrence, development, or progression of one or more symptoms associated with a disorder; detect a disorder; or enhance or improve the prophylactic or therapeutic effect(s) of another therapy (e.g., prophylactic or therapeutic agent).

Production of Anti-PD-1 and Anti-LAG-3 Antibodies

Anti-PD-1 and anti-LAG-3 antibodies of the present invention may be produced by any of a number of techniques known in the art. For example, expression from host cells, wherein expression vector(s) encoding the heavy and light chains is (are) transfected into a host cell by

standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection, and the like. Although it is possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells is preferable, and most preferable in mammalian host cells, because such eukaryotic cells (and in particular mammalian cells) are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody.

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including $dhfr^-$ CHO cells, described in Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216-4220 (1980), used with a DHFR selectable marker, e.g., as described in Kaufman and Sharp, *J. Mol. Biol.*, 159: 601-621 (1982)), NS0 myeloma cells, COS cells, and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce functional antibody fragments, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding functional fragments of either the light chain and/or the heavy chain of an antibody of this invention. Recombinant DNA technology may also be used to remove some, or all, of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to the antigens of interest. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than the antigens

of interest by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In an exemplary system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr$^-$ CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transfected host cells are cultured to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transfectants, culture the host cells and recover the antibody from the culture medium. Still further the invention provides a method of making a recombinant anti-PD-1 or anti-LAG-3 antibody of the invention by culturing a transfected host cell of the invention in a suitable culture medium until a recombinant antibody of the invention is produced. The method can further comprise isolating the recombinant antibody from the culture medium.

Production of Bispecific FIT-Igs Binding PD-1 and LAG-3

Clinical studies using immune checkpoint inhibitors such as antibodies targeting PD-1, PD-L1, and CTLA-4 have led to promising results, however it has been observed that only a subset of patients initially respond to these inhibitors, and increasing clinical evidence indicates that a substantial proportion of initial responders ultimately relapse, with lethal, drug-resistant disease months or years later. Syn et al., *The Lancet Oncology*, 18(12):e731–e741 (2017). Both LAG-3 and PD-1 are co-expressed on tolerized tumor infiltrated lymphocytes (TILS), contributing to immune suppression in tumors; and dual blockade of LAG-3 and PD-1 has been proposed as a means to restore antitumor function in CD8+ T cells. Matsuzaki et al., *Proc. Natl. Acad. Sci. USA*, 107(17): 7875-7880 (2010). Accordingly, the design of LAG-3/PD-1 bispecific

binding proteins that can block both targets on immune suppressed T cells simultaneously, may provide an advance in this therapeutic area.

This invention provides Fabs-in-Tandem immunoglobulin binding proteins (FIT-Igs) that bind to both PD-1 and LAG-3. An exemplary embodiment of such FIT-Ig molecules comprises (1) a heavy polypeptide chain that comprises either the structural formula (i) VL_A-CL-VH_B-CH1-Fc wherein CL is directly fused to VH_B, or the structural formula (ii) VH_B-CH1-VL_A-CL-Fc wherein CH1 is directly fused to VL_A; (2) a light polypeptide chain of the formula VH_A-CH1; and (3) another light polypeptide chain of the formula VL_B-CL,

wherein VL is a light chain variable domain, CL is a light chain constant domain, VH is a heavy chain variable domain, CH1 is a heavy chain constant domain, Fc is an immunoglobulin Fc region, A is an epitope of PD-1 or LAG-3 and B is an epitope of PD-1 or LAG-3, with the proviso that A and B are different. In accordance with the present invention, such FIT-Ig binding proteins bind to both PD-1 and LAG-3.

A FIT-Ig may comprise two such heavy chains (1), two such light chains (2), and two such light chains (3), forming a six-chain binding protein monomer exhibiting four functional Fab antigen binding sites. Such a FIT-Ig binding protein comprises two identical subunits, wherein each subunit comprises one heavy chain (1), one light chain (2), and one light chain (3) that together form a pair of Fab binding sites arranged in tandem. Pairing of the Fc regions of two such subunits yields a six-chain, bispecific, FIT-Ig binding protein of the invention having a total of four functional Fab binding units.

It is possible to use a peptide linker on the heavy chain to separate the tandemly connected Fab moieties, however for bispecific FIT-Igs according to the invention the omission of such linker sequences is preferred. Whereas in multivalent engineered immunoglobulin formats having tandem binding sites, it was commonly understood in the field that the adjacent binding sites would interfere with each other unless a flexible linker was used to separate the binding sites spatially. It has been discovered for the PD-1/LAG-3 FIT-Igs of the present invention, however, that the arrangement of the immunoglobulin domains according to the chain formulas given above results in polypeptide chains that are well-expressed in transfected

mammalian cells, assemble appropriately, and are secreted as bispecific, multivalent immunoglobulin-like binding proteins that bind the target antigens PD-1 and LAG-3. See, Example 10, *infra*. Despite the absence of any linker sequences between the Fab binding sites, the PD-1/LAG-3 FIT-Igs of the invention retain the binding affinities for the target antigens, exhibiting comparable binding affinities to the parental mAbs. Moreover, omission of synthetic linker sequences from the binding proteins can avoid the creation of antigenic sites recognizable by mammalian immune systems, and in this way the elimination of linkers decreases possible immunogenicity of the FIT-Igs and leads to a half-life in circulation that is like a natural antibody, that is, the FIT-Ig is not rapidly cleared through immune opsonization and capture in the liver.

Each variable domain (VH or VL) in a FIT-Ig may be obtained from one or more "parental" monoclonal antibodies that bind one of the target antigens, i.e., PD-1 or LAG-3. FIT-Ig binding proteins are advantageously produced using variable domain sequences of anti-PD-1 and anti-LAG-3 monoclonal antibodies as disclosed herein. Preferably, the parental antibodies are humanized antibodies.

An aspect of the present invention pertains to selecting parental antibodies with at least one or more properties desired in the FIT-Ig molecule. In an embodiment, the antibody properties are selected from the group consisting of antigen specificity, affinity to antigen, potency, biological function, epitope recognition, stability, solubility, production efficiency, lack of immunogenicity, pharmacokinetics, bioavailability, tissue cross-reactivity, and orthologous antigen binding. PD-1 and LAG-3 are both cell surface proteins, and interaction with their respective ligands PD-L1 (cell surface receptor) and MHC Class II (surface proteins on antigen presenting cells) lead to intracellular signaling involved with T cell suppression and immune response. Accordingly, the ability of anti-PD-1 antibodies, anti-LAG-3 antibodies, and PD-1/LAG-3 FIT-Ig binding proteins according to the invention to inhibit PD-1/PD-L1 and/or MHC Class II/LAG-3 interaction makes them potent regulators of immune cell activation and immune effector cell activity.

Antibodies, functional fragments thereof, and binding proteins according to the invention

may be purified (for an intended use) by using one or more of a variety of methods and materials available in the art for purifying antibodies and binding proteins. Such methods and materials include, but are not limited to, affinity chromatography (e.g., using resins, particles, or membranes conjugated to Protein A, Protein G, Protein L, or a specific ligand of the antibody, functional fragment thereof, or binding protein), ion exchange chromatography (for example, using ion exchange particles or membranes), hydrophobic interaction chromatography ("HIC"; for example, using hydrophobic particles or membranes), ultrafiltration, nanofiltration, diafiltration, size exclusion chromatography ("SEC"), low pH treatment (to inactivate contaminating viruses), and combinations thereof, to obtain an acceptable purity for an intended use. A non-limiting example of a low pH treatment to inactivate contaminating viruses comprises reducing the pH of a solution or suspension comprising an antibody, functional fragment thereof, or binding protein of the invention to pH 3.5 with 0.5 M phosphoric acid, at 18°C - 25°C, for 60 to 70 minutes.

Uses of Antibodies and Binding Proteins of the Invention

Given their ability to bind to human PD-1 and/or LAG-3, the antibodies described herein, functional fragments thereof, and bispecific multivalent binding proteins described herein can be used to detect PD-1 or LAG-3, or both, e.g., in a biological sample containing cells that express one or both of those target antigens. The antibodies, functional fragments, and binding proteins of the invention can be used in a conventional immunoassay, such as an enzyme linked immunosorbent assay (ELISA), a radioimmunoassay (RIA), or tissue immunohistochemistry. The invention provides a method for detecting PD-1 or LAG-3 in a biological sample comprising contacting a biological sample with an antibody, antigen-binding portion thereof, or binding protein of the invention and detecting whether binding to a target antigen occurs, thereby detecting the presence or absence of the target in the biological sample. The antibody, functional fragment, or binding protein may be directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound antibody/fragment/binding protein. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include

horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase. Examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include ^{3}H, ^{14}C, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I, ^{177}Lu, ^{166}Ho, or ^{153}Sm.

The antibodies, functional fragments thereof, and binding proteins of the invention preferably are capable of neutralizing human PD-1 and/or human LAG-3 activity both *in vitro* and *in vivo*. Accordingly, the antibodies, functional fragments thereof, and binding proteins of the invention can be used to inhibit human PD-1 and/or human LAG-3 activity, e.g., inhibit cell signaling mediated by PD-1/PD-L1 (or PD-1/PD-L2) interaction and/or MHC Class II/LAG-3 interaction in a cell culture containing PD-1-expressing and/or LAG-3-expressing cells, in human subjects, or in other mammalian subjects having PD-1 or LAG-3 with which an antibody, functional fragment thereof, or binding protein of the invention cross-reacts. In one embodiment, the invention provides a method for restoring the activity of activated T cells (reversing suppression) comprising contacting human PD-1-expressing cells with an anti-PD-1 antibody or PD-1 binding protein of the invention such that PD-1 activity is inhibited. In another embodiment, the invention provides a method for restoring the activity of activated T cells (reversing suppression) comprising contacting human LAG-3-expressing cells with an anti-LAG-3 antibody or LAG-3 binding protein of the invention such that LAG-3 activity is inhibited.

In another embodiment, the invention provides a method for treating a subject suffering from a disease or disorder in which PD-1 and/or LAG-3 activity is detrimental, such method comprising administering to the subject an antibody or binding protein of the invention in an effective amount, such that activity mediated by PD-1/PD-L1 or PD-1/PD-L2 binding and/or MHC Class II/LAG-3 binding in the subject is reduced.

As used herein, the term "a disorder in which PD-1 and/or LAG-3 activity is detrimental" is intended to include diseases and other disorders in which the interaction of PD-1 with one or both of its ligands (PD-L1, PD-L2) or the interaction of LAG-3 with its ligand (MHC Class II) in

a subject suffering from the disorder is either responsible for the pathophysiology of the disorder or is a factor that contributes to a worsening of the disorder. Accordingly, a disorder in which PD-1 and/or LAG-3 activity is detrimental is a disorder in which inhibition of PD-1 and/or LAG-3 activity is expected to alleviate the symptoms and/or progression of the disorder.

In another embodiment, the present invention provides methods for treating an autoimmune disease or a cancer in a subject in need thereof, comprising administering to the subject an antibody, functional fragment thereof, or a binding protein described herein that is capable of binding LAG-3, PD-1, or both LAG-3 and PD-1, and wherein the autoimmune disease or cancer is a disease that is responsive to immunotherapy. In another embodiment, a method of the invention is used for treating an autoimmune disease or cancer that has not been associated with immunotherapy. In another embodiment, a method of the invention is used for treating a cancer that is a refractory or a recurring malignancy. In another embodiment, a LAG-3 or PD-1 antibody, functional fragment thereof, or a LAG-3/PD-1 bispecific binding protein of the invention is used in a method that inhibits the growth or survival of tumor cells.

In another embodiment, the invention provides a method for treating cancer in a subject comprising the step of administering to the subject an antibody to PD-1 or LAG-3 described herein, a functional fragment thereof, or a LAG-3/PD-1 bispecific binding protein described herein, e.g., such as a Fabs-in-tandem immunoglobulin (FIT-Ig) binding protein, wherein the cancer is selected from any of a group consisting of: a melanoma (e.g., metastatic malignant melanoma), a renal cancer (e.g., clear cell carcinoma), a prostate cancer (e.g. hormone refractory prostate adenocarcinoma), a pancreatic adenocarcinoma, a breast cancer, a colon cancer, a lung cancer (e.g. non-small cell lung cancer), an esophageal cancer, a squamous cell carcinoma of the head and neck, a liver cancer, an ovarian cancer, a cervical cancer, a thyroid cancer, a glioblastoma, a glioma, a leukemia, a lymphoma, a primary bone cancer (e.g., osteosarcoma, Ewing sarcoma, malignant fibrous histiocytoma, and chondrosarcoma), a metastatic cancer, and other neoplastic malignancies.

The invention also provides pharmaceutical compositions comprising an antibody, or antigen-binding portion thereof, or a bispecific multivalent binding protein of the invention (i.e.,

the primary active ingredient) and a pharmaceutically acceptable carrier. The pharmaceutical compositions comprising proteins of the invention are for use in, but not limited to, diagnosing, detecting, or monitoring a disorder; treating, managing, or ameliorating a disorder or one or more symptoms thereof; and/or research. In a specific embodiment, a composition comprises one or more antibodies or binding proteins of the invention. In another embodiment, the pharmaceutical composition comprises one or more antibodies or binding proteins of the invention and one or more prophylactic or therapeutic agents other than antibodies or binding proteins of the invention for treating a disorder in which PD-1 and/or LAG-3 activity is detrimental. In an embodiment, the prophylactic or therapeutic agents are known to be useful for or have been or currently are being used in the prevention, treatment, management, or amelioration of a disorder or one or more symptoms thereof. In accordance with these embodiments, the composition may further comprise a carrier, diluent. or excipient. An excipient is generally any compound or combination of compounds that provides a desired feature to a composition other than that of the primary active ingredient (i.e., other than an antibody, functional portion thereof, or binding protein of the invention).

The antibodies (including functional fragments thereof) and binding proteins of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises an antibody or binding protein of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols (such as, mannitol or sorbitol), or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives, or buffers, which enhance the shelf life or effectiveness of the antibody or binding protein present in the

composition.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include, but are not limited to, parenteral (e.g., intravenous, intradermal, subcutaneous, intramuscular), oral, intranasal (e.g., inhalation), transdermal (e.g., topical), intratumoral, transmucosal, and rectal administration. In a specific embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous, subcutaneous, intramuscular, oral, intranasal, or topical administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic, such as lidocaine (xylocaine, lignocaine), to ease pain at the site of the injection.

The method of the invention may comprise administration of a composition formulated for parenteral administration by injection (e.g., by bolus injection or continuous infusion). Formulations for injection may be presented in unit dosage form (e.g., in ampoules or in multi-dose containers) with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the primary active ingredient may be in powder form for constitution with a suitable vehicle (e.g., sterile pyrogen-free water) before use.

The methods of the invention may additionally comprise administration of compositions formulated as depot preparations. Such long acting formulations may be administered by implantation (e.g., subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compositions may be formulated with suitable polymeric or hydrophobic materials (e.g., as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives (e.g., as a sparingly soluble salt).

An antibody, functional fragment thereof, or binding protein of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of various diseases. Antibodies, functional fragments thereof, and binding proteins described herein can be

used alone or in combination with an additional agent, e.g., a therapeutic agent, said additional agent being selected by the skilled artisan for its intended purpose. For example, the additional agent can be a therapeutic agent art-recognized as being useful to treat the disease or condition being treated by the antibody or binding protein of the present invention. The additional agent also can be an agent that imparts a beneficial attribute to the therapeutic composition, e.g., an agent that affects the viscosity of the composition.

Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included for purposes of illustration only and are not intended to be limiting of the invention.

Examples

Example 1: Generation of Anti-human PD-1 Monoclonal Antibodies

Anti-human PD-1 monoclonal antibodies were generated as follows:

Example 1.1: Immunization with Human PD-1 Antigen

50 μg of recombinant purified human PD-1 extracellular domain (ECD) polypeptide mixed with Complete Freund's adjuvant were injected intraperitoneally into five 6-8 week-old Balb/C and five SJL mice on Day 1. On days 16 and 26, 25 μg of recombinant purified human PD-1 ECD immunogen mixed with Incomplete Freund's adjuvant were injected intraperitoneally into the same mice. A final boost with 25 μg of the immunogen was given 3-4 days before fusion.

Example 1.2: Generation of Hybridomas

Splenocytes obtained from the immunized mice described in Example 1.1 were fused with SP2/0-Ag-14 cells at a ratio of 5:1 according to the established method described in Kohler and Milstein, *Nature*, 256: 495-497 (1975) to generate hybridomas. Fusion products were plated in selection media containing hypoxanthine-aminopterin-thymidine (HAT) in 96-well plates at a density of 1×10^5 spleen cells per well. Seven to ten days post-fusion, macroscopic hybridoma colonies were observed.

Example 1.3: Assessment of PD-1 Binding Activity by ELISA and FACS

The presence of PD-1 specific antibodies was assayed by Enzyme-Linked Immunosorbent Assay (ELISA), as follows:

First, synthetic targets for anti-human PD-1, anti-cynomolgus PD-1 and anti-murine PD-1 were made to order by Synbio Technologies (Suzhou, China). Each target consisted of a polypeptide segment of the extracellular domain (ECD) of human, cynomolgus, or murine PD-1 protein fused to a human IgG Fc region. Synthetic genes encoding each ECD-Fc fusion protein were subcloned into a pCP expression vector (Chempartner, Shanghai, China) and the expression plasmids were transiently transfected into HEK 293E cells in 1-3 liters of medium and cultured for seven days in a CO_2 shaker. The ECD sequences used for each fusion are set forth in Table 1, below. The PD-1 ECD portion of each fusion protein is underlined.

Table 1: Amino Acid Sequences for PD-1 ECD-Fc Fusion Protein Targets

SEQ ID NO.	PD-1 Source	Amino acid sequences 12345678901234567890123456789012345678 90
1	human	LDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVL NWYRMSPSNQTDKLAAFPEDRSQPGQDCRFRVTQLPNGRDF HMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQIEGRMDPKSCDKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY TQKSLSLSPGK
2	cynomolgus monkey	LESPDRPWNAPTFSPALLLVTEGDNATFTCSFSNASESFVL NWYRMSPSNQTDKLAAFPEDRSQPGQDCRFRVTRLPNGRDF HMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQIEGRMDPKSCDKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY TQKSLSLSPGK
3	mouse	LEVPNGPWRSLTFYPAWLTVSEGANATFTCSLSNWSEDLML NWNRLSPSNQTEKQAAFCNGLSQPVQDARFQIIQLPNRHDF

SEQ ID NO.	PD-1 Source	Amino acid sequences 12345678901234567890123456789012345678 90
		HMNILDTRRNDSGIYLCGAISLHPKAKIEESPGAELVVTER ILETSTRYPSPSPKPEGRFQIEGRMDPKSCDKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY TQKSLSLSPGK

The supernatants of HEK 293E transfectants containing recombinant ECD/Fc fusions were harvested by centrifugation at 4000 × g for 30 minutes, followed by protein A purification using MabSelect SuRe™ affinity resin (GE Healthcare). The fusion products were dialyzed against phosphate buffered saline (PBS) pH 7.4 and stored at -80° C.

ELISA plates were incubated overnight at 4° C with 50 µl of the synthetic human PD-1 ECD/Fc fusion protein targets described above diluted in PBS buffer, pH 7.4, at 1 µg/ml. Plates were washed four times in washing buffer (PBS containing 0.05% Tween 20), and blocked for 1 hour at 37° C with 200 µl per well blocking buffer (1% BSA in PBS containing 0.05% Tween 20). After blocking buffer was removed, hybridoma supernatant (or later diluted purified mAbs) were added to the wells at 100 µl per well and incubated at 37° C for 1 hour. The wells were washed four times with washing buffer, and anti-mouse HRP (Sigma) for mouse anti-human PD-1 antibody characterization were diluted 1:5000 and added to the wells at 100 µl per well. The plates were incubated for 1 hour at 37° C and washed four times in washing buffer. 100 µl of tetramethylbenzidine (TMB) chromogenic solution were added per well. Following color development, the reaction was stopped with 1 Normal HCl and absorbance at 450 nm was measured on a SpectraMax® M5e plate reader (Molecular Devices; San Jose, California, US).

Example 1.4: Preparation of PD-1-Expressing Cell Lines and FACS Analysis

Stable cell lines overexpressing human PD-1 or cynomolgus PD-1 were generated by transfection of CHO-K1 cells (obtained from ATCC) with pLvx lentiviral plasmid vectors (Clontech) having inserted genes encoding either human PD-1 or cynomolgus PD-1. Single

clones were isolated by limiting dilution. Clones were screened for expression level by FACS analysis using anti-PD-1 antibodies produced recombinantly from known antibody sequences (Chempartner), and clones having highest expression of PD-1 were selected for use in a FACS binding assays and functional assays, as described below.

Binding Assay for Cell Surface Targets: The ability of the purified antibodies to bind to cell membrane human PD-1 or cynomolgus monkey PD-1 was determined by FACS analysis. CHO-K1 cells stably expressing human PD-1 (CHO-K1-hPD-1 cells) or cynomolgus PD-1 (CHO-K1-cynoPD-1) were resuspended in PBS containing 2% FBS (FACS buffer) and seeded at 2×10^4 cells/well into 96-well round-bottomed plates (Corning; Cat. No. 3799). Supernatants of hybridomas producing anti-PD-1 antibodies were added to the wells and detected with AlexaFluor® 488 Donkey Anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (Invitrogen; Cat. No. A-21202), and the assay plate was then read on a flow cytometer. Hybridomas producing supernatant signaling positive against human PD-1 expressing targets were further characterized using CHO-K1/cynoPD-1 cells to determine cross-reactivity of the antibodies with cynomolgus PD-1.

Example 1.5: Receptor Blocking Assay (RBA)

Supernatants displaying PD-1 specific activity were tested for the ability to block PD-1 binding to its ligands PD-L1 and PD-L2 using immobilized human PD-1 ECD/Fc as a target and PD-L1/Fc and PD-L2/Fc fusion proteins, prepared in the same manner as the PD-1 ECD/Fc binding proteins described in Example 1.3 above. To determine the relative potency of the antibody-containing supernatants, their ability to inhibit the binding of a human PD-1 ligand (PD-L1 or PD-L2) to human PD-1 protein was evaluated. ELISA plates were coated with 100 µl of 50 ng/ml of huPD-1/Fc (i.e., the extracellular domain of PD-1 grafted onto the N-terminus of a human Fc region, recovered as a homodimer) in PBS and incubated overnight at 4° C. Plates were washed four times in washing buffer (PBS containing 0.05% Tween 20) and blocked for 1 hour at 37° C with 200 µl per well of blocking buffer (1% BSA in PBS containing 0.05% Tween 20). After blocking buffer was removed, hybridoma supernatant (50 µl) was added to the wells, mixed with either 50 µl biotinylated human PD-L1/Fc (1.0 mg/ml final concentration)

in blocking buffer or 50 µl biotinylated human PD-L2/Fc (final concentration 50 µg/ml) in blocking buffer, then incubated at 37° C for 1 hour. Signal was developed by adding streptavidin-HRP (Sigma, Cat. No. S2468) (100 µl/well of streptavidin-HRP at 1:5000 dilution) and incubating for 40 minutes at 37° C and washed four times in washing buffer. 100 µl of TMB solution were added per well. Following color development, the reaction was stopped with 1 Normal HCl and absorbance at 450 nm was measured.

Example 1.6: Expression and Purification of Anti-PD-1 Monoclonal Antibodies

Murine monoclonal antibody-producing hybridoma cells were cultured in FreeStyle™ 293 Expression Medium (Gibco/Life Technologies) in a CO_2 shaker at 37° C for 5 to 7 days. The conditioned medium was collected through centrifugation at 4000 × g for 30 minutes to remove all cells and cell debris, then filtered through a 0.22 µm membrane before purification. Murine antibodies were applied and bound to a MabSelect™ SuRe (GE Healthcare) protein A resin column according to the manufacturer's guidelines, washed with PBS, eluted with buffer containing 20 mM citrate, 150 mM NaCl, pH 3.5. The eluted materials were neutralized with 1 M Tris at pH 8.0 immediately and dialyzed against PBS. One-step purified antibodies usually have above 90% purity, as detected by SEC-HPLC. Protein concentrations were determined by measuring absorbance at 280 nm or by NanoDrop™ microvolume spectrophotometer (Thermo Scientific). The purified antibodies were stored in aliquots in a -80° C freezer.

Example 2: Binding Activity of Purified Anti-PD-1 Antibodies

Examples 2.1: Characterization by ELISA

A binding ELISA was performed in the same way as described in Example 1.3 above. Each purified antibody was 10-fold serially diluted and duplicated. After blocking of the 96-well assay plate with wells containing immobilized PD-1 ECD/Fc fusion protein targets, the purified antibody samples with diluted concentrations were added to wells of assay plates. The HRP-linked anti-mouse IgG antibody (A0168, Sigma) and TMB reagent were used to detect and develop the ELISA signal, which were read on a SpectraMax® M5e plate reader at wavelength of 450 nm. Curves were fitted using GraphPad software, and EC50 were calculated. Similarly, a receptor blocking assay (RBA) was also performed as described in Example 1.5 with

titrated, purified antibodies, and top blocking percentages and IC50 values were determined.

Example 2.2: Characterization by FACS

CHO-K1/huPD-1 or CHO-K1/cynoPD-1 cells, described above, were charged at 2×10^4 cell per well into 96-well assay round-bottomed assay plates (Cat. No. 3799; Corning) and stained with purified anti-PD-1 antibodies. PD-1 antibodies were detected with AlexaFluor® Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, (Cat. No. A21202; Invitrogen), and cell fluorescence was monitored using a flow cytometer. The data were processed by GraphPad software, and EC50 values were calculated.

The results of these binding characterization assays are shown in Table 2 below.

Table 2: Binding Activity of Purified Anti-PD-1 Antibodies

	ELISA	**FACS**		**Receptor Blocking Assay (RBA)**			
mAb Identifier	Coating huPD-1/Fc	CHO-K1/ huPD-1	CHO-K1/ cynoPD-1	Ligand 1 (huPD-L1)		Ligand 2 (huPD-L2)	
	EC50 (nM)	EC50 (nM)	EC50 (nM)	TOP inhibition (%)	IC50 (nM)	TOP inhibition (%)	IC50 (nM)
mAb701	0.10	3.0	3.8	87.1	5.32	94.1	2.67
mAb703	0.05	0.5	0.7	96.0	3.00	95.7	2.02
mAb707	0.10	27.4	4.3	87.7	13.94	23.1	9.91
mAb709	0.01	0.2	0.2	91.6	0.80	93.7	0.52
mAb711	0.03	18.6	3.0	89.3	11.56	27.4	6.77
mAb713	0.08	1.1	0.9	94.4	2.95	93.5	2.52
mAb714	0.05	1.6	0.8	92.3	2.95	93.1	2.24
mAb715	0.04	1.1	0.9	86.9	2.91	88.0	2.17
mAb716	0.02	0.7	0.7	95.8	1.56	96.9	1.05
mAb718	0.02	3.2	4.2	96.6	4.19	96.6	1.91
mAb719	0.02	1.7	2.1	96.5	3.39	95.2	1.85
Human IgG1 (control)	63.95			0.0	NA	9.9	NA

Example 2.3: Affinity measurement by Surface Plasmon Resonance (SPR)

The binding kinetics of purified antibodies were measured by surface plasmon resonance using a Biacore T200 instrument (GE Healthcare) using standard procedures. Briefly, goat anti-

mouse IgG Fc polyclonal antibody (Genway) was directly immobilized across a biosensor chip, and antibodies samples were injected over reaction matrices at a flow rate of 5 µl/min. The association and dissociation rate constants, k_{on} ($M^{-1}s^{-1}$) and k_{off} (s^{-1}) respectively, were determined with a continuous flow rate of 30 µl/min. Rate constants were derived by making kinetic binding measurements at five different concentrations of human PD-1/Fc protein. The equilibrium dissociation constant K_D (M) of the reaction between antibodies and related target proteins was then calculated from the kinetic rate constants using the formula $K_D = k_{off}/k_{on}$. Affinities for eleven murine anti-PD-1 antibodies were measured, as set forth in Table 3, below.

Table 3: Affinity Measurements for 11 Anti-PD-1 Monoclonal Antibodies

mAb Identifier	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
mAb701	7.52×10^{4}	5.12×10^{-4}	6.81×10^{-9}
mAb703	3.47×10^{5}	8.50×10^{-4}	2.45×10^{-9}
mAb707	5.26×10^{4}	3.10×10^{-4}	5.89×10^{-9}
mAb709	1.11×10^{5}	1.04×10^{-4}	9.39×10^{-9}
mAb711	4.80×10^{4}	2.52×10^{-4}	5.24×10^{-9}
mAb713	1.45×10^{5}	2.85×10^{-4}	1.96×10^{-9}
mAb714	9.94×10^{4}	2.10×10^{-4}	2.11×10^{-9}
mAb715	1.58×10^{5}	2.37×10^{-4}	1.50×10^{-9}
mAb716	1.26×10^{5}	1.40×10^{-4}	1.11×10^{-9}
mAb718	5.84×10^{4}	2.83×10^{-4}	4.84×10^{-9}
mAb719	7.15×10^{4}	2.15×10^{-4}	3.00×10^{-9}

Example 3: Functional Activity of Anti-PD-1 Antibodies

A mixed lymphocyte reaction (MLR) assay was performed using monocyte-derived dendritic cells from one donor and allogeneic CD4+ T cells from another donor. Whole blood samples were collected from healthy donors, and PBMC were isolated from whole blood using Ficoll-Pague gradient centrifugation. On day 1, PBMC from one donor were isolated and diluted with serum-free RPMI 1640 at 1×10^{6} cells/ml. The diluted PBMC were seeded into a 6-well tissue culture plate at 3 ml/well and incubated for 3 hours. Supernatant was removed and unattached cells were washed off. The attached monocytes were polarized into dendritic cells

with 250 U/ml of IL-4 and 500 U/ml of GM-CSF in RPMI 1640 with 10 % FBS. The medium was replaced with fresh medium containing IL-4 and GM-CSF on day 4. At day 7, immature dendritic cells were collected and treated with 1 μg/ml bacterial lipopolysaccharide (LPS) (Sigma) in RPMI 1640 with 10% FBS for an additional 24 hrs. for maturation. At Day 8, CD4+ T cells were isolated from PBMC from another donor by negative selection and adjusted to final concentration at 2×10^6 cells/ml. Mature dendritic cells were treated with mitomycin C at 37° C for 1.5 hr., then dendritic cells were washed with PBS and adjusted to a final concentration at 1×10^6 cells/ml. CD4+ T cells (responder cells) were added into 96-well plates at 100 μl/well and pre-treated with test antibody at diluted concentration for 30 minutes. Mature dendritic cells (stimulator cells) were added into the wells at 100 μl/well. The final volume of each well is 200 μl. The mixed lymphocytes were incubated at 37° C. IL-2 production was measured after 72 hours (see Fig. 1A and 1B); IFN-γ was measured at 120 hours (see Fig. 2A and 2B).

Example 4: C1oning and Sequence Analysis of anti-PD-1 mAbs

Total RNA of each hybridoma clone was isolated from > 5×10^6 cells with TRIzol reagent (Cat. No. 15596; Invitrogen). cDNA was synthesized by SuperScript™ III First-Strand Synthesis SuperMix (Cat. No. 18080; Invitrogen) and applied as a PCR template of Mouse Ig-Primer Set (Cat. No. 69831-3; Novagen). PCR products were analyzed by electrophoresis on a 1.2% agarose gel with SYBR™ Safe DNA gel stain (Invitrogen). DNA fragments with correct size were purified with NucleoSpin® Gel and PCR Clean-up (Cat. No. 740609; Macherey-Nagel GmbH) according to manufacturer's instructions and subcloned to pMD18-T vector (Sino Biological Inc.) individually. Fifteen colonies from each transformation were selected and sequences of insert fragments were analyzed by DNA sequencing. Sequences were confirmed if at least 8 match consensus sequences for VH and VL. The protein sequences of murine anti-PD-1 mAbs variable regions were analyzed by sequence homology alignment and listed in Table 4. Complementarity determining regions (CDRs) were identified based on Kabat numbering and appear underlined in Table 4 below.

Table 4: Amino Acid Sequences of 8 Anti-PD-1 Murine Monoclonal Antibodies

antibody	domain	SEQ ID NO.	amino acid sequences 12345678901234567890123456789012345678901234567890
mAb701	VH	4	EVLLVESGGGLVKPGGSLKLSCAASGFTFSSYMMSWIRQT PERRLEWVASMSGGGRDTYYPDSVKGRFTISRDNAKNTLY LQMSSLRSEDTALYYCARRGTYAMDYWGQGTSVTVSS
	VL	5	DIQMTQSPASQSASLGESVTITCLASQTIGTWLTWYQQKP GKSPQLLIYAATSLADGVPSRFSGSGSGTKFSFKISSLQA EDFVSYYCQQLYSTPWTFGGGTKLEIK
mAb703	VH	6	DVQLQESGPGLVKPSQSLSLTCSVTGYSITTGYYWNWIRQ FPGNKLEWMGYMSYDGNNNYNPSLKNRISITRDTSKNQFL LRLNSVTTEDTATYFCARDRGTTILGGTMDYWGQGTSVTV S
	VL	7	SIVMTQTPKFLFVSAGDRVTIACKASQSVSNDVAWYQQKP GQSPKLLIYYAFYRYTGVPDRFTGSGYGTDFTFTISTVQA EDLAVYFCQQDYSSPWTFGGGTKLEIK
mAb709	VH	8	EVKLVESGGGLVKPGGSLKLSCAASGFTFSFYTMSWVRQT PEKRLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNTLY LHMSSLRSEDTALYYCAGQGGNYLFAYWGQGTLVTVSA
	VL	9	DIVMTQSHKFMSTSVGDSVTITCKASQDVNTVVAWYQQKP GQSLKVLISWASTRHTGVPARFTGSGSGTDYTLTISSVQA EDLALYYCQQHYTTPYTFGGGTQLEIK
mAb713	VH	10	EVKLVESGGGLVKPGGSLELSCAASGFTSSDYGMHWVRQA PEKGLEWVAYISSGSYTIYYADTVKGRFTISRDNAKNTLF LQMTSLRSEDTAMYYCAKRGGSSHVNVMDYWGQGTSVTVS S
	VL	11	DIQMTQSSSYLSVSLGGRVTITCKASDHINNWLAWYQQKP GNAPRLLISGATSLETGVPSRFSGSGSGKDYTLSITSLQT EDVATYYCQQYWSPPYTFGGGTKLEIK
mAb714	VH	12	EVHLQQSGPELVKPGASVKIFCKASGYTFTDNNVEWVKQS HGKSLEWIGDINPNNGDTLYSQYFKDKATLTVDKSSTTAY MELRSLTSEDTGLYYCARGKSDQFDYWGQGTTLTVSS
	VL	13	DIQMTQSPASQSASLGESVTITCLASQTIGTWLAWYQQKP GKSPQLLIYAATSLADGVPSRFSGSGSGTKFSFKISSLQG EDFVSYYCQQLYSSPWTFGGGTKLEIK
mAb715	VH	14	EVMLVESGGGLLKPGGSLKLSCAASGFTFSSYAMSWVRQT PEKRLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNTLY LQMTSLRSEDTAFYYCAGQGGTYLFASWGQGTLVTVSA
	VL	15	DIVMTQSHKFMSTSVGDSVTITCKASQDVNTAVAWYQQKP GQPPKVLIYWASTRHTGVPDRFTGSGSGTDYTLTISSVQA EDLALYYCQQHYTTPYTFGGGTKLEIK

antibody	domain	SEQ ID NO.	amino acid sequences 1234567890123456789012345678901234567890
mAb718	VH	16	QVQLQQSGAELVRPGASVTLSCKASGYTFTDYEMHWAKQT PVHGLEWIGVIEPESGGTVYNQKFKGKAKLTADKSSRTAY MELRSLTSEDSAVYYCTREGFNSDHYFDYWGQGTTLTVSS
	VL	17	DVLMTQTPLSLPVSLGDQASISCRSSQNIVHSNGNTYLEW YLQKPGQSPKLLIYKVFNRFSGVPDRFSGSGSGTDFTLKI SRVEAEDLGVYYCFQGSHVPYTFGGGTKLEIK
mAb719	VH	18	EVKLVESGGGLVKPGGSLKLSCTASGFSFSSHLMSWVRQT PEKRLEWVAAISGGGADTYYPDSVKGRFTISRDNAKNTLY LQMRSLRSEDTALYYCTRQILAFDSWGQGTTLTVSS
	VL	19	DIQMNQSPSSLSVSLGDTITITCHASQNIYVWLNWYQQKP GNIPKLLIYKASNLHTGVPSRFSGGGSGTGFTLTISSLQP EDIATYYCQQGQSYPWTFGGGTKLEIK

Example 5: Humanization of Murine anti-PD-1 Antibodies

Based on the human PD-1 binding activity, cynomolgus PD-1 cross-reactivity similarly to human, almost 100% blocking activity in the RBA assay, functional activity in MLR and at least nanomole affinity as measured by Biacore, four anti-PD-1 antibodies, mAb709, mAb713, mAb703, and mAb719, were selected for humanization.

Example 5.1: Humanization of Murine Antibody mAb709

The mAb709 variable region genes were employed to create a humanized antibody. In the first step of this process, the amino acid sequences of the VH and VL of mAb709 were compared against the available database of human Ig V-gene sequences in order to find the overall best-matching human germline Ig V-gene sequences. Additionally, the framework 4 segment of VH or VL was compared against the J-region database to find the human framework having the highest homology to the murine VH and VL regions, respectively. For the light chain, the closest human V-gene match was the O12 gene; and for the heavy chain, the closest human match was the VH3-7 gene. Humanized variable domain sequences were then designed where the CDR-L1, CDR-L2, and CDR-L3 of the mAb709 light chain were grafted onto framework sequences of the O12 gene with JK4 framework 4 sequence after CDR-L3; and the CDR-H1, CDR-H2, and CDR-H3 of the mAb709 heavy chain were grafted onto framework

sequences of the VH3-7 with JH1 framework 4 sequence after CDR-H3. A 3-dimensional Fv model of mAb709 was then generated to determine if there were any framework positions where mouse amino acids were critical to support loop structures or the VH/VL interface. These residues in humanized sequences should be back-mutated to mouse residues at the same position to retain affinity/activity. In the case of the light chain, a Phe to Tyr back mutation at position 71 (F71Y, Kabat numbering), a Tyr to Ser back mutation at position 49 (Y49S, Kabat numbering), a Gln to Val back mutation at position 3 (Q3V, Kabat numbering), a Leu to Val back mutation at position 46 (L46V, Kabat numbering), a Ser to Thr at position 63 (S63T, Kabat numbering), an Ala to Ser back mutation at position 43 (A43S, Kabat numbering), and a Pro to Leu back mutation at position 44 (P44L, Kabat numbering) were identified as desirable back mutations. In the case of the heavy chain, an Arg to Gly mutation at position 98 (R94G, by Kabat numbering), and a Gly to Arg mutation at position 44 (G44R, by Kabat numbering), were identified as desirable back mutations. Mutated variable domains containing one or more of these back mutations were constructed. See Table 5 below. (Back mutated framework amino acid residues are indicated with double underscore; murine CDRs from the original parental antibody are underlined.)

Table 5: Humanization VH/VL Design for mAb709 w/ Back Mutations to Murine Residues

Humanized mAb709 VH or VL Identifier	SEQ ID NO.	Amino acid sequences 12345678901234567890123456789012345678 90
mAb709 VH.1	20	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCARQGGNYLFAYWGQGTLVTVSS
mAb709 VH.1A	21	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSS
mAb709 VH.1B	22	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKRLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSS
mAb709 VK.1A	23	DIQMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKLLIYWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIK

Humanized mAb709 VH or VL Identifier	SEQ ID NO.	Amino acid sequences 1234567890123456789012345678901234567890
mAb709 VK.1B	24	DIQMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKLLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIK
mAb709 VK.1C	25	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIK
mAb709 VK.1D	26	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFTGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIK
mAb709 VK.1E	27	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKSLKVLISWASTRHTGVPSRFTGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIK

The humanized VH and VK genes were produced synthetically and then respectively cloned into vectors containing the human IgG1 and human kappa constant domains. (See Table 6, below.)

Table 6: Human Constant Region Sequence Used in Antibody Humanization

Constant Region	SEQ ID NO.	Amino Acid Sequences 1234567890123456789012345678901234567890
human constant Ig gamma 1 mutant	28	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT YICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT QKSLSLSPGK
human constant kappa	29	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQ WKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE KHKVYACEVTHQGLSSPVTKSFNRGEC

The pairing of the humanized VH and the humanized VK chains created 15 humanized antibodies, named HumAb709-1 to HumAb709-15 (Table 7). A chimeric antibody with parental mouse VH/VL and human constant region sequences (mAb709c) was also produced as a

positive control, for affinity comparison. All recombinant mAbs were expressed and purified.

Table 7: Production List of anti-PD-1 Humanized mAb709 Antibodies

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
HumAb709-1	mAb709 VH.1	mAb709 VK.1A
HumAb709-2	mAb709 VH.1A	mAb709 VK.1A
HumAb709-3	mAb709 VH.1B	mAb709 VK.1A
HumAb709-4	mAb709 VH.1	mAb709 VK.1B
HumAb709-5	mAb709 VH.1A	mAb709 VK.1B
HumAb709-6	mAb709 VH.1B	mAb709 VK.1B
HumAb709-7	mAb709 VH.1	mAb709 VK.1C
HumAb709-8	mAb709 VH.1A	mAb709 VK.1C
HumAb709-9	mAb709 VH.1B	mAb709 VK.1C
HumAb709-10	mAb709 VH.1	mAb709 VK.1D
HumAb709-11	mAb709 VH.1A	mAb709 VK.1D
HumAb709-12	mAb709 VH.1B	mAb709 VK.1D
HumAb709-13	mAb709 VH.1	mAb709 VK.1E
HumAb709-14	mAb709 VH.1A	mAb709 VK.1E
HumAb709-15	mAb709 VH.1B	mAb709 VK.1E
HumAb709c	SEQ ID NO:8	SEQ ID NO:9

All 15 humanized antibodies and the chimeric antibody (mAb709c) were characterized by binding ELISA and cell-based RBA. For cell-based RBA, 2×10^5 cells/well of CHO-K1-huPD1 cells were added to a pre-blocked 96-well round-bottomed plate and after washing, 50 µl antibodies with diluted concentration ranging from 0.064 nM to 200 nM were added to each well. Next, 50 µl of 60 µg/ml biotinylated PD-L1/Fc or biotinylated PD-L2/Fc protein were added. After gentle mixing and incubation at 4° C, the cells were washed and stained by Alexa Fluor™ 488 streptavidin solution (1:1000, ThermoFisher Scientific; Cat. No. S32354). Signals were

read out by FACS and curves were fitted by GraphPad software. Calculated IC50 values are shown in Table 8 below. Antibodies having positive (low) IC50 values (i.e., below about 1.0 nM for at least one PD-1 ligand) were further analyzed for binding affinity by surface plasmon resonance measurements using a Biacore T200 instrument. Briefly, goat anti-human IgG Fc polyclonal antibody was directly immobilized across a biosensor chip, and anti-PD-1 humanized antibody or chimeric antibody samples were injected over reaction matrices at a flow rate of 5 µl/min. The association and dissociation rate constants, k_{on} ($M^{-1}s^{-1}$) and k_{off} (s^{-1}), respectively, were determined by making kinetic binding measurements at five different concentrations of human PD-1-His protein at a continuous flow rate of 30 µl/min. The equilibrium dissociation constant K_D (M) of the reaction between antibodies and related target proteins was calculated from the kinetic rate constants using the formula $K_D = k_{off}/k_{on}$. Affinities for five of the mAb709 humanized anti-PD-1 derivatives are shown in Table 8. HumAb709-8 had minimal back-mutation(s) while maintaining to the greatest extent the affinity of the parental variable domains on chimeric mAb709c.

Table 8: RBA Values and Binding Affinities for Humanized mAb709 anti-PD-1 Antibodies

Humanized Antibody ID	PD-L1 RBA IC50 (nM)	PD-L2 RBA IC50 (nM)	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
HumAb709-1	1.02	1.64	1.95×10^{5}	2.145×10^{-3}	1.10×10^{-8}
HumAb709-2	0.47	0.99	8.03×10^{4}	5.5×10^{-5}	6.84×10^{-10}
HumAb709-3	1.25	1.64			
HumAb709-4	0.78	1.68			
HumAb709-5	0.67	0.97			
HumAb709-6	1.23	1.26			
HumAb709-7	0.40	0.84	1.41×10^{5}	3.36×10^{-4}	2.36×10^{-9}
HumAb709-8	0.44	1.00	1.27×10^{5}	4.69×10^{-5}	3.68×10^{-10}
HumAb709-9	1.04	1.76			
HumAb709-10	0.29	0.80	1.46×10^{5}	2.97×10^{-4}	2.04×10^{-9}

Humanized Antibody ID	PD-L1 RBA IC50 (nM)	PD-L2 RBA IC50 (nM)	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
HumAb709-11	0.55	0.92			
HumAb709-12	0.45	1.35			
HumAb709-13	0.50	0.78			
HumAb709-14	0.51	0.92			
HumAb709-15	0.90	1.21			
mAb709c	0.62	0.56	1.21×10^{5}	6.88×10^{-5}	5.67×10^{-10}

Example 5.2: Humanization of Murine Antibody mAb713

The variable region genes for anti-PD-1 mAb713 were employed to create a humanized antibody. The amino acid sequences of the VH and VK of mAb713 were compared against the available database of human Ig V-gene sequences in order to find the overall best-matching human germline Ig V-gene sequences. Additionally, the framework 4 segment of VH or VL was compared against the J-region database to find the framework having the highest homology to the murine VH and VL regions, respectively. For the light chain, the closest human V-gene match was the O18 gene; and for the heavy chain, the closest human match was the VH3-48 gene. Humanized variable domain sequences were then designed where the CDR-L1, CDR-L2, and CDR-L3 of the mAb713 light chain were grafted onto framework sequences of the O18 gene with JK4 framework 4 sequence after CDR-L3; and the CDR-H1, CDR-H2, and CDR-H3 of the mAb713 heavy chain were grafted onto framework sequences of the VH3-48 with JH6 framework 4 sequence after CDR-H3. A 3-dimensional Fv model of mAb709 was then generated to determine if there were any framework positions where mouse amino acids were critical to support loop structures or the VH/VL interface. These residues in humanized sequences should be back-mutated to mouse residues at the same position to retain affinity/activity. In the case of the light chain, a Phe to Tyr back mutation at position 71 (F71Y, Kabat numbering), a Tyr to Ser back mutation at position 49 (Y49S, Kabat numbering), and a Thr to Lys back mutation at position 69 (T69K, Kabat numbering) were identified as desirable back

mutations. In the case of the heavy chain, an Arg to Lys mutation at position 98 (R94K, by Kabat numbering), a Phe to Ser back mutation at position 29 (F29S, Kabat numbering), and a Ser to Ala back mutation at position 49 (S49A, by Kabat numbering), were identified as desirable back mutations. Mutated variable domains containing one or more of these back mutations were constructed. See Table 9 below. (Back mutated framework amino acid residues are indicated with double underscore; murine CDRs from the original parental antibody are underlined.)

Table 9: Variable Domain Sequence Variants for mAb713 VH and VL

mAb713 VH/VL variants	SEQ ID NO.	Amino acid sequence 1234567890123456789012345678901234567890
mAb713 VH.1	30	EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYGMHWVRQA PGKGLEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCARRGGSSHVNVMDYWGQGTTVTVS S
mAb713 VH.1A	31	EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYGMHWVRQA PGKGLEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVS S
mAb713 VH.1B	32	EVQLVESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQA PGKGLEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVS S
mAb713 VH.1C	33	EVQLVESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQA PGKGLEWVAYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVS S
mAb713 VK.1	34	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLIYGATSLETGVPSRFSGSGSGTDFTFTISSLQP EDIATYYCQQYWSPPYTFGGGTKVEIK
mAb713 VK.1A	35	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLIYGATSLETGVPSRFSGSGSGTDYTFTISSLQP EDIATYYCQQYWSPPYTFGGGTKVEIK
mAb713 VK.1B	36	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLISGATSLETGVPSRFSGSGSGTDYTFTISSLQP EDIATYYCQQYWSPPYTFGGGTKVEIK

mAb713 VH/VL variants	SEQ ID NO.	Amino acid sequence 1234567890123456789012345678901234567890
mAb713 VK.1C	37	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLISGATSLETGVPSRFSGSGSGKDYTFTISSLQP EDIATYYCQQYWSPPYTFGGGTKVEIK

The humanized VH and VK genes were produced synthetically and then individually cloned into vectors containing the human IgG1 and human kappa constant domains (see Table 6, *supra*). The pairing of the human VH variants and the human VK variants created 16 humanized antibodies, named HumAb713-1 to HumAb713-16 (Table 10). A chimeric antibody (mAb713c) with parental mouse VH/VL and human constant sequences was also produced as a positive control, for affinity comparison.

Table 10: Production List for Humanized mAb713 Anti-PD-1 Antibodies

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
HumAb713-1	mAb713 VH.1	mAb713 VK.1
HumAb713-2	mAb713 VH.1A	mAb713 VK.1
HumAb713-3	mAb713 VH.1B	mAb713 VK.1
HumAb713-4	mAb713 VH.1C	mAb713 VK.1
HumAb713-5	mAb713 VH.1	mAb713 VK.1A
HumAb713-6	mAb713 VH.1A	mAb713 VK.1A
HumAb713-7	mAb713 VH.1B	mAb713 VK.1A
HumAb713-8	mAb713 VH.1C	mAb713 VK.1A
HumAb713-9	mAb713 VH.1	mAb713 VK.1B
HumAb713-10	mAb713 VH.1A	mAb713 VK.1B
HumAb713-11	mAb713 VH.1B	mAb713 VK.1B
HumAb713-12	mAb713 VH.1C	mAb713 VK.1B
HumAb713-13	mAb713 VH.1	mAb713 VK.1C
HumAb713-14	mAb713 VH.1A	mAb713 VK.1C

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
HumAb713-15	mAb713 VH.1B	mAb713 VK.1C
HumAb713-16	mAb713 VH.1C	mAb713 VK.1C
mAb713c	SEQ ID NO:10	SEQ ID NO:11

All 16 humanized antibodies and the chimeric antibody (mAb713c) were characterized by binding ELISA, cell-based RBA, and Biacore affinity testing. The results are summarized in Table 11.

Table 11: RBA Values and Binding Affinities for Humanized mAb713 anti-PD-1 Antibodies

Humanized Antibody ID	PD-L1 RBA IC50 (nM)	PD-L2 RBA IC50 (nM)	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
HumAb713-1	0.50	1.20	9.010×10^{4}	1.003×10^{-3}	1.113×10^{-8}
HumAb713-2	1.71	3.13			
HumAb713-3	0.77	1.24	8.447×10^{4}	2.082×10^{-4}	2.465×10^{-9}
HumAb713-4	1.06	2.24			
HumAb713-5	0.91	2.95			
HumAb713-6	1.04	1.46			
HumAb713-7	0.76	1.40	1.237×10^{5}	3.500×10^{-4}	2.829×10^{-9}
HumAb713-8	1.05	1.91			
HumAb713-9	1.20	2.00			
HumAb713-10	0.80	1.23			
HumAb713-11	0.51	0.97	1.591×10^{5}	3.776×10^{-4}	2.373×10^{-9}
HumAb713-12	0.94	1.59			
HumAb713-13	0.70	2.13			
HumAb713-14	0.91	1.45			
HumAb713-15	0.88	1.65			
HumAb713-16	0.65	1.63			

Humanized Antibody ID	PD-L1 RBA IC50 (nM)	PD-L2 RBA IC50 (nM)	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
mAb713c	0.91	2.20	2.182×10^{5}	2.839×10^{-4}	1.301×10^{-9}

HumAb713-7 had minimal back-mutation(s) while maintaining the affinity characteristics of the parental variable domains of the chimeric antibody, mAb713c. Functional activity of mAb713 humanized antibodies were validated in an MLR assay as described in Example 3. As seen in Figure 5, HumAb713-7 exhibited comparable activity with the chimeric antibody mAb713c in MLR, in line with its retained binding properties.

Example 5.3: Humanization of Murine Antibody mAb703

Following the same procedure as in Example 5.1 and 5.2, murine anti-PD-1 antibody mAb703 was selected and humanized. Humanized variable domains, some containing one or more back mutations, were constructed, and the amino acid sequences are set forth in Table 12 below. (Back mutated framework amino acid residues are indicated with double underscore; murine CDRs from the original parental antibody are underlined.)

Table 12: Variable Domain Sequence Variants for mAb703 VH and VL

mAb703 VH/VL variants	SEQ ID NO.	Amino acid sequence 12345678901234567890123456789012345678 90
mAb703 VH.1A	38	EVQLQESGPGLVKPSETLSLTCAVSGYSISTGYYWNWIRQ PPGKGLEWIGYMSYDGNNNYNPSLKNRVTISRDTSKNQFS LKLSSVTAADTAVYYCARDRGTTILGGTMDYWGQGTTVTV SS
mAb703 VH.1B	39	EVQLQESGPGLVKPSETLSLTCAVSGYSISTGYYWNWIRQ PPGKGLEWIGYMSYDGNNNYNPSLKNRITISRDTSKNQFS LKLSSVTAADTAVYYCARDRGTTILGGTMDYWGQGTTVTV SS
mAb703 VH.1C	40	EVQLQESGPGLVKPSETLSLTCAVSGYSISTGYYWNWIRQ PPGKGLEWMGYMSYDGNNNYNPSLKNRITISRDTSKNQFS LKLSSVTAADTAVYYCARDRGTTILGGTMDYWGQGTTVTV SS

mAb703 VH/VL variants	SEQ ID NO.	Amino acid sequence 12345678901234567890123456789012345678 90
mAb703 VH.1D	41	EVQLQESGPGLVKPSETLSLTCAVSGYSITTGYYWNWIRQ PPGKGLEWMGYMSYDGNNNYNPSLKNRITISRDTSKNQFS LKLSSVTAADTAVYYCARDRGTTILGGTMDYWGQGTTVTV SS
mAb703 VH.1E	42	EVQLQESGPGLVKPSETLSLTCAVSGYSITTGYYWNWIRQ PPGKKLEWMGYMSYDGNNNYNPSLKNRITISRDTSKNQFS LKLSSVTAADTAVYFCARDRGTTILGGTMDYWGQGTTVTV SS
mAb703 VK.1	43	DIQMTQSPSSLSASVGDRVTITCKASQSVSNDVAWYQQKP GKAPKLLIYYAFYRYTGVPSRFSGSGSGTDFTLTISSLQP EDFATYYCQQDYSSPWTFGGGTKVEIK
mAb703 VK.1A	44	DIQMTQSPSSLSASVGDRVTITCKASQSVSNDVAWYQQKP GKAPKLLIYYAFYRYTGVPSRFSGSGYGTDFTLTISSLQP EDFATYYCQQDYSSPWTFGGGTKVEIK
mAb703 VK.1B	45	DIQMTQSPSSLSASVGDRVTITCKASQSVSNDVAWYQQKP GKAPKLLIYYAFYRYTGVPDRFSGSGYGTDFTLTISSLQP EDFATYYCQQDYSSPWTFGGGTKVEIK
mAb703 VK.1C	46	DIQMTQSPSSLSASVGDRVTITCKASQSVSNDVAWYQQKP GKSPKLLIYYAFYRYTGVPDRFSGSGYGTDFTLTISSLQP EDFATYFCQQDYSSPWTFGGGTKVEIK
mAb703 VK.1D	47	SIVMTQSPSSLSASVGDRVTITCKASQSVSNDVAWYQQKP GKSPKLLIYYAFYRYTGVPDRFTGSGYGTDFTLTISSLQP EDFATYFCQQDYSSPWTFGGGTKVEIK

The humanized VH and VK genes were produced synthetically and then individually cloned into vectors containing the human IgG1 and human kappa constant domains (see Table 6, *supra*). The pairing of the human VH variants and the human VK variants created 25 humanized antibodies, named HumAb703-1 to HumAb703-25 (Table 13). A chimeric antibody with parental mouse VH/VL and human constant sequences was also produced as a positive control, for affinity comparison.

Table 13: Production List for Humanized mAb703 Anti-PD-1 Antibodies

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
HumAb703-1	mAb703 VH.1A	mAb703 VK.1
HumAb703-2	mAb703 VH.1B	mAb703 VK.1
HumAb703-3	mAb703 VH.1C	mAb703 VK.1
HumAb703-4	mAb703 VH.1D	mAb703 VK.1
HumAb703-5	mAb703 VH.1E	mAb703 VK.1
HumAb703-6	mAb703 VH.1A	mAb703 VK.1A
HumAb703-7	mAb703 VH.1B	mAb703 VK.1A
HumAb703-8	mAb703 VH.1C	mAb703 VK.1A
HumAb703-9	mAb703 VH.1D	mAb703 VK.1A
HumAb703-10	mAb703 VH.1E	mAb703 VK.1A
HumAb703-11	mAb703 VH.1A	mAb703 VK.1B
HumAb703-12	mAb703 VH.1B	mAb703 VK.1B
HumAb703-13	mAb703 VH.1C	mAb703 VK.1B
HumAb703-14	mAb703 VH.1D	mAb703 VK.1B
HumAb703-15	mAb703 VH.1E	mAb703 VK.1B
HumAb703-16	mAb703 VH.1A	mAb703 VK.1C
HumAb703-17	mAb703 VH.1B	mAb703 VK.1C
HumAb703-18	mAb703 VH.1C	mAb703 VK.1C
HumAb703-19	mAb703 VH.1D	mAb703 VK.1C
HumAb703-20	mAb703 VH.1E	mAb703 VK.1C
HumAb703-21	mAb703 VH.1A	mAb703 VK.1D
HumAb703-22	mAb703 VH.1B	mAb703 VK.1D
HumAb703-23	mAb703 VH.1C	mAb703 VK.1D
HumAb703-24	mAb703 VH.1D	mAb703 VK.1D
HumAb703-25	mAb703 VH.1E	mAb703 VK.1D

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
mAb703c	SEQ ID NO:6	SEQ ID NO:7

All 25 humanized antibodies and the chimeric antibody (mAb703c) were characterized by binding ELISA and Biacore affinity testing. Affinity results for the positive binders are summarized in Table 14.

Table 14: Binding Affinities for Selected Humanized mAb703 anti-PD-1 Antibodies

Humanized Antibody ID	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
HumAb703-11	1.874×10^{5}	1.757×10^{-3}	9.374×10^{-9}
HumAb703-12	1.770×10^{5}	1.594×10^{-3}	9.003×10^{-9}
HumAb703-13	1.454×10^{5}	1.537×10^{-3}	1.057×10^{-8}
HumAb703-18	6.572×10^{4}	1.242×10^{-3}	1.890×10^{-8}
HumAb703-22	2.294×10^{5}	1.593×10^{-3}	6.942×10^{-9}
mAb703c	3.594×10^{5}	9.664×10^{-4}	2.684×10^{-9}

The functional activity of humanized mAb703 antibodies was validated in MLR assays conducted as described in Example 3, as shown in Figures 2A and 3.

Example 5.4: Humanization of Murine Antibody mAb719

Following the same procedure as in Example 5.1 and 5.2, murine anti-PD-1 antibody mAb719 was selected and humanized. Humanized variable domains, some containing one or more back mutations, were constructed, and the amino acid sequences are set forth in Table 15 below. (Back mutated framework amino acid residues are indicated with double underscore; murine CDRs from the original parental antibody are underlined.) In addition, an Asp→Ala substitution in CDR-H2 and a Ser→Ala substitution in CDR-H3 were made to avoid possible isomerization of Asp often seen recombinant antibodies. (See, mAb719 VH.1E and mAb719 VH.1F sequences in Table 15.)

Table 15: Variable Domain Sequence Variants for mAb719 VH and VL

mAb719 VH/VL variants	SEQ ID NO.	Amino acid sequence 1234567890123456789012345678901234567890
mAb719 VH.1	48	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSHLMSWVRQA PGKGLEWVSAISGGGADTYYPDSVKGRFTISRDNSKNTLY LQMNSLRAEDTAVYYCAKQILAFDS-WGQGTTVTVSS
mAb719 VH.1A	49	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSHLMSWVRQA PGKGLEWVSAISGGGADTYYPDSVKGRFTISRDNSKNTLY LQMNSLRAEDTAVYYCTRQILAFDS-WGQGTTVTVSS
mAb719 VH.1B	50	EVQLLESGGGLVQPGGSLRLSCAASGFSFSSHLMSWVRQA PGKGLEWVSAISGGGADTYYPDSVKGRFTISRDNSKNTLY LQMNSLRAEDTAVYYCTRQILAFDS-WGQGTTVTVSS
mAb719 VH.1C	51	EVQLLESGGGLVQPGGSLRLSCAASGFSFSSHLMSWVRQA PGKGLEWVAAISGGGADTYYPDSVKGRFTISRDNSKNTLY LQMNSLRAEDTAVYYCTRQILAFDS-WGQGTTVTVSS
mAb719 VH.1D	52	EVQLLESGGGLVQPGGSLRLSCAASGFSFSSHLMSWVRQA PGKRLEWVAAISGGGADTYYPDSVKGRFTISRDNSKNTLY LQMNSLRAEDTAVYYCTRQILAFDS-WGQGTTVTVSS
mAb719 VH.1E	53	EVQLLESGGGLVQPGGSLRLSCAASGFSFSSHLMSWVRQA PGKGLEWVAAISGGGADTYYPASVKGRFTISRDNSKNTLY LQMNSLRAEDTAVYYCTRQILAFDA-WGQGTTVTVSS
mAb719 VH.1F	54	EVQLLESGGGLVQPGGSLRLSCAASGFSFSSHLMSWVRQA PGKRLEWVAAISGGGADTYYPASVKGRFTISRDNSKNTLY LQMNSLRAEDTAVYYCTRQILAFDA-WGQGTTVTVSS
mAb719 VK.1	55	DIQMTQSPSSLSASVGDRVTITCHASQNIYVWLNWYQQKP GKAPKLLIYKASNLHTGVPSRFSGSGSGTDFTFTISSLQP EDIATYYCQQGQSYPWTFGGGTKVEIK
mAb719 VK.1A	56	DIQMNQSPSSLSASVGDRVTITCHASQNIYVWLNWYQQKP GKAPKLLIYKASNLHTGVPSRFSGSGSGTDFTFTISSLQP EDIATYYCQQGQSYPWTFGGGTKVEIK
mAb719 VK.1B	57	DIQMNQSPSSLSASVGDRVTITCHASQNIYVWLNWYQQKP GKIPKLLIYKASNLHTGVPSRFSGSGSGTDFTFTISSLQP EDIATYYCQQGQSYPWTFGGGTKVEIK

The humanized VH and VK genes were produced synthetically and then individually cloned into vectors containing the human IgG1 and human kappa constant domains (see Table 6, *supra*). The pairing of the human VH variants and the human VK variants created 21

humanized antibodies, named HumAb719-1 to HumAb719-21 (Table 16). A chimeric antibody with parental mouse VH/VL and human constant sequences was also produced as a positive control, for affinity comparison. All recombinant mAbs were expressed and purified.

Table 16: Production List for Humanized mAb719 Anti-PD-1 Antibodies

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
HumAb719-1	mAb719 VH.1	mAb719 VK.1
HumAb719-2	mAb719 VH.1A	mAb719 VK.1
HumAb719-3	mAb719 VH.1B	mAb719 VK.1
HumAb719-4	mAb719 VH.1C	mAb719 VK.1
HumAb719-5	mAb719 VH.1D	mAb719 VK.1
HumAb719-6	mAb719 VH.1E	mAb719 VK.1
HumAb719-7	mAb719 VH.1F	mAb719 VK.1
HumAb719-8	mAb719 VH.1	mAb719 VK.1A
HumAb719-9	mAb719 VH.1A	mAb719 VK.1A
HumAb719-10	mAb719 VH.1B	mAb719 VK.1A
HumAb719-11	mAb719 VH.1C	mAb719 VK.1A
HumAb719-12	mAb719 VH.1D	mAb719 VK.1A
HumAb719-13	mAb719 VH.1E	mAb719 VK.1A
HumAb719-14	mAb719 VH.1F	mAb719 VK.1A
HumAb719-15	mAb719 VH.1	mAb719 VK.1B
HumAb719-16	mAb719 VH.1A	mAb719 VK.1B
HumAb719-17	mAb719 VH.1B	mAb719 VK.1B
HumAb719-18	mAb719 VH.1C	mAb719 VK.1B
HumAb719-19	mAb719 VH.1D	mAb719 VK.1B
HumAb719-20	mAb719 VH.1E	mAb719 VK.1B
HumAb719-21	mAb719 VH.1F	mAb719 VK.1B
mAb719c	SEQ ID NO:18	SEQ ID NO:19

All 21 humanized antibodies and the chimeric antibody (mAb719c) were characterized by binding ELISA and affinity determination using Octet® RED96 biolayer interferometry system (Pall FortéBio LLC), using a biosensor having immobilized human PD-1/Fc as the antibody target. Rate constants were derived by making kinetic binding measurements at five different concentrations of antibody. The affinities showed higher than previous Biacore testing due to the bivalent binding target. Affinity results for the positive binders are summarized in Table 17.

Table 17: Binding Affinities for Selected Humanized mAb719 anti-PD-1 Antibodies

Humanized Antibody ID	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
HumAb719-8	1.066×10^{5}	4.905×10^{-5}	4.602×10^{-10}
HumAb719-11	5.944×10^{4}	2.270×10^{-4}	3.819×10^{-9}
HumAb719-12	6.882×10^{4}	5.805×10^{-5}	8.435×10^{-10}
HumAb719-21	1.042×10^{5}	6.256×10^{-5}	6.005×10^{-10}
mAb719c	9.735×10^{4}	$<1.00 \times 10^{-5}$	$<1.027 \times 10^{-10}$

The functional activity of humanized mAb719 antibodies was validated in MLR assays, as shown in Figures 2B and 3.

Example 6: Pharmacokinetic Properties of Lead Anti-PD-1 Antibodies.

Pharmacokinetic properties of HumAb709-8 and HumAb713-7 were assessed in male Sprague-Dawley (SD) rats. Antibodies were administered to male SD rats at a single intravenous dose of 5 mg/kg. Serum samples were collected at different time points over a period of 28 days with sampling at 0, 5, 15, and 30 minutes; 1, 2, 4, 8, and 24 hours; and 2, 4, 7, 10, 14, 21, and 28 days serial bleeding via tail vein, and analyzed by general ELISAs. Briefly, ELISA plates were coated with 125 ng/well of goat anti-human IgG Fc antibody (Rockland, Cat#: 609-101-017) at 4°C overnight, blocked with 1X PBS/1% BSA/0.05% Tween-20/0.05% ProClin™ 300. All serum samples were diluted 20-fold in blocking buffer first. Additional dilution was made in 5% pooled rat serum and incubated on the plate for 60 minutes at 37°C. Detection was carried out with Anti-human IgG (Fab fragment) peroxidase conjugated (Sigma;

Cat. No. A0293) and concentrations were determined with the help of standard curves using the four- parameter logistic fit. Values for the pharmacokinetic parameters were determined by non-compartmental model using WinNonlin software (Pharsight Corporation, Mountain View, Calif.). As demonstrated by these results (Table 18), the properties of HumAb09-8 and HumAb13-7 are stable.

Table 18: Pharmacokinetic properties of HumAb709-8 and HumAb713-7

PK parameters	CL	Vss	Beta $t_{1/2}$	AUC	MRT
Antibody	mL/day/kg	mL/kg	day	day*μg/mL	day
HumAb709-8	8.6	129.6	10.9	594.2	15.4
HumAb713-7	6.4	114.4	12.7	789.3	18.1

Example 7: Generation of anti-LAG-3 Monoclonal Antibodies

Anti-LAG-3 monoclonal antibodies (mAbs) were generated by hybridoma fusion.

Example 7.1: Immunization, hybridoma fusion and cloning.

Immunization of Balb/C mice was performed in the same manner as described above for anti-PD-1 antibody generation (Example 1), except using human LAG-3 D1-D2/murine Fc homodimer as the immunogen. Immunized animals were boosted 2-4 times at 2-3-week intervals. Three days after final boosting, the splenocytes from immunized mice were isolated and fused with the murine myeloma cell line, SP2/0, using standard techniques.

Example 7.2: Identification and Characterization of Anti-LAG-3 Antibodies

Synthetic targets for anti-human LAG-3 and anti-cynomolgus LAG-3 were made to order by Synbio Technologies (Suzhou, China). Each target consisted of a polypeptide segment of the extracellular domain of human or cynomolgus LAG-3 protein fused to a human IgG Fc region. Synthetic genes encoding each LAG-3 ECD/Fc fusion protein were subcloned into a pCP expression vector (Chempartner, Shanghai, CN) and the expression plasmids were transiently transfected into HEK 293E cells in 1-3 liters of medium and cultured for seven days in a CO_2 shaker. The ECD sequences used for each fusion are set forth in Table 19, below. The LAG-3 ECD portion of each fusion protein is underlined.

Table 19: Amino Acid Sequences for LAG-3 ECD/Fc Fusion Protein Targets

SEQ ID NO.	LAG-3 Source	amino acid sequences 12345678901234567890123456789012345678901234567890
58	human	LQPGAEVPVVWAQEGAPAQLPCSPTIPLQDLSLLRRAGVT WQHQPDSGPPAAAPGHPLAPGPHPAAPSSWGPRPRRYTVL SVGPGGLRSGRLPLQPRVQLDERGRQRGDFSLWLRPARRA DAGEYRAAVHLRDRALSCRLRLRLGQASMTASPPGSLRAS DWVILNCSFSRPDRPASVHWFRNRGQGRVPVRESPHHHLA ESFLFLPQVSPMDSGPWGCILTYRDGFNVSIMYNLTVLGL EPPTPLTVYAGAGSRVGLPCRLPAGVGTRSFLTAKWTPPG GGPDLLVTGDNGDFTLRLEDVSQAQAGTYTCHIHLQEQQL NATVTLAIITVTPKSFGSPGSLGKLLCEVTPVSGQERFVW SSLDTPSQRSFSGPWLEAQEAQLLSQPWQCQLYQGERLLG AAVYFTELSSPGAQRSGRAPGALPAGHLIEGRMDPKSCDK THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGK
59	cynomolgus monkey	PQPGAEISVVWAQEGAPAQLPCSPTIPLQDLSLLRRAGVT WQHQPDSGPPAXAPGHPPVPGHRPAAPYSWGPRPRRYTVL SVGPGGLRSGRLPLQPRVQLDERGRQRGDFSLWLRPARRA DAGEYRATVHLRDRALSCRLRLRVGQASMTASPPGSLRTS DWVILNCSFSRPDRPASVHWFRSRGQGRVPVQGSPHHHLA ESFLFLPHVGPMDSGLWGCILTYRDGFNVSIMYNLTVLGL EPATPLTVYAGAGSRVELPCRLPPAVGTQSFLTAKWAPPG GGPDLLVAGDNGDFTLRLEDVSQAQAGTYICHIRLQGQQL NATVTLAIITVTPKSFGSPGSLGKLLCEVTPASGQEHFVW SPLNTPSQRSFSGPWLEAQEAQLLSQPWQCQLHQGERLLG AAVYFTELSSPGAQRSGRAPGALRAGHLIEGRMDPKSCDK THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGK

The supernatants of hybridoma clones were primarily screened by ELISA. Briefly, 50 µl/well of 1 µg/ml human LAG-3 ECD/Fc in $NaHCO_3$ were directly coated in each well of 96-well plate overnight. Plates were washed 3 times with 1X PBST, 300 µl per well. After blocking with 1% BSA in PBST at 250 µl per well and incubating at room temperature for 1 hour, the hybridoma supernatants were added at 50 µl per well and incubated at 37° C for 1 hour. After washing, an HRP-linked goat anti-mouse IgG Fc secondary antibody (Cat. No. A0168, Sigma) was added at 100 µl/well and the plates were incubated at room temperature for 1 hour. TMB reagent (InnoReagents) was used to detect and develop the ELISA signal at 100 µl/well for 15 minutes, the the reaciont was stopped with 1 Normal HCl. The plates were read with a plate reader (SpectraMax® M5e, Molecular Devices, USA) at a wavelength of 450 nm. The ELISA-positive antibody producer clones were further verified by FACS analysis using methods similar to Example 1.4, above, except that stable HEK 293F cell lines expressing either human LAG-3 or cynomolgus LAG-3 were used. Hybridomas producing LAG-3 binding activity were selected and further characterized in a receptor blocking assay (RBA).

Example 7.3: Receptor Blocking Assay (RBA)

Supernatants displaying LAG-3 specific activity were tested for the ability to block LAG-3 receptor binding to MHC Class II. Raji human B cell lymphoblasts express high levels of MCH Class II and were used as binding targets for LAG-3 ECD/Fc proteins described above. Briefly, Raji cells were harvested and resuspend in FACS buffer and plated in 96-well plates (2×10^5 cells/well). Anti-LAG-3 hybridoma supernatants were mixed with soluble LAG-3 ECD/Fc and the mixture was added to wells at a final volume of 100 µl/well. After adding the mixture to cells, the plates were incubated at room temperature for 30 minutes. After washing twice with PBS, the cells were incubated with anti-human IgG Alexa Fluor® 488 secondary antibody at 4° C for 1 hour, washed twice with PBS, then fluorescence was measured on a flow cytometer.

Example 7.4: Expression and Purification of Anti-LAG-3 Monoclonal Antibodies

Murine monoclonal antibody-producing hybridoma cells were cultured in FreeStyle™ 293 Expression Medium (Gibco/Life Technologies) in a CO_2 shaker at 37° C for 5 to 7 days. The conditioned medium was collected through centrifugation at 4000 × g for 30 minutes to remove

all cells and cell debris, then filtered through a 0.22 μm membrane before purification. Murine antibodies were applied and bound to a MabSelect™ SuRe (GE Healthcare) protein A resin column according to the manufacturer's guidelines, washed with PBS, eluted with buffer containing 20 mM citrate, 150 mM NaCl, pH3.5. The eluted materials were neutralized with 1 M Tris at pH 8.0 immediately and dialyzed against PBS. One-step purified antibodies usually have above 90% purity, as detected by SEC-HPLC. Protein concentrations were determined by measuring absorbance at 280 nm or by NanoDrop™ microvolume spectrophotometer (Thermo Scientific). The purified antibodies were stored in aliquots in a -80° C freezer.

Example 7.5: Binding Activity of Purified Anti-LAG-3 Antibodies

Characterization by ELISA

A binding ELISA was performed in the same way as described in Example 7.2 above. Each purified antibody was 10-fold serially diluted. After blocking of a 96-well assay plate with wells containing immobilized LAG-3 ECD/Fc fusion protein targets, the purified antibody samples with diluted concentrations were added to wells of the assay plate. The HRP-linked anti-mouse IgG antibody (A0168, Sigma) and TMB reagent were used to detect and develop the ELISA signal, which were read on a SpectraMax® M5e plate reader at wavelength of 450 nm. Curves were fitted using GraphPad software, and EC50 values were calculated. Similarly, a RBA was also performed as described in Example 7.3 with titrated, purified antibodies, and maximum inhibition percentages and IC50 values were determined.

Characterization by FACS

FACS analysis was performed using methods similar to Example 1.4, above, except that stable HEK 293F cell lines expressing either human LAG-3 or cynomolgus LAG-3 were used. LAG-3 expressing cells were charged at 2×10^4 cell per well into 96-well assay round-bottomed assay plates (Cat. No. 3799; Corning) and stained with purified anti-LAG-3 antibodies. LAG-3 antibodies were detected with AlexaFluor® Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (Cat. No. A21202; Invitrogen), and cell fluorescence was monitored using a flow cytometer. The data were processed by GraphPad software, and EC50 values were calculated.

The results of these binding characterization assays are shown in Table 20 below.

Table 20: Binding Activity of Purified Murine Anti-LAG-3 Antibodies

mAb Identifier	Binding to Human LAG-3			Binding to Cynomolgus LAG-3		
	ELISA	FACS		ELISA	FACS	
	EC50 (nM)	EC50 (nM)	Max-MFI	EC50 (nM)	EC50 (nM)	Max-MFI
mAb742	0.22	2.8	102.9	0.32	56.4	48.5
mAb743	0.26	30.4	115.7	0.26	111.5	36.9
mAb744	0.19	9.5	135.0	0.21	67.0	37.8
mAb745	0.27	32.2	54.8	0.30	224.3	26.0
mAb746	0.31	1.3	120.8	0.21	4.1	66.2
mAb747	0.25	1.1	104.4	0.34	3.1	65.2
mAb748	0.24	13.4	73.1	0.25	79.6	31.9
mAb749	0.55	3.3	123.6	0.33	14.3	67.4
mAb750	0.25	24.1	88.7	0.32	113.6	36.7
mAb751	0.22	26.2	88.9	0.27	79.3	33.1
mAb757	0.23	25.3	91.8	0.30	77.2	35.1
mAb758	0.87	9.8	64.8	3.18	15.0	17.3
mAb759	0.43	3.0	60.2	0.53	6.3	18.6
mAb760	N/A	N/A	12.3	N/A	N/A	4.9
mAb761	0.1.18	17.8	105.2	1.24	34.6	39.5
Human IgG1 (control)	0.13	0.9	190.6	78.23	63.7	28.1

In this table, "N/A" denotes no binding activity measured.

Example 7.6: Characterization by RBA and Antigen-Dependent Activation Assay

Purified anti-LAG-3 antibodies also were tested in a RBA in the same manner as described in Example 7.3. Antibodies were also tested in an antigen-specific T cell activation assay, as follows: A huLAG-3 expressing murine T hybridoma cell line was generated to order by ChemPartner (Shanghai, CN). Mouse splenocytes from the same strain of mice were used as effector cells. The hybridoma expressing the huLAG-3 receptor protein is capable of binding to MHC Class II-positive mouse splenocytes, with inhibitory effect via engagement of Class II. The assay tests for anti-LAG-3 antibody-mediated reversal of the inhibitory effect, as measured by increased production of IL-2. Mouse spleenocytes were harvested from 6-8 week-old female C57BL/6 mice, red blood cells were lysed using Red Blood Cell Lysis Buffer (Sigma-Aldrich;

R7757) according to the maker's instructions. Next, 50 µl T hybridoma-huLAG-3 cells (2×10^6 cells/ml) were seeded in each well of a 96-well culture plate, and then a series of anti-LAG-3 monoclonal antibodies in solution at 50 µl/well were added and incubated at 37°C for 30 min. Mouse splenocytes (4×10^6 cells/ml) and the antigen (20 µg/ml) were mixed and incubated at 37°C for 30 min. The mixture (100 µl/well) was added into each well that was already seeded with T hybridoma-huLAG-3 cells and anti-LAG-3 mAbs. The mixture of antibodies, T hybridoma-huLAG-3 cells, mouse splenocytes, and the antigen was cultured for 3 days. After 72 hours, 100 µl of cell culture supernatant were aspirated and diluted into appropriate concentrations for performing a mouse IL-2 quantitative ELISA using an R&D Systems ELISA kit according to the manufacturer's protocol. The ELISA plate was read on a SpectraMax M5 plate reader (Molecular Devices) using the ELISA-Endpoint-TMB & HRP protocol. RBA and Antigen-dependent Activation assay results are shown in Table 21.

Table 21: Characterization of Murine Anti-LAG-3 Antibodies

	FACS RBA		Antigen-dependent Activation	
	Raji 1 µg/ml HuLAG-3 ECD/Fc		Mouse IL-2	
mAb Identifier	IC50 (nM)	Max. Inhib. (%)	EC50 (nM)	Max. IL-2 (pg/ml)
mAb742	3.84	96.5	++	732.8
mAb743	11.66	96.6	++	583.2
mAb744	10.77	96.5	++	612.3
mAb745	12.38	95.0	+	357.1
mAb746	2.92	96.2	1.28	653.5
mAb747	2.84	96.3	1.27	729.2
mAb748	4.80	96.1	++	539.2
mAb749	4.73	93.9	+++	513.0
mAb750	7.15	96.6	+++	552.5
mAb751	6.59	96.0	++	447.0
mAb757	7.80	96.8	++	570.9
mAb758	N/A	7.9	-	182.9
mAb759	86.46	34.2		
mAb760	N/A	3.4		

mAb761	17.98	96.0		
Human IgG1 (control)	2.30	94.7	0.71	785.7

Example 7.7: Binding Affinity Determination by Biacore

For antibodies with high binding affinity in ELISA and FACS assays, as well as potent functional activity, binding affinities were determined based on measurement of binding kinetic constants in real time binding reactions using Biacore surface plasmon resonance. Briefly, the binding assay of antibody to antigen was performed using a Biacore T200 system through an antibody capture approach. Anti-mouse IgG Fc antibody was immobilized on a CM5 sensor chip according to the manufacturer's instructions. The test anti-LAG-3 murine monoclonal antibody was injected and captured by the immobilized anti-mouse IgG Fc. Then serial concentrations of LAG-3 antigen were individually injected, and the binding profile was recorded for each concentration of antigen analyte. The assay temperature was 25°C, and the association and dissociation times were 180 and 1200 seconds, respectively. The Biacore data were fitted using Biacore T200 evaluation software 1.0 according to a 1:1 binding model to calculate the association (k_{on}) and dissociation (k_{off}) rate constants and from these calculations the equilibrium dissociation constant (K_D) was determined. The affinities (K_D) for four selected anti-LAG-3 antibodies are shown in Table 22, below.

Table 22: Binding Affinity for Selected Anti-LAG-3 Antibodies

Antibody Identifier	Affinity for LAG-3 Antigen (K_D)
mAb746	3.774×10^{-8} M
mAb747	5.201×10^{-8} M
mAb749	1.893×10^{-7} M
mAb750	7.506×10^{-8} M

Example 7.8: Comparison of Anti-LAG-3 Antibody Function in PBMC Assay

To further verify the anti-LAG-3 antibodies function in human PBMC, a bacterial toxin stimulation assay using superantigen *Staphylococcus aureus* enterotoxin B (SEB) was conducted. SEB is a known superantigen for activating the immune system by stimulation of human T cells, which in turn causes an over-production of several cytokines. PBMC were isolated from a blood sample from a healthy human donor. PBMC were seeded into a 96-well assay plate with at 2×10^5 cells/well, then various anti-LAG-3 test antibodies were added into the plates and incubated with the PBMC at 37° C for 30 min. An SEB solution was added, to a final concentration of 10 ng/ml. The plates were then incubated for 96 hours. At the end of this incubation, 100 µl of cell culture supernatant were collected and IL-2 production was measured using an ELISA IL-2 detection kit (R&D Systems; Cat. No. DY202). Results are shown in Figure 6.

Example 8: Sequencing of Murine Anti-LAG-3 Antibody Variable Regions

To amplify heavy and light chain variable regions, total RNA of selected hybridoma clones was isolated from > 5×10^6 cells with TRIzol® RNA isolation reagent (Invitrogen; Cat. No. 15596). cDNA was synthesized by SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen; Cat. No. 18080) and applied as a PCR template of Mouse Ig-Primer Set (Novagen; Cat. No. 69831-3). PCR products were analyzed by electrophoresis on a 1.2% agarose gel with SYBR™ Safe DNA gel stain (Invitrogen). DNA fragments of correct size were purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH; Cat. No. 740609) according to manufacturer's instructions and subcloned into pMD18-T cloning vectors individually. Fifteen colonies from each transformation were selected and sequences of insert fragments were analyzed by DNA sequencing. Sequences were confirmed if at least 8 matches for consensus sequences for VH and VL were found. The variable region sequences of seven murine mAbs analyzed by sequence homology alignment are listed in Table 23. Complementarity determining regions (CDRs) were identified based on Kabat numbering.

Table 23: VH/VL Amino Acid Sequences of 7 Murine Anti-LAG-3 Antibodies

α-LAG-3 mAb ID	Domain	SEQ ID NO.	amino acid sequences 1234567890123456789012345678901234567890
mAb746	VH	60	EVQLQQSGAELVRPGASVKLSCTASDFNIKDDYMHWVKQR PEQGLDWIGWIVPENGNTEYASKFQGKATITADTSSNTAY LQLSSLTSEDTAVYYCTVYGDYWGQGTTLTVSS
	VL	61	DIQMTQSPSSLSASLGERVSLNCRASQEISGYLSWLQQKS DGTIKRLIYAASTLDSGVPKRFSGSRSGSDYSLTISSLES EDFADYYCLQYASYPLTFGAGTKLELK
mAb747	VH	60	EVQLQQSGAELVRPGASVKLSCTASDFNIKDDYMHWVKQR PEQGLDWIGWIVPENGNTEYASKFQGKATITADTSSNTAY LQLSSLTSEDTAVYYCTVYGDYWGQGTTLTVSS
	VL	62	DIQMTQSPSSLSASLGERVSLNCRASQEISGYLSWLQQKP DGTIKRLIYAASTLDSGVPKRFSGSRSGSDYSLTISSLES EDFAAYYCLQYASYPLTFGAGTKLELK
mAb742	VH	63	QGQLQQSGAELVRPGASVTLSCKASGYTFNDYEMHWVKQT PVHGLEWIGAIDPETGGTAYNQKFKGKAILTADKSSSTAY MELRSLTSEDSAVYYCIRWGSTVFPYWGQGTLVTVS
	VL	64	DGVLTQTPLSLPVNIGDQASISCKSTKSLLNSDGFTYLDW YLQKPGQSPQLLIYLVSNRFSGVPDRFSGSGSGTDFTLKI SRVEAEDLGVYYCFQSNYLPWTFGGGTKLEIK
mAb744	VH	65	QVQLQQSGAELVRPGTSVTLSCKASGYTFTDYEMHWMKQT PVHGLEWIGAIDPATGGTAYNQKFKGKAILTADKSSSTAY MDFRSLTSEDSAVYYCIRWGTTVFPYWGQGTLVTVS
	VL	66	DVVLTQTPLSLPVNIGDQASISCKSTKSLLNSDGFTYLDW YLQKPGQSPQLLIYLVSNRFSGVPDRFSGSGSGTDFTLKI SRVEAEDLGVYYCFQSNYLPWTFGGGTKLEIK
mAb748	VH	67	EVQMQQSGAELVRPGASVKLSCTVSGFNIKDDYMHWVKQR PEQGLEWIGWIDPENGDTEYASKFQGKATITADTSSNTAY LQLNSLTSEDTAVYYCTYFDYWGQGTTLTVSS
	VL	68	DVVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNW LLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKI SRVEAEDLGVYYCWQGSHFPQTFGGGTKLEIK
mAb749	VH	69	EVQLQQSGAELVRPGASVKVSCTASDFNIKDDYVHWVKQR PEQGLEWIGWIDPENGDTEYASKFQGKATITADTSSNTAY LQLSSLTSEDTAVYFCSTWDAEENYWGQGTTLSVSS
	VL	70	DIVLTQAAPSVPVTPGESVSISCRSSKSLLHSNGNTYLYW FLQRPGQSPQVLIYRMSNLASGVPVRFSGSGSGTAFTLRI SRVEAEDVGVYYCMQHLEYPFTFGSGTKLEIK

α-LAG-3 mAb ID	Domain	SEQ ID NO.	amino acid sequences 1234567890123456789012345678901234567890
mAb750	VH	71	EVQLQQSGAELVRPGASVKLSCTPSGLNIKDDYIHWVKQR PEQGLEWIGWIDPENGDTEYASKFQGKATITADTSSNTAY LQLSSLTSEDSAVYYCCTADYRNWYWGQGTTLTVSS
	VL	68	DVVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNW LLQRPGQSPKRLIYLVSKLDSGVPDRFTGSGSGTDFTLKI SRVEAEDLGVYYCWQGSHFPQTFGGGTKLEIK

Example 9: Humanization of Murine Anti-LAG-3 Antibody mAb747

Based on the antigen binding activity, cynomolgus LAG-3 protein cross-reactivity, functional activity, and affinity, mAb747 was selected for humanization.

Example 9.1: Humanization of mAb747

The anti-LAG-3 mAb747 variable region genes were employed to create a humanized mAb. In the first step of this process, the amino acid sequences of the VH and VK of mAb747 (SEQ ID NO:60 and SEQ ID NO:62) were compared against the available database of human Ig V-gene sequences in order to find the overall best-matching human germline Ig V-gene sequences. Additionally, the framework 4 sequence of VH or VL was compared against the J-region database to find the human framework having the highest homology to the murine VH and VL regions, respectively. For the light chain, the closest human V-gene match was the A1 gene, and for the heavy chain the closest human match was the VH1-f gene. Humanized variable domain sequences were then designed where the CDR-L1, CDR-L2, and CDR-L3 of the mAb747 light chain were grafted onto framework sequences of the A1 gene with JK4 framework 4 sequence after CDR-L3; and the CDR-H1, CDR-H2, and CDR-H3 sequences of the mAb747 heavy chain were grafted onto framework sequences of the VH1-f with JH1 framework 4 sequence after CDR-H3. A 3-dimensional Fv model of mAb747 was then generated to determine if there were any framework positions where mouse amino acids were critical to support loop structures or the VH/VL interface. Such residues in humanized sequences should be back-mutated to mouse residues at the same position to retain affinity/activity. Several desirable back-mutations were indicated for mAb747 VH and VL, and three alternative VH and

VL designs were constructed, as shown in Table 24, below. (Back mutated framework amino acid residues are indicated with double underscore; murine CDRs from the original parental antibody are underlined.)

Table 24: Humanization VH/VL Design for mAb747 – Back Mutations to Murine Residues

Humanized VH/VL Identifier	SEQ ID NO.	amino acid sequences 12345678901234567890123456789012345678901234567890
mAb747 VH.2A	72	EVQLVQSGAEVKKPGASVKVSCKASDFNIKDDYMHWVRQA PGQGLEWIGWIVPENGNTEYASKFQGRVTITADTSINTAY MELSRLRSDDTAVYYCTVYGDY----WGQGTTVTVSS
mAb747 VH.2B	73	EVQLVQSGAEVKKPGASVKVSCKASDFNIKDDYMHWVRQA PGQGLEWIGWIVPENGNTEYASKFQGKATITADTSINTAY MELSRLRSDDTAVYYCTVYGDY----WGQGTTVTVSS
mAb747 VH.1G	74	EVQLVQSGAEVKKPGATVKISCKASDFNIKDDYMHWVQQA PGKGLEWIGWIVPENGNTEYASKFQGRVTITADTSTNTAY MELSSLRSEDTAVYYCTVYGDY----WGQGTTVTVSS
mAb747 VK.1E	75	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKP GKTIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFATYYCLQYASYPLTFGGGTKVEIK
mAb747 VK.2A	76	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKP EKTIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFATYYCLQYASYPLTFGGGTKVEIK
mAb747 VK.2B	77	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKP EGTIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFATYYCLQYASYPLTFGGGTKVEIK

The humanized VH and VK genes were produced synthetically and then cloned into vectors containing the human IgG1 and human kappa constant domains, respectively. The pairing of the human VH and the human VK created 9 humanized anti-LAG-3 antibodies, named HumAb747-34 to -42 (Table 25). A chimeric antibody with parental mouse VH/VL and human constant sequences was also produced (mAb747c) as a positive control, for affinity comparison.

Table 25: Production List Humanized mAb747 Anti-LAG-3 Antibodies

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
HumAb747-34	mAb747 VH.1G	mAb747 VK.1E
HumAb747-35	mAb747 VH.1G	mAb747 VK.2A

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
HumAb747-36	mAb747 VH.1G	mAb747 VK.2B
HumAb747-37	mAb747 VH.2A	mAb747 VK.1E
HumAb747-38	mAb747 VH.2A	mAb747 VK.2A
HumAb747-39	mAb747 VH.2A	mAb747 VK.2B
HumAb747-40	mAb747 VH.2B	mAb747 VK.1E
HumAb747-41	mAb747 VH.2B	mAb747 VK.2A
HumAb747-42	mAb747 VH.2B	mAb747 VK.2B

All 9 humanized antibodies (Table 25) and a chimeric antibody having the parental murine VH and VL domains (mAb747c) were ranked by dissociation rate constant (k_{off}). Briefly, antibodies were characterized for affinities and binding kinetics by Octet® RED96 biolayer interferometry (Pall FortéBio LLC). Antibodies were captured by Anti-HIgG Fc Capture (AHC) Biosensors (Pall) at a concentration of 100 nM for 30 seconds. Sensors were then dipped into running buffer (1X pH7.2 PBS, 0.05% Tween 20, 0.1% BSA) for 60 seconds to check baseline. Binding was measured by dipping sensors into a single concentration of recombinant human LAG-3-his protein (Novoprotein). Dissociation was followed by dipping sensors into running buffer for 1200 seconds. The association and dissociation curves were fitted to a 1:1 Langmuir binding model using FortéBio Data Analysis software (Pall). Results are shown in Table 26.

Table 26: off-rate ranking of humanized anti-LAG-3 antibodies

Antibody	Off-rate (k_{off}) (1/s)
mAb747c	3.77×10^{-4}
HumAb747-42	8.30×10^{-4}
HumAb747-39	1.14×10^{-3}

HumAb747-42 showed an off-rate constant only 2.2-fold greater than that of the chimeric control having the parental variable domains.

Example 10: Production of PD-1/LAG-3 Fabs-in-Tandem Immunoglobulins (FIT-Igs)

Bispecific Fabs-in-Tandem Immunoglobulin binding proteins recognizing both human PD-1 and human LAG-3 were constructed.

For each of the FIT-Ig constructs described in the following tables, the signal sequence used in the expression vector for each of the three component polypeptide chains is shown. Either MDMRVPAQLLGLLLLWFPGSRC (SEQ ID NO:79) or MEFGLSWLFLVAILKGVQC (SEQ ID NO:84) was used in the production of the FIT-Ig proteins described below, although many alternative signal peptides will be known to those skilled in the art and may be used as well. It will be understood that such signal sequences are cleaved during secretion of the polypeptides by the expressing host cell, and thus the signal sequences are not part of the final FIT-Ig binding proteins.

Example 10.1: FIT107-1-2a

A PD-1/LAG-3 FIT-Ig designated FIT107-1-2a was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies mAb709 (murine anti-PD-1, see Table 4 *supra*) and mAb746 (murine anti-LAG-3, see Table 23 *supra*). FIT-Ig FIT107-1-2a is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of mAb709 fused directly to VH-CH1 of mAb746 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1 (see Table 6, *supra*);

Polypeptide chain #2 has the domain formula: VH-CH1 of mAb709; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of mAb746.

The amino acid sequences for the three expressed FIT107-1-2a polypeptide chains are shown in Table 27 below.

Table 27: Amino Acid Sequences of FIT107-1-2a Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-2a	78	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSHKFMSTSVGDS** **VTITCKASQDVNTVVAWYQQKPGQSLKVLISWASTRHTGV**

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT-Ig Polypeptide Chain #1		**PARFTGSGSGTDYTLTISSVQAEDLALYYCQQHYTTPYTF GGGTQLEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL QQSGAELVRPGASVKLSCTASDFNIKDDYMHWVKQRPEQG LDWIGWIVPENGNTEYASKFQGKATITADTSSNTAYLQLS SLTSEDTAVYYCTVYGDYWGQGTTLTVSS**ASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb murine mAb709 (VL underlined)	80	DIVMTQSHKFMSTSVGDSVTITCKASQDVNTVVAWYQQKP GQSLKVLISWASTRHTGVPARFTGSGSGTDYTLTISSVQA EDLALYYCQQHYTTPYTFGGGTQLEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb murine mAb746 (VH underlined)	81	EVQLQQSGAELVRPGASVKLSCTASDFNIKDDYMHWVKQR PEQGLDWIGWIVPENGNTEYASKFQGKATITADTSSNTAY LQLSSLTSEDTAVYYCTVYGDYWGQGTTLTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-2a FIT-Ig Polypeptide	83	MEFGLSWLFLVAILKGVQC**EVKLVESGGGLVKPGGSLKLS CAASGFTFSFYTMSWVRQTPEKRLEWVATISGGGRDTYYP DSVKGRFTISRDNAKNTLYLHMSSLRSEDTALYYCAGQGG**

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
Chain #2		**NYLFAYWGQGTLVTVSA**ASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of murine mAb709 (VH underlined)	85	EVKLVESGGGLVKPGGSLKLSCAASGFTFSFYTMSWVRQT PEKRLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNTLY LHMSSLRSEDTALYYCAGQGGNYLFAYWGQGTLVTVSAAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC
FIT107-1-2a FIT-Ig Polypeptide Chain #3	86	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASLGER VSLNCRASQEISGYLSWLQQKSDGTIKRLIYAASTLDSGV PKRFSGSRSGSDYSLTISSLESEDFADYYCLQYASYPLTF GAGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of murine mAb746 (VL underlined)	87	DIQMTQSPSSLSASLGERVSLNCRASQEISGYLSWLQQKS DGTIKRLIYAASTLDSGVPKRFSGSRSGSDYSLTISSLES EDFADYYCLQYASYPLTFGAGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 10.2: FIT107-1-2b

Another bispecific Fabs-in-Tandem Immunoglobulin recognizing both human PD-1 and human LAG-3 was constructed. This PD-1/LAG-3 FIT-Ig was designated FIT107-1-2b.

Construction of FIT107-1-2b binding protein utilized coding sequences for immunoglobulin domains from the parental murine antibodies mAb709 and mAb746, but in this FIT-Ig construct, the LAG-3-binding domain was in the N-terminal (outer) position, and the PD-1-binding domain was in the inner position, fused C-terminal to the VL-CL domains of the LAG-3 binding region. FIT-Ig FIT107-1-2b is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of mAb746 fused directly to VH-CH1 of mAb709 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1 (see Table 6, *supra*);

Polypeptide chain #2 has the domain formula: VH-CH1 of mAb746; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of mAb709.

The amino acid sequences for the three expressed FIT107-1-2b polypeptide chains are shown in Table 28 below:

Table 28: Amino Acid Sequences of FIT107-1-2b Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-2b FIT-Ig Polypeptide Chain #1	88	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASLGER VSLNCRASQEISGYLSWLQQKSDGTIKRLIYAASTLDSGV PKRFSGSRSGSDYSLTISSLESEDFADYYCLQYASYPLTF GAGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVKL VESGGGLVKPGGSLKLSCAASGFTFSFYTMSWVRQTPEKR LEWVATISGGGRDTYYPDSVKGRFTISRDNAKNTLYLHMS SLRSEDTALYYCAGQGGNYLFAYWGQGTLVTVSA**ASTKGP SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN HKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of murine mAb746 (VL underlined)	89	DIQMTQSPSSLSASLGERVSLNCRASQEISGYLSWLQQKS DGTIKRLIYAASTLDSGVPKRFSGSRSGSDYSLTISSLES EDFADYYCLQYASYPLTFGAGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
VH-CH1 of murine mAb709 (VH underlined)	90	EVKLVESGGGLVKPGGSLKLSCAASGFTFSFYTMSWVRQT PEKRLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNTLY LHMSSLRSEDTALYYCAGQGGNYLFAYWGQGTLVTVSAAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-2b FIT-Ig Polypeptide Chain #2	91	MEFGLSWLFLVAILKGVQC**EVQLQQSGAELVRPGASVKLS CTASDFNIKDDYMHWVKQRPEQGLDWIGWIVPENGNTEYA SKFQGKATITADTSSNTAYLQLSSLTSEDTAVYYCTVYGD YWGQGTTLTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of murine mAb746 (VH underlined)	92	EVQLQQSGAELVRPGASVKLSCTASDFNIKDDYMHWVKQR PEQGLDWIGWIVPENGNTEYASKFQGKATITADTSSNTAY LQLSSLTSEDTAVYYCTVYGDYWGQGTTLTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
FIT107-1-2b FIT-Ig Polypeptide Chain #3	93	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSHKFMSTSVGDS VTITCKASQDVNTVVAWYQQKPGQSLKVLISWASTRHTGV PARFTGSGSGTDYTLTISSVQAEDLALYYCQQHYTTPYTF GGGTQLEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
VL-CL of murine mAb709 (VL underlined)	94	DIVMTQSHKFMSTSVGDSVTITCKASQDVNTVVAWYQQKP GQSLKVLISWASTRHTGVPARFTGSGSGTDYTLTISSVQA EDLALYYCQQHYTTPYTFGGGTQLEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 10.3: FIT107-1-5a

A PD-1/LAG-3 FIT-Ig designated FIT107-1-5a was constructed utilizing coding sequences for immunoglobulin domains from the parental humanized antibodies HumAb709-8 (anti-PD-1, SEQ ID NO:21 and SEQ ID NO:25) and HumAb747-42 (SEQ ID NO:73 and SEQ ID NO:77). FIT-Ig FIT107-1-5a is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb709-8 fused directly to VH-CH1 of HumAb747-42 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1 (see Table 6, *supra*);

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb709-8; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb747-42.

The amino acid sequences for the three expressed FIT107-1-5a polypeptide chains are shown in Table 29 below:

Table 29: Amino Acid Sequences of FIT107-1-5a Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-5a FIT-Ig Polypeptide Chain #1	95	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSPSSLSASVGDR** **VTITCKASQDVNTVVAWYQQKPGKAPKVLISWASTRHTGV** **PSRFSGSGSGTDYTLTISSLQPEDFATYYCQQHYTTPYTF** **GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL** **VQSGAEVKKPGASVKVSCKASDFNIKDDYMHWVRQAPGQG**

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
		LEWIGWIVPENGNTEYASKFQGKATITADTSINTAYMELS **RLRSDDTAVYYCTVYGDYWGQGTTVTVSS**ASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HumAb709-8 (VL underlined)	96	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb747-42 (VH underlined)	97	EVQLVQSGAEVKKPGASVKVSCKASDFNIKDDYMHWVRQA PGQGLEWIGWIVPENGNTEYASKFQGKATITADTSINTAY MELSRLRSDDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-5a FIT-Ig Polypeptide Chain #2	98	MEFGLSWLFLVAILKGVQC**EVQLVESGGGLVQPGGSLRLS** **CAASGFTFSFYTMSWVRQAPGKGLEWVATISGGGRDTYYP** **DSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAGQGG** **NYLFAYWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
VH-CH1 of HumAb709-8 (VH underlined)	99	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC
FIT107-1-5a FIT-Ig Polypeptide Chain #3	100	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTINCRASQEISGYLSWLQQKPEGTIKRLIYAASTLDSGV PSRFSGSRSGSDYTLTISSLQPEDFATYYCLQYASYPLTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb747-42 (VL underlined)	101	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKP EGTIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFATYYCLQYASYPLTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 10.4: FIT107-1-5b

Another bispecific Fabs-in-Tandem Immunoglobulin recognizing both human PD-1 and human LAG-3 was constructed. This PD-1/LAG-3 FIT-Ig was designated FIT107-1-5b. Construction of FIT107-1-5b binding protein utilized coding sequences for immunoglobulin domains from the parental humanized antibodies HumAb709-8 and HumAb747-42, but in this FIT-Ig construct, the LAG-3-binding domain was in the N-terminal (outer) position, and the PD-1-binding domain was in the inner position, fused C-terminal to the VL-CL domains of the N-terminal LAG-3 binding region. FIT-Ig FIT107-1-5b is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb747-42 fused directly to VH-CH1 of HumAb709-8 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1 (see

Table 6, *supra*);

Polypeptide chain #2 has the domain formula: VH-CH1 of mAb747-42; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb709-8.

The amino acid sequences for the three expressed FIT107-1-5b polypeptide chains are shown in Table 30 below:

Table 30: Amino Acid Sequences of FIT107-1-5b Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678 90
FIT107-1-5b FIT-Ig Polypeptide Chain #1	102	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTINCRASQEISGYLSWLQQKPEGTIKRLIYAASTLDSGV PSRFSGSRSGSDYTLTISSLQPEDFATYYCLQYASYPLTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL VESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQAPGKG LEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLYLQMN SLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSS**ASTKGP SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN HKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb747-42 (VL underlined)	103	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKP EGTIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFATYYCLQYASYPLTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of HumAb709-8	104	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
(VH underlined)		SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-5b FIT-Ig Polypeptide Chain #2	105	MEFGLSWLFLVAILKGVQC**EVQLVQSGAEVKKPGASVKVS CKASDFNIKDDYMHWVRQAPGQGLEWIGWIVPENGNTEYA SKFQGKATITADTSINTAYMELSRLRSDDTAVYYCTVYGD YWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb747-42 (VH underlined)	106	EVQLVQSGAEVKKPGASVKVSCKASDFNIKDDYMHWVRQA PGQGLEWIGWIVPENGNTEYASKFQGKATITADTSINTAY MELSRLRSDDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
FIT107-1-5b FIT-Ig Polypeptide Chain #3	107	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSPSSLSASVGDR VTITCKASQDVNTVVAWYQQKPGKAPKVLISWASTRHTGV PSRFSGSGSGTDYTLTISSLQPEDFATYYCQQHYTTPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HuMAb709-8 (VL underlined)	108	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 10.5: Expression and Purification of FIT-Igs

The four PD-1/LAG-3 FIT-Ig constructs FIT107-1-2a, FIT107-1-2b, FIT107-1-5a and FIT107-1-5b are a type of bispecific, multivalent binding protein known as a Fabs-in-Tandem Immunoglobulin (or FIT-Ig) described generally in WO 2015/103072 and WO 2017/136820. The binding proteins were produced by co-expression of three component polypeptide chains in a mammalian host cell transfected with expression vectors for all three chains. The design of the binding protein calls for the long polypeptide chain (Chain #1) to pair with both the short polypeptide chains (Chains #2 and #3) to form functional tandem Fab moieties, and also the long chain is designed to dimerize via the Fc region (hinge-CH2-CH3), such that a six-chain binding protein exhibiting four intact Fab binding sites is formed. In the binding proteins FIT107-1-2a and FIT107-1-5a, the N-terminal or "outer" Fab binding sites bind PD-1 and the adjacent "inner" Fab binding sites bind LAG-3. The outer Fab fragment (anti-PD-1) of FIT107-1-2a and FIT107-1-5a is joined to the inner Fab fragment (anti-LAG-3) only through the long chain (Chain #1) by direct fusion of VL-CL_{mAb709} or VL-$CL_{HumAb709-8}$ as the case may be at its C-terminus to the N-terminus of VH-$CH1_{mAb746}$ or VH-$CH1_{HumAb747-42}$, respectively, without the use of linkers connecting the immunoglobulin domains. Similarly, the outer Fab fragment (anti-LAG-3) of FIT107-1-2b and FIT107-1-5b is joined to the inner Fab fragment (anti-PD-1) only through the long chain (Chain #1) by direct fusion of VL-CL_{mAb746} or VL-$CL_{mAb747-42}$ as the case may be at its C-terminus to the N-terminus of VH-$CH1_{mAb709}$ or VH-$CH1_{mAb709-8}$, respectively, without the use of linkers connecting the immunoglobulin domains.

Expression vectors coding for polypeptide Chains #1, #2, and #3 of each FIT-Ig (FIT107-1-2a, FIT107-1-2b, FIT107-1-5a and FIT107-1-5b) were transiently co-expressed using polyethyleneimine (PEI) as a transfection agent in human embryonic kidney 293E cells. Briefly, DNA in FreeStyle™ 293 Expression Medium was mixed with the PEI with the final concentration of DNA to PEI ratio of 1:2, incubated for 15-20 minutes at room temperature, and then added to the HEK293E cells (1.0–1.2 × 10^6/ml, cell viability > 95%) at 60μg DNA/120ml culture. After 6-24 hours culture in shaker, peptone was added to the transfected cells at a final concentration of 5%, with shaking at 125 rpm/min., at 37℃, 8% CO_2. On the 6th - 7th day,

supernatant was harvested by centrifugation and filtration, and FIT-Ig protein was purified using Protein A chromatography (Pierce, Rockford, IL) according to the manufacturer's instructions.

For the expression FIT107-1-2a, FIT107-1-2b, FIT107-1-5a and FIT107-1-5b, the DNA coding for expression of Chains #1, #2, and #3 were transfected using a molar ratio for Chain #1: Chain #2: Chain #3 of 1:3:3. This was designed to cause proportionally more of the short chains #2 and #3 to be expressed relative to the long chain (Chain #1), which in turn would decrease the occurrence of VL-CL and VH-CH1 segments on the long chain (Chain #1) that were not paired with corresponding light chains and thus would fail to form a functional Fab fragment. FIT-Ig protein expression products were purified by Protein A chromatography. The composition and purity of the purified FIT-Igs were analyzed by size exclusion chromatography (SEC). Purified FIT-Ig, in PBS, was applied on a TSKgel SuperSW3000, 300 × 4.6 mm column (TOSOH). An HPLC instrument, Model U3000 (DIONEX) was used for SEC using UV detection at 280 nm and 214 nm. See Table 31, below.

Table 31: Expression and SEC analysis of PD-1/LAG-3 FIT-Ig binding proteins

FIT-Ig protein	DNA molar ratio: Chain #1 : #2 : #3	Expression level (mg/L)	% Peak Monomeric Fraction by SEC
FIT107-1-2a	1:3:3	5.40	>90%
FIT107-1-2b	1:3:3	5.04	>80%
FIT107-1-5a	1:3:3	7.95	>80%
FIT107-1-5b	1:3:3	8.52	>80%

The lower monomeric fraction contents for FIT107-1-2b, -5a, and -5b indicate some possible aggregation.

Example 11: Binding Affinities of FIT-Igs for Target Antigens

The kinetics of FIT-Ig binding to PD-1 and LAG-3 targets was determined by Biacore SPR measurements. Binding affinities of FIT107-1-2a and FIT107-1-2b for both target antigens PDL-1 and LAG-3 are shown in Table 32, below.

Table 32: Binding Affinities for FIT107-1-2a and FIT107-1-2b

Target immobilized on sensor chip	Analyte	Analyte Specificity	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
HuPD-1 ECD/Fc and HuLAG-3 ECD/Fc	FIT107-1-2a	outer: PD-1 inner: LAG-3	6.97×10^{4}	1.17×10^{-5}	1.68×10^{-10}
	FIT107-1-2b	outer: LAG-3 inner: PD-1	1.68×10^{5}	8.76×10^{-5}	5.23×10^{-10}
	mAb709c	PD-1	2.88×10^{5}	9.72×10^{-6}	3.37×10^{-11}
	mAb746c	LAG-3	9.51×10^{3}	1.54×10^{-4}	1.62×10^{-8}
	mAb709c + mAb746c	PD-1 and LAG-3	2.82×10^{5}	2.08×10^{-5}	7.35×10^{-11}
HuPD-1 ECD/Fc	mAb709c	PD-1	2.52×10^{5}	8.82×10^{-6}	3.50×10^{-11}
HuLAG-3 ECD/Fc	mAb746c	LAG-3	5.58×10^{3}	1.81×10^{-4}	3.25×10^{-8}

Example 12: FIT-Ig Specificity and Function Determinations

The anti-PD1 and anti-LAG-3 bispecificity and biological activity of the FIT107-1-2a binding protein was tested in a PBMC activation assay using Staphylococcal enterotoxin B (SEB) as a superantigen (see, Example 7.8). Briefly, PBMC were isolated from a healthy donor, then seeded into a 96-well plate with 50 µl/well at 2×10^{5} cells/well. Test binding proteins (i.e., FIT107-1-2a, a combination of commercially available anti-PD-1 and anti-LAG-3 monoclonal antibodies, or a monoclonal anti-PD-1 antibody) were added into the plates and incubated with PBMC at 37° C for 30 min. SEB solution was added to a final concentration of 10 ng/ml. The plates were incubated for 96 hours, then 100 µl cell culture supernatant were collected and IL-2 production was measured using an ELISA IL-2 detection kit (R&D Systems; Cat. No. DY202). The results are shown in Figure 7. The FIT107-1-2a bispecific FIT-Ig protein was able to enhance T cell activation, as indicated by IL-2 production, in comparison to an anti-PD-1

antibody alone or a mixture of anti-LAG-3 and anti-PD-1 antibodies.

In addition, a mixed lymphocyte reaction (MLR) assay was performed in a similar manner as described in Example 3, to further verify anti-PD-1 and anti-LAG-3 function for FIT107-1-2a. The mixed lymphocyte reaction is an *ex vivo* cellular immune response that occurs between two allogeneic lymphocyte populations when mixed together. Allogeneic lymphocytes undergo blast transformation, DNA synthesis and cellular proliferation in response to the major histocompatibility antigen (MHC Class I and II) differences between the two cell populations, which designated as Responder and Stimulator cells. In the MLR for testing FIT107-1-2a, at day 1, PBMC were purified from healthy donors and CD14+ monocytes were isolated. Monocytes were seeded into 6-well plates and treated with 35 ng/ml IL-4 (R&D Systems) and 50 ng/ml GM-CSF (R&D Systems) in RPMI 1640 medium plus 10% FBS. The medium was exchanged after day 4. At Day 7, monocytes differentiated into immature dendritic cells were collected and further processed for two days in maturation medium with 20 ng/ml TNF-α (R&D Systems), 50 μg/ml Poly I:C (Sigma), 35 ng/ml IL-4 and 50 ng/ml GM-CSF. For MLR co-culture assays, X-VIVO™ 15 serum free medium was used to avoid serum interference in antibody efficacy. 96-well U-bottom plates were seeded with allogeneic CD4+ T cells (responder cells) at 1×10^5 cells/well and pre-treated with test binding protein (i.e., FIT107-1-2a, a combination of commercially available anti-PD-1 and anti-LAG-3 monoclonal antibodies, or a monoclonal anti-PD-1 antibody) for 30 min. Then mature dendritic cells (stimulator cells) were seeded into the wells at 1×10^4 cells/well, and co-cultured with the responder cells for five days, at which time 100 μl of supernatant was collected and IFN-γ production was measured by ELISA. The results are shown in Figure 8. The FIT-Ig bispecific binding protein showed an EC50 of 0.5084 nM, as compared with EC50 values of 16.84 nM for a combination of anti-PD-1 and anti-LAG-3 antibodies or an anti-PD-1 antibody alone. Thus, the FIT-Ig binding protein enhanced IFN-γ (gamma interferon) production in the MLR at a concentration over 30-fold lower than the single antibody or antibody combination.

Example 13: New Batch Humanization of mAb747

The anti-LAG-3 mAb747 variable region genes were employed to create a further anti-

LAG-3 humanized mAb. In the first step of this process, the amino acid sequences of the VH and VK of mAb747 (SEQ ID NO:60 and SEQ ID NO:62) were compared against the available database of human Ig V-gene sequences in order to find best-matching human germline Ig V-gene sequences. Additionally, the framework 4 (FW4) sequence of VH or VL was compared against the J-region database to find the human framework having the highest homology to the murine VH and VL regions, respectively. For the light chain, the best human V-gene match was the A30 gene, and for the heavy chain the best human match was the VH1-69-2 gene. Humanized variable domain sequences were then designed where the CDR-L1, CDR-L2, and CDR-L3 of the mAb747 light chain were grafted onto framework sequences of the A30 gene with JK4 framework 4 sequence after CDR-L3; and the CDR-H1, CDR-H2, and CDR-H3 sequences of the mAb747 heavy chain were grafted onto framework sequences of the VH1-69-2 with JH6 framework 4 sequence after CDR-H3. A 3-dimensional Fv model of mAb747 was then generated to determine if there were any framework positions where mouse amino acids were critical to support loop structures or the VH/VL interface. Such residues in humanized sequences should be back-mutated to mouse residues at the same position to retain affinity/activity. Several desirable back-mutations were indicated for mAb747 VH and VL, and three alternative VH and VL designs were constructed, as shown in Table 33, below. (Back mutated framework amino acid residues are indicated with double underscore; CDRs are underlined according to Kabat numbering system except VH CDR1 defined with ABM numbering system.)

Table 33: Humanization VH/VL Design for mAb747 – Back Mutations to Murine Residues

Humanized VH/VL Identifier	SEQ ID NO.	Amino acid sequences 12345678901234567890123456789012345678901234567890
huEpi001-VHv1	109	EVQLVQSGAEVKKPGATVKISCKVSDFNIKDDYMHWVQQA PGKGLEWIGWIVPENGNTEYASKFQGRVTITADTSTDTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS
huEpi001-VHv2	110	EVQLVQSGAEVKKPGATVKLSCKASDFNIKDDYMHWVQQA PGKGLEWIGWIVPENGNTEYASKFQGRVTITADTSTDTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS

Humanized VH/VL Identifier	SEQ ID NO.	Amino acid sequences 12345678901234567890123456789012345678901234567890
huEpi001-VHv3	111	EVQLVQSGAEVKKPGATVKLSCTASDFNIKDDYMHWVQQA PGKGLEWIGWIVPENGNTEYASKFQGRVTITADTSTDTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS
huEpi001-VHv4	112	EVQLVQSGAEVKKPGATVKLSCTASDFNIKDDYMHWVKQA PGKGLEWIGWIVPENGNTEYASKFQGRATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS
huEpi001-VHv5	113	EVQLVQSGAEVKKPGATVKLSCTASDFNIKDDYMHWVKQR PEQGLEWIGWIVPENGNTEYASKFQGRATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS
huEpi001-VHv6	114	EVQLVQSGAEVKKPGATVKLSCTASDFNIKDDYMHWVKQR PEQGLDWIGWIVPENGNTEYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS
huEpi001 VLv1	115	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GKAIKSLIYAASTLDSGVPSRFSGSRSGTDFTLTISSLQP EDFATYYCLQYASYPLTFGQGTKLEIK
huEpi001 VLv2	116	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GKAIKRLIYAASTLDSGVPSRFSGSRSGTDFTLTISSLQP EDFATYYCLQYASYPLTFGQGTKLEIK
huEpi001 VLv3	117	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELK
huEpi001 VLv4	118	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLEP EDFADYYCLQYASYPLTFGQGTKLELK

The humanized VH and VK genes were produced synthetically and then cloned into vectors containing the human IgG1 and human kappa constant domains, respectively. The pairing of the human VH and the human VK created 9 humanized anti-LAG-3 antibodies, named HumAb747-43 to HumAb747-60 (Table 34). The chimeric antibody with parental mouse VH/VL and human constant sequences described above was also used (mAb747c) as a positive control, for affinity comparison.

Table 34: Production List Humanized mAb747 Anti-LAG-3 Antibodies

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
HumAb747-43	huEpi001-VHv1	huEpi001 VLv1
HumAb747-44	huEpi001-VHv2	huEpi001 VLv1
HumAb747-45	huEpi001-VHv3	huEpi001 VLv1
HumAb747-46	huEpi001-VHv4	huEpi001 VLv1
HumAb747-47	huEpi001-VHv1	huEpi001 VLv2
HumAb747-48	huEpi001-VHv2	huEpi001 VLv2
HumAb747-49	huEpi001-VHv3	huEpi001 VLv2
HumAb747-50	huEpi001-VHv4	huEpi001 VLv2
HumAb747-51	huEpi001-VHv1	huEpi001 VLv3
HumAb747-52	huEpi001-VHv2	huEpi001 VLv3
HumAb747-53	huEpi001-VHv3	huEpi001 VLv3
HumAb747-54	huEpi001-VHv4	huEpi001 VLv3
HumAb747-55	huEpi001-VHv1	huEpi001 VLv4
HumAb747-56	huEpi001-VHv2	huEpi001 VLv4
HumAb747-57	huEpi001-VHv3	huEpi001 VLv4
HumAb747-58	huEpi001-VHv4	huEpi001 VLv4
HumAb747-59	huEpi001-VHv5	huEpi001 VLv3
HumAb747-60	huEpi001-VHv6	huEpi001 VLv3

All 18 humanized antibodies (Table 34) and a chimeric antibody having the parental murine VH and VL domains (mAb747c) were ranked by dissociation rate constant (k_{off}). Briefly, antibodies were characterized for affinities and binding kinetics by Octet®RED96 biolayer interferometry (Pall FortéBio LLC). Antibodies were captured by Anti-hIgG Fc Capture (AHC) Biosensors (Pall) at a concentration of 100 nM for 30 seconds. Sensors were then dipped into running buffer (1X pH7.2 PBS, 0.05% Tween 20, 0.1% BSA) for 60 seconds to

check baseline. Binding was measured by dipping sensors into a single concentration of recombinant human LAG-3-his protein (Novoprotein). Dissociation was followed by dipping sensors into running buffer for 1200 seconds. The association and dissociation curves were fitted to a 1:1 Langmuir binding model using FortéBio Data Analysis software (Pall). Results are shown in Table 35. In each test group, the off-rates of antibodies were able to be compared with that of mAb747c. The off-rate ratios were calculated by the off-rate of antibody to that of mAb747c of its group and were compared all together. The lower the ratio was, the higher was the affinity of the antibody.

Table 35: Off-rate Ranking of Humanized Anti-LAG-3 Antibodies

Test group	Antibody	Off-rate (k_{off}) (1/s)	off-rate ratio to that of mAb747c
1	HumAb747-43	9.25×10^{-3}	682%
	HumAb747-44	1.10×10^{-2}	809%
	HumAb747-45	1.08×10^{-2}	797%
	HumAb747-46	1.09×10^{-2}	801%
	HumAb747-47	1.44×10^{-2}	1062%
	HumAb747-48	8.03×10^{-3}	592%
	mAb747c	1.36×10^{-3}	100%
2	HumAb747-49	7.73×10^{-3}	575%
	HumAb747-50	7.19×10^{-3}	534%
	HumAb747-51	1.19×10^{-2}	888%
	HumAb747-52	4.36×10^{-3}	324%
	HumAb747-53	4.30×10^{-3}	319%
	HumAb747-54	4.23×10^{-3}	314%
	mAb747c	1.35×10^{-3}	100%
3	HumAb747-55	1.19×10^{-2}	972%
	HumAb747-56	4.31×10^{-3}	352%
	HumAb747-57	4.11×10^{-3}	335%
	HumAb747-58	4.05×10^{-3}	331%
	mAb747c	1.23×10^{-3}	100%
4	HumAb747-59	1.20×10^{-3}	226%
	HumAb747-60	8.10×10^{-4}	153%

Test group	Antibody	Off-rate (k_{off}) (1/s)	off-rate ratio to that of mAb747c
	mAb747c	5.30×10^{-4}	100%

HumAb747-60 showed an off-rate constant only 1.5-fold greater than that of the chimeric control having the parental variable domains.

To further verify the anti-LAG-3 antibodies function in human PBMC, a bacterial toxin stimulation assay using superantigen *Staphylococcus aureus* enterotoxin B (SEB) was conducted, in the manner described in Example 7.8 *supra*. IL-2 production was measured using a PerkinElmer IL-2 detection kit (PerkinElmer; Cat. No. TRF1221M). HumAb747-60 was able to enhance IL-2 secretion of SEB-stimulated PBMC by blocking LAG-3 signal pathway. Results are shown in Figure 9. HumAb747-60 was thus proven functional and was selected for further engineering.

Example 14: Affinity Maturation of HumAb747-60

Example 14.1: Affinity Maturation Library Construction and Screening

Although HumAb747-60 showed an off-rate constant only 1.5-fold greater than that of the chimeric control mAb747c, the PBMC-SEB assay results indicated HumAb747-60 has a slightly weaker functional activity. To further improve the affinity, the CDR residues (in ABM numbering system) were optimized by affinity maturation based on HumAb747-39 (see Tables 24, 25). Two phage libraries were designed and constructed. One was designed to mutate CDR-L1, CDR-L3 and CDR-H3 (ABM numbering), each of which had one randomly mutated residue. The other was designed to mutate CDR-L2, CDR-H1 and CDR-H2 (ABM numbering), each of which had one randomly mutated residue.

The phage display libraries were constructed using the method reported in *Journal of Immunological Methods*, 201:35–55 (1997). Briefly, VH-CH1 and VL-CL were amplified with degenerated primers that introduce mutations, and then cloned into two multiple cloning sites (MCS) of a phagemid vector sequentially. The phagemid vectors were then electro-transformed into TG1 (Cat.No. 60502-1, Lucigen), resulting in libraries of approximately 1.2×10^8 clones, respectively, showing high sequence diversity. The libraries were rescued with M13K07 helper

phage (Cat. No. N0315S) at approximately a 1:20 ratio (cell to phage). Phage display library selections were performed with recombinant human LAG-3 protein, followed by washing steps. A Fab fragment of HumAb747-39 was also constructed in a phagemid vector as a positive control. Selected phage were used for infection of host cells. The binding ability of Fab supernatants from single clones were screened by ELISA. Briefly, the Fab supernatants of single clones were prepared by overnight culturing at 30° C with 1mM IPTG. 2 μg/ml of human LAG-3 protein in 100 μL of phosphate buffered saline (PBS) were directly coated in each well of 96-well plates. The HRP-linked anti-c-myc antibody (Cat. No. Ab1261, Abcam) and TMB reagent were used to detect and develop the ELISA signal, which was read out by a plate reader (SpectraMax® Plus 384 absorbance plate reader, Molecular Devices) at wavelength of 450 nm.

Positive clones in the screening ELISA were picked for sequencing. Considering sequence redundancy and signal strength in the screening ELISA, the following 7 clones were selected for further evaluation. The VH/VL sequences are shown in Table 36. (Mutated amino acid residues identified by affinity maturation are indicated with double underscore; CDRs are underlined according to Kabat numbering system.)

Table 36: VH/VL Amino Acid Sequences of 7 Antibodies with affinity matured mutations

Affinity matured clones	Domain	SEQ ID NO.	protein sequences 1234567890123456789012345678901234567890
B2-53	VH	119	EVQLVQSGAEVKKPGASVKVSCKASGFNIKDDYMHWVRQAPG QGLEWIGWIVPENGNTVYASKFQGRVTITADTSINTAYMELS RLRSDDTAVYYCTVYGDYWGQGTTVTVSS
	VL	120	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKPEG TIKRLIYAASALDSGVPSRFSGSRSGSDYTLTISSLQPEDFA TYYCLQYASYPLTFGGGTKVEIK
B3-21	VH	121	EVQLVQSGAEVKKPGASVKVSCKASDFNIKDDYMHWVRQAPG QGLEWIGWIVPENGNTEYASKFQGRVTITADTSINTAYMELS RLRSDDTAVYYCTVYGDVWGQGTTVTVSS
	VL	122	DIQMTQSPSSLSASVGDRVTINCRAMQEISGYLSWLQQKPEG TIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQPEDFA TYYCLQYAYYPLTFGGGTKVEIK

Affinity matured clones	Domain	SEQ ID NO.	protein sequences 12345678901234567890123456789012345678901234567890
B3-43	VH	123	EVQLVQSGAEVKKPGASVKVSCKASGFNIKDDYMHWVCQAPG QGLEWIGWIVPENGNTEYASKFQGRVTITADTSINTAYMELS RLRSDDTAVYYCTVYGDYWGQGTTVTVSS
	VL	124	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKPEG TIKRLIYAASHLDSGVPSRFSGSRSGSDYTLTISSLQPEDFA TYYCLQYASYPLTFGGGTKVEIK
B3-46	VH	125	EVQLVQSGAEVKKPGASVKVSCKASGFNIKDDYMHWVRQAPG QGLEWIGWIVPENGLTEYASKFQGRVTITADTSINTAYMELS RLRSDDTAVYYCTVYGDYWGQGTTVTVSS
	VL	126	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKPEG TIKRLIYATSTLDSGVPSRFSGSRSGSDYTLTISSLQPEDFA TYYCLQYASYPLTFGGGTKVEIK
B3-48	VH	127	EVQLVQSGAEVKKPGASVKVSCKASDFSIKDDYMHWVRQAPG QGLEWIGWIVPENGKTEYASKFQGRVTITADTSINTAYMELS RLRSDDTAVYYCTVYGDYWGQGTTVTVSS
	VL	128	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKPEG TIKRLIYAAMTLDSGVPSRFSGSRSGSDYTLTISSLQPEDFA TYYCLQYASYPLTFGGGTKVEIK
B3-69	VH	129	EVQLVQSGAEVKKPGASVKVSCKASGFNIKDDYMHWVRQAPG QGLEWIGWIVPENGNTHYASKFQGRVTITADTSINTAYMELS RLRSDDTAVYYCTVYGDYWGQGTTVTVSS
	VL	130	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKPEG TIKRLIYEASTLDSGVPSRFSGSRSGSDYTLTISSLQPEDFA TYYCLQYASYPLTFGGGTKVEIK
D1-70	VH	131	EVQLVQSGAEVKKPGASVKVSCKASGFNIKDDYMHWVRQAPG QGLEWIGWIVPRNGNTMYASKFQGRVTITADTSINTAYMELS RLRSDDTAVYYCTVYGDYWGQGTTVTVSS
	VL	132	DIQMTQSPSSLSASVGDRVTINCRASQEISGYLSWLQQKPEG TIKRLIYAASTLDLGVPSRFSGSRSGSDYTLTISSLQPEDFA TYYCLQYASYPLTFGGGTKVEIK

Example 14.2: IgG Conversion and Characterization of Positive Clones

The seven Fab clones were converted to full IgG proteins. Briefly, the VH and VL genes were produced synthetically and then cloned into vectors containing coding sequences for the human IgG1 and human kappa constant domains, respectively. The heavy chain and

cognate light chain plasmids were co-transfected into HEK 293E cells, individually. After approximately six days of post-transfection cell culture, the supernatants were harvested and subjected to Protein A affinity chromatography. The affinity of purified antibodies was ranked by Octet® RED96 biolayer interferometry (see, Example 9.1, *supra*). Results are shown in Table 37.

Table 37: Off-Rate Ranking of Anti-LAG-3 Antibodies After Affinity Maturation

Test group	Full-length Antibody	Off-rate (k_{off}) (1/s)	off-rate ratio to that of mAb747c
1	B3-21-IgG	1.98×10^{-3}	132%
	B3-43-IgG	5.88×10^{-3}	392%
	B3-46-IgG	2.48×10^{-3}	165%
	B3-48-IgG	2.85×10^{-3}	190%
	B3-69-IgG	3.59×10^{-3}	239%
	B2-53-IgG	1.80×10^{-3}	120%
	mAb747c	1.50×10^{-3}	100%
2	D1-70-IgG	2.60×10^{-3}	160%
	HumAb747-42	4.28×10^{-3}	263%
	HumAb747-39	3.91×10^{-3}	240%
	mAb747c	1.63×10^{-3}	100%

D1-70-IgG and B2-53-IgG showed an off-rate constant with minimal increase compared to HumAb747-39, reflecting the most affinity increase after mutations. Therefore, the mutations in D1-70-IgG and B2-53-IgG were introduced to the sequence of HumAb747-60 that was the best candidate after humanization.

Example 14.3: Generation and Characterization of Further Engineered Antibodies

The mutations in D1-70 identified by the affinity maturation process were D26G, E53R and E58M in the VH domain, and S56L in the VL domain (residue position as determined by Kabat numbering system). The mutations in B2-53 identified by the affinity maturation process were D26G and E58V in the VH domain, and T53A in the VL domain (residue position as determined by Kabat numbering). These mutations were incorporated into the VH/VL sequences of HumAb747-60, separately or in combination.

There was a NG pattern in CDR-H2 of HumAb747-60, which may have resulted in heterogeneity during manufacturing because of deamination reactions, therefore a mutation from NG to NA was also evaluated. The G55A mutation in the VH domain was calculated not to disturb the activity of HumAb747-60 while breaking the NG pattern. Amino acid sequences for the antibody variants including the mutations discussed above are shown in Table 38. (CDRs are underlined according to Kabat numbering.)

Table 38: Engineered VH/VL Design for HumAb747-60

Engineered VH/VL Identifier	SEQ ID NO.	amino acid sequences 12345678901234567890123456789012345678 90
huEpi001-VHv6(G55A)	133	EVQLVQSGAEVKKPGATVKLSCTASDFNIKDDYMHWVKQR PEQGLDWIGWIVPENANTEYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS
huEpi001-VHv6.1	134	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNGNTMYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS
huEpi001-VHv6.2	135	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPENGNTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS
huEpi001-VHv6.3	136	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNGNTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSS
huEpi001-VLv3.4	137	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASTLDLGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELK
huEpi001-VLv3.5	138	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASALDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELK
huEpi001-VLv3.6	139	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASALDLGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELK

The engineered VH and VK genes were produced synthetically and then cloned into vectors containing the human IgG1 and human kappa constant domains, respectively. The pairing of the human VH and the human VK created 13 engineered anti-LAG-3 antibodies, named HumAb747V-61 to HumAb747V-73 (Table 39). The chimeric antibody with parental mouse

VH/VL and human constant sequences (mAb747c) was used as a positive control for affinity comparison.

Table 39: Production List Engineered Anti-LAG-3 Antibodies

Antibody Identifier	VH Region in Heavy Chain	VL Region in Light κ Chain
HumAb747V-61	huEpi001-VHv6(G55A)	huEpi001 VLv3
HumAb747V-62	huEpi001-VHv6.1	huEpi001 VLv3.4
HumAb747V-63	huEpi001-VHv6.1	huEpi001 VLv3.5
HumAb747V-64	huEpi001-VHv6.1	huEpi001 VLv3.6
HumAb747V-65	huEpi001-VHv6.1	huEpi001 VLv3
HumAb747V-66	huEpi001-VHv6.2	huEpi001 VLv3.4
HumAb747V-67	huEpi001-VHv6.2	huEpi001 VLv3.5
HumAb747V-68	huEpi001-VHv6.2	huEpi001 VLv3.6
HumAb747V-69	huEpi001-VHv6.2	huEpi001 VLv3
HumAb747V-70	huEpi001-VHv6.3	huEpi001 VLv3.4
HumAb747V-71	huEpi001-VHv6.3	huEpi001 VLv3.5
HumAb747V-72	huEpi001-VHv6.3	huEpi001 VLv3.6
HumAb747V-73	huEpi001-VHv6.3	huEpi001 VLv3

All 13 humanized antibodies (Table 39) and chimeric anti-LAG-3 antibody mAb747c having the parental murine VH and VL domains were ranked by dissociation rate constant (k_{off}) in the same manner described in Example 9.1 *supra*. Results are shown in Table 40. In each test group, the off-rates of antibodies were able to be compared with that of mAb747c. The off-rate ratios were calculated by the off-rate of antibody to that of mAb747c of its group and were compared all together. The lower the ratio was, the higher was the affinity of the antibody.

Table 40: Off-Rate Ranking of Anti-LAG-3 Antibodies with Further Engineering

Test group	Full length Antibody	Off-rate (k_{off}) (1/s)	off-rate ratio to that of mAb747c
1	HumAb747V-61	6.86×10^{-4}	112%
	HumAb747V-62	4.80×10^{-4}	78%
	HumAb747V-63	5.22×10^{-4}	85%
	HumAb747V-64	4.50×10^{-4}	73%
	HumAb747V-65	2.92×10^{-4}	47%
	HumAb747V-66	5.84×10^{-4}	95%
	mAb747c	6.15×10^{-4}	100%
2	HumAb747V-67	3.19×10^{-4}	47%
	HumAb747V-68	2.95×10^{-4}	44%
	HumAb747V-69	3.57×10^{-4}	53%
	HumAb747V-70	2.73×10^{-4}	40%
	HumAb747V-71	2.92×10^{-4}	43%
	HumAb747V-72	2.47×10^{-4}	37%
	mAb747c	6.76×10^{-4}	100%
3	HumAb747V-73	3.21×10^{-4}	51%
	mAb747c	6.31×10^{-4}	100%

On the basis of HumAb747-60, most antibodies with CDR mutations adopted after affinity maturation showed improved off-rate in comparison to the off-rate of mAb747c, predicting improved affinity. HumAb747V-61 had a similar off-rate to mAb747c, which indicated that the VH G55A amino acid substitution did not disturb the affinity. The antibodies that had off-rate ratios less than 60% that of mAb747c in their test group were further evaluated in a cell-based functional assay.

The anti-LAG-3 activity was tested in a PBMC activation assay using Staphylococcal enterotoxin B (SEB) as a superantigen. Briefly, PBMC were seeded into a 96-well plate with 2 $\times 10^5$ cells/well. Test proteins (anti-LAG-3 monoclonal antibodies) were added into the plates and incubated with PBMC at 37° C for 30 min. SEB solution was added to a final concentration of 10 ng/ml. The plates were incubated for 96 hours, then 100 µl cell culture supernatant were collected and IL-2 production was measured using a PerkinElmer IL-2

detection kit (PerkinElmer; Cat. No. TRF1221M). Results are shown in Figure 10. The results show that the engineered antibody variants can enhance IL-2 production from SEB-stimulated PBMC by blocking LAG-3-mediated signaling.

Based on the PBMC-SEB assay results, HumAb747V-67, HumAb747V-72, and HumAb747V-73 demonstrated superior LAG-3-blocking activity; therefore, these three antibodies were used for generating FIT-Ig binding proteins targeting LAG-3 and also PD-1.

Example 15: Generation of PD-1/LAG-3 FIT-Igs Using New anti-LAG-3 Antibody Sequences

The anti-LAG-3 antibodies HumAb747V-67, HumAb747V-72 and HumAb747V-73 generated as described above, and two anti-PD-1 antibodies, HumAb709-8 (see Tables 5 and 7, *supra*) and HumAb713-7 (see Tables 9 and 10, *supra*), were used to generate FIT-Ig binding proteins, following the procedures described in Example 10, *supra*. The G55A mutation was included in sequence design of all the VH domains of the anti-LAG-3 Fab moieties.

Example 15.1: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-6a-1

A PD-1/LAG-3 FIT-Ig designated FIT107-1-6a-1 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies mAb709-8 (humanized anti-PD-1, see Tables 5 and 6, *supra*) and HumAb747V-67 (humanized anti-LAG-3, see Tables 38 and 39, *supra*). FIT-Ig FIT107-1-6a-1 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb709-8 fused directly to VH-CH1 of HumAb747V-67 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1 (see Table 6, *supra*);

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb709-8; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb747V-676.

The amino acid sequences for the three expressed FIT107-1-6a-1 polypeptide chains are shown in Table 41 below.

Table 41: Amino Acid Sequences of FIT107-1-6a-1 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678901234567890
FIT107-1-6a-1 FIT-Ig Polypeptide Chain #1	140	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSPSSLSASVGDR VTITCKASQDVNTVVAWYQQKPGKAPKVLISWASTRHTGV PSRFSGSGSGTDYTLTISSLQPEDFATYYCQQHYTTPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL VQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQRPEQG LDWIGWIVPENANTVYASKFQGKATITADTSTNTAYLELS SLRSEDTAVYYCTVYGDYWGQGTTVTVSS**ASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HumAb709-8 (VL underlined)	141	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb747V-67 (VH underlined)	142	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPENANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678901234567890
FIT107-1-6a-1 FIT-Ig Polypeptide Chain #2	143	MEFGLSWLFLVAILKGVQC**EVQLVESGGGLVQPGGSLRLS CAASGFTFSFYTMSWVRQAPGKGLEWVATISGGGRDTYY PDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAGQG GNYLFAYWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGT AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS C
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb709-8 (VH underlined)	144	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC
FIT107-1-6a-1 FIT-Ig Polypeptide Chain #3	145	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASALDSGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb747V-67 (VL underlined)	146	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASALDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.2: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-6b-1

Another bispecific Fabs-in-Tandem Immunoglobulin recognizing both human PD-1 and human LAG-3 was constructed. This PD-1/LAG-3 FIT-Ig was designated FIT107-1-6b-1. Construction of FIT107-1-6b-1 binding protein utilized coding sequences for immunoglobulin

domains from parental antibodies HumAb747V-67 (anti-LAG-3) and HumAb709-8 (anti-PD-1). This FIT-Ig construct exhibited a LAG-3-binding domain in the N-terminal (outer) position and a PD-1-binding domain in the inner position fused C-terminal to the VL-CL domains of the LAG-3 binding region. FIT-Ig FIT107-1-6b-1 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb747V-67 fused directly to VH-CH1 of HumAb709-8 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1 (see Table 6, *supra*);

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb747V-67; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb709-8.

The amino acid sequences for the three expressed FIT107-1-6b-1 polypeptide chains are shown in Table 42 below:

Table 42: Amino Acid Sequences of FIT107-1-6b-1 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678901234567890
FIT107-1-6b-1 FIT-Ig Polypeptide Chain #1	147	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASALDSGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL VESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQAPGKG LEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLYLQMN SLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSS**ASTKGP SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN HKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
VL-CL of mAb HumAb747V-67 (VL underlined)	148	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASALDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb709-8 (VH underlined)	149	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-6b-1 FIT-Ig Polypeptide Chain #2	150	MEFGLSWLFLVAILKGVQC**EVQLVQSGAEVKKPGATVKLS CTASGFNIKDDYMHWVKQRPEQGLDWIGWIVPENANTVY ASKFQGKATITADTSTNTAYLELSSLRSEDTAVYYCTVYG DYWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGC LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb747V-67 (VH underlined)	151	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPENANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
FIT107-1-6b-1 FIT-Ig Polypeptide Chain #3	152	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSPSSLSASVGDR VTITCKASQDVNTVVAWYQQKPGKAPKVLISWASTRHTGV PSRFSGSGSGTDYTLTISSLQPEDFATYYCQQHYTTPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
		LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb709-8 (VL underlined)	153	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.3: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-6a-2

A PD-1/LAG-3 FIT-Ig designated FIT107-1-6a-2 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb709-8 (humanized anti-PD-1) and HumAb747V-72 (humanized anti-LAG-3). FIT-Ig FIT107-1-6a-2 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb709-8 fused directly to VH-CH1 of HumAb747V-72 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1 (see Table 6, *supra*);

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb709-8; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb747V-72.

The amino acid sequences for the three expressed FIT107-1-6a-2 polypeptide chains are shown in Table 43 below.

Table 43: Amino Acid Sequences of FIT107-1-6a-2 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-6a-2 FIT-Ig Polypeptide Chain #1	154	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSPSSLSASVGDR** **VTITCKASQDVNTVVAWYQQKPGKAPKVLISWASTRHTGV** **PSRFSGSGSGTDYTLTISSLQPEDFATYYCQQHYTTPYTF** **GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL**

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
		VQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQRPEQG **LDWIGWIVPRNANTVYASKFQGKATITADTSTNTAYLELS** **SLRSEDTAVYYCTVYGDYWGQGTTVTVSS**ASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HumAb709-8 (VL underlined)	155	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb747V-72 (VH underlined)	156	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-6a-2 FIT-Ig Polypeptide Chain #2	157	MEFGLSWLFLVAILKGVQC**EVQLVESGGGLVQPGGSLRLS** **CAASGFTFSFYTMSWVRQAPGKGLEWVATISGGGRDTYY** **PDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAGQG** **GNYLFAYWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGT AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS C

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678901234567890
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb709-8 (VH underlined)	158	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC
FIT107-1-6a-2 FIT-Ig Polypeptide Chain #3	159	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASALDLGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb747V-72 (VL underlined)	160	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASALDLGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.4: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-6b-2

A PD-1/LAG-3 FIT-Ig designated FIT107-1-6b-2 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb709-8 (humanized anti-PD-1) and HumAb747V-72 (humanized anti-LAG-3). FIT-Ig FIT107-1-6b-2 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb747V-72 fused directly to VH-CH1 of HumAb709-8 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1;

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb747V-72; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb709-8.

The amino acid sequences for the three expressed FIT107-1-6b-2 polypeptide chains are shown in Table 44 below.

Table 44: Amino Acid Sequences of FIT107-1-6b-2 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678901234567890
FIT107-1-6b-2 FIT-Ig Polypeptide Chain #1	161	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASALDLGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL VESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQAPGKG LEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLYLQMN SLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSS**ASTKGP SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN HKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HumAb747V-72 (VL underlined)	162	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASALDLGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb709-8 (VH underlined)	163	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678901234567890
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-6b-2 FIT-Ig Polypeptide Chain #2	164	MEFGLSWLFLVAILKGVQC**EVQLVQSGAEVKKPGATVKLS CTASGFNIKDDYMHWVKQRPEQGLDWIGWIVPRNANTVY ASKFQGKATITADTSTNTAYLELSSLRSEDTAVYYCTVYG DYWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGC LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb747V-72 (VH underlined)	165	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
FIT107-1-6b-2 FIT-Ig Polypeptide Chain #3	166	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSPSSLSASVGDR VTITCKASQDVNTVVAWYQQKPGKAPKVLISWASTRHTGV PSRFSGSGSGTDYTLTISSLQPEDFATYYCQQHYTTPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb709-8 (VL underlined)	167	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.5: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-6a-3

A PD-1/LAG-3 FIT-Ig designated FIT107-1-6a-3 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb709-8 (humanized anti-PD-1) and HumAb747V-73 (humanized anti-LAG-3). FIT-Ig FIT107-1-6a-3 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb709-8 fused directly to VH-CH1 of HumAb747V-73 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1;

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb709-8; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb747V-73.

The amino acid sequences for the three expressed FIT107-1-6a-3 polypeptide chains are shown in Table 45 below.

Table 45: Amino Acid Sequences of FIT107-1-6a-3 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-6a-3 FIT-Ig Polypeptide Chain #1	168	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSPSSLSASVGDR VTITCKASQDVNTVVAWYQQKPGKAPKVLISWASTRHTGV PSRFSGSGSGTDYTLTISSLQPEDFATYYCQQHYTTPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL VQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQRPEQG LDWIGWIVPRNANTVYASKFQGKATITADTSTNTAYLELS SLRSEDTAVYYCTVYGDYWGQGTTVTVSS**ASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb		DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
HumAb709-8 (VL underlined)	169	EDFATYYCQQHYTTPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb747V-73 (VH underlined)	170	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-6a-3 FIT-Ig Polypeptide Chain #2	171	MEFGLSWLFLVAILKGVQC**EVQLVESGGGLVQPGGSLRLS CAASGFTFSFYTMSWVRQAPGKGLEWVATISGGGRDTYY PDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAGQG GNYLFAYWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGT AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS C
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb709-8 (VH underlined)	172	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC
FIT107-1-6a-3 FIT-Ig Polypeptide Chain #3	173	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASTLDSGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
		LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb747V-73 (VL underlined)	174	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.6: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-6b-3

A PD-1/LAG-3 FIT-Ig designated FIT107-1-6b-3 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb709-8 (humanized anti-PD-1) and HumAb747V-73 (humanized anti-LAG-3). FIT-Ig FIT107-1-6b-3 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb747V-73 fused directly to VH-CH1 of HumAb709-8 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1;

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb747V-73; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb709-8.

The amino acid sequences for the three expressed FIT107-1-6b-3 polypeptide chains are shown in Table 46 below.

Table 46: Amino Acid Sequences of FIT107-1-6b-3 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-6b-3 FIT-Ig Polypeptide Chain #1	175	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR** **VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASTLDSGV** **PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF** **GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL** **VESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQAPGKG**

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
		LEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLYLQMN **SLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSS**ASTKGP SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN HKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HumAb747V-73 (VL underlined)	176	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb709-8 (VH underlined)	177	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYTMSWVRQA PGKGLEWVATISGGGRDTYYPDSVKGRFTISRDNAKNSLY LQMNSLRAEDTAVYYCAGQGGNYLFAYWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-6b-3 FIT-Ig Polypeptide Chain #2	178	MEFGLSWLFLVAILKGVQC**EVQLVQSGAEVKKPGATVKLS** **CTASGFNIKDDYMHWVKQRPEQGLDWIGWIVPRNANTVY** **ASKFQGKATITADTSTNTAYLELSSLRSEDTAVYYCTVYG** **DYWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGC LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb747V-73 (VH underlined)	179	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
FIT107-1-6b-3 FIT-Ig Polypeptide Chain #3	180	MDMRVPAQLLGLLLLWFPGSRC**DIVMTQSPSSLSASVGDR VTITCKASQDVNTVVAWYQQKPGKAPKVLISWASTRHTGV PSRFSGSGSGTDYTLTISSLQPEDFATYYCQQHYTTPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb709-8 (VL underlined)	181	DIVMTQSPSSLSASVGDRVTITCKASQDVNTVVAWYQQKP GKAPKVLISWASTRHTGVPSRFSGSGSGTDYTLTISSLQP EDFATYYCQQHYTTPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.7: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-7a-1

A PD-1/LAG-3 FIT-Ig designated FIT107-1-7a-1 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb713-7 (humanized anti-PD-1; see Tables 9 and 10, *supra*) and HumAb747V-67 (humanized anti-LAG-3). FIT-Ig FIT107-1-7a-1 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb713-7 fused directly to VH-CH1 of HumAb747V-67 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1;

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb713-7; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb747V-67.

The amino acid sequences for the three expressed FIT107-1-7a-1 polypeptide chains are shown in

Table 47 below.

Table 47: Amino Acid Sequences of FIT107-1-7a-1 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-7a-1 FIT-Ig Polypeptide Chain #1	182	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR** **VTITCKASDHINNWLAWYQQKPGKAPKLLIYGATSLETGV** **PSRFSGSGSGTDYTFTISSLQPEDIATYYCQQYWSPPYTF** **GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL** **VQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQRPEQG** **LDWIGWIVPENANTVYASKFQGKATITADTSTNTAYLELS** **SLRSEDTAVYYCTVYGDYWGQGTTVTVSS**ASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HumAb713-7 (VL underlined)	183	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLIYGATSLETGVPSRFSGSGSGTDYTFTISSLQP EDIATYYCQQYWSPPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb747V-67 (VH underlined)	184	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPENANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-7a-1 FIT-Ig Polypeptide Chain #2	185	MEFGLSWLFLVAILKGVQC**EVQLVESGGGLVQPGGSLRLS CAASGFTSSDYGMHWVRQAPGKGLEWVSYISSGSYTIYY ADTVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCAKRG GSSHVNVMDYWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTS GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE PKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb713-7 (VH underlined)	186	EVQLVESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQA PGKGLEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVS SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSC
FIT107-1-7a-1 FIT-Ig Polypeptide Chain #3	187	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASALDSGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb747V-67 (VL underlined)	188	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASALDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.8: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-7b-1

A PD-1/LAG-3 FIT-Ig designated FIT107-1-7b-1 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb713-7 (humanized anti-PD-1) and HumAb747V-67 (humanized anti-LAG-3). FIT-Ig FIT107-1-7b-1 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb747V-67 fused directly to VH-CH1 of HumAb713-7 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1;

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb747V-67; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb713-7.

The amino acid sequences for the three expressed FIT107-1-7b-1 polypeptide chains are shown in Table 48 below.

Table 48: Amino Acid Sequences of FIT107-1-7b-1 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-7b-1 FIT-Ig Polypeptide Chain #1	189	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASALDSGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL VESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQAPGKG LEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLYLQMN SLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVSS**AST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS LSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb		DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
HumAb747V-67 (VL underlined)	190	GGAIKRLIYAASALDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb713-7 (VH underlined)	191	EVQLVESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQA PGKGLEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVS SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-7b-1 FIT-Ig Polypeptide Chain #2	192	MEFGLSWLFLVAILKGVQC**EVQLVQSGAEVKKPGATVKLS CTASGFNIKDDYMHWVKQRPEQGLDWIGWIVPENANTVY ASKFQGKATITADTSTNTAYLELSSLRSEDTAVYYCTVYG DYWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGC LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb747V-67 (VH underlined)	193	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPENANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
FIT107-1-7b-1 FIT-Ig Polypeptide Chain #3	194	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCKASDHINNWLAWYQQKPGKAPKLLIYGATSLETGV PSRFSGSGSGTDYTFTISSLQPEDIATYYCQQYWSPPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678901234567890
		LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb713-7 (VL underlined)	195	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLIYGATSLETGVPSRFSGSGSGTDYTFTISSLQP EDIATYYCQQYWSPPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.9: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-7a-2

A PD-1/LAG-3 FIT-Ig designated FIT107-1-7a-2 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb713-7 (humanized anti-PD-1) and HumAb747V-72 (humanized anti-LAG-3). FIT-Ig FIT107-1-7a-2 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb713-7 fused directly to VH-CH1 of HumAb747V-72 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1 (see Table 6, *supra*);

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb713-7; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb747V-72.

The amino acid sequences for the three expressed FIT107-1-7a-2 polypeptide chains are shown in Table 49 below.

Table 49: Amino Acid Sequences of FIT107-1-7a-2 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678901234567890
FIT107-1-7a-2 FIT-Ig Polypeptide Chain #1	196	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR** **VTITCKASDHINNWLAWYQQKPGKAPKLLIYGATSLETGV** **PSRFSGSGSGTDYTFTISSLQPEDIATYYCQQYWSPPYTF** **GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL**

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
		VQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQRPEQG **LDWIGWIVPRNANTVYASKFQGKATITADTSTNTAYLELS** **SLRSEDTAVYYCTVYGDYWGQGTTVTVSS**ASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HumAb713-7 (VL underlined)	197	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLIYGATSLETGVPSRFSGSGSGTDYTFTISSLQP EDIATYYCQQYWSPPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb747V-72 (VH underlined)	198	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-7a-2 FIT-Ig Polypeptide Chain #2	199	MEFGLSWLFLVAILKGVQC**EVQLVESGGGLVQPGGSLRLS** **CAASGFTSSDYGMHWVRQAPGKGLEWVSYISSGSYTIYYA** **DTVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCAKRGG** **SSHVNVMDYWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSG GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP KSC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb713-7 (VH underlined)	200	EVQLVESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQA PGKGLEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVS SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSC
FIT107-1-7a-2 FIT-Ig Polypeptide Chain #3	201	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASALDLGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb747V-72 (VL underlined)	202	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASALDLGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.10: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-7b-2

A PD-1/LAG-3 FIT-Ig designated FIT107-1-7b-2 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb713-7 (humanized anti-PD-1) and HumAb747V-72 (humanized anti-LAG-3). FIT-Ig FIT107-1-7b-2 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb747V-72 fused directly to VH-CH1 of HumAb713-7 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1;

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb747V-72; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb713-7.

The amino acid sequences for the three expressed FIT107-1-7b-2 polypeptide chains are shown in Table 50 below.

Table 50: Amino Acid Sequences of FIT107-1-7b-2 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-7b-2 FIT-Ig Polypeptide Chain #1	203	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASALDLGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL VESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQAPGKG LEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLYLQMN SLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVSS**AST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS LSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HumAb747V-72 (VL underlined)	204	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASALDLGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb713-7 (VH underlined)	205	EVQLVESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQA PGKGLEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVS SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 12345678901234567890123456789012345678901234567890
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-7b-2 FIT-Ig Polypeptide Chain #2	206	MEFGLSWLFLVAILKGVQC**EVQLVQSGAEVKKPGATVKLS CTASGFNIKDDYMHWVKQRPEQGLDWIGWIVPRNANTVY ASKFQGKATITADTSTNTAYLELSSLRSEDTAVYYCTVYG DYWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGC LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb747V-72 (VH underlined)	207	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
FIT107-1-7b-2 FIT-Ig Polypeptide Chain #3	208	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCKASDHINNWLAWYQQKPGKAPKLLIYGATSLETGV PSRFSGSGSGTDYTFTISSLQPEDIATYYCQQYWSPPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb713-7 (VL underlined)	209	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLIYGATSLETGVPSRFSGSGSGTDYTFTISSLQP EDIATYYCQQYWSPPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.11: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-7a-3

A PD-1/LAG-3 FIT-Ig designated FIT107-1-7a-3 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb713-7 (humanized anti-PD-1) and HumAb747V-73 (humanized anti-LAG-3). FIT-Ig FIT107-1-7a-3 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb713-7 fused directly to VH-CH1 of HumAb747V-73 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1;

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb713-7; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb747V-73.

The amino acid sequences for the three expressed FIT107-1-7a-3 polypeptide chains are shown in Table 51 below.

Table 51: Amino Acid Sequences of FIT107-1-7a-3 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-7a-3 FIT-Ig Polypeptide Chain #1	210	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCKASDHINNWLAWYQQKPGKAPKLLIYGATSLETGV PSRFSGSGSGTDYTFTISSLQPEDIATYYCQQYWSPPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL VQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQRPEQG LDWIGWIVPRNANTVYASKFQGKATITADTSTNTAYLELS SLRSEDTAVYYCTVYGDYWGQGTTVTVSS**ASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb	211	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLIYGATSLETGVPSRFSGSGSGTDYTFTISSLQP

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
HumAb713-7 (VL underlined)		EDIATYYCQQYWSPPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb747V-73 (VH underlined)	212	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-7a-3 FIT-Ig Polypeptide Chain #2	213	MEFGLSWLFLVAILKGVQC**EVQLVESGGGLVQPGGSLRLS CAASGFTSSDYGMHWVRQAPGKGLEWVSYISSGSYTIYY ADTVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCAKRG GSSHVNVMDYWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTS GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE PKSC
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb713-7 (VH underlined)	214	EVQLVESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQA PGKGLEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVS SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSC
FIT107-1-7a-3 FIT-Ig Polypeptide Chain #3	215	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASTLDSGV PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
		LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb747V-73 (VL underlined)	216	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 15.12: Production of PD-1/LAG-3 FIT-Ig Binding Protein FIT107-1-7b-3

A PD-1/LAG-3 FIT-Ig designated FIT107-1-7b-3 was constructed utilizing coding sequences for immunoglobulin domains from the parental antibodies HumAb713-7 (humanized anti-PD-1) and HumAb747V-73 (humanized anti-LAG-3). FIT-Ig FIT107-1-7b-3 is a hexamer comprised of three component polypeptide chains:

Polypeptide chain #1 has the domain formula: VL-CL of HumAb747V-73 fused directly to VH-CH1 of HumAb713-7 fused directly to hinge-CH2-CH3 of a mutant human constant IgG1;

Polypeptide chain #2 has the domain formula: VH-CH1 of HumAb747V-73; and

Polypeptide chain #3 has the domain formula: light chain (VL-CL) of HumAb713-7.

The amino acid sequences for the three expressed FIT107-1-7b-3 polypeptide chains are shown in Table 52 below.

Table 52: Amino Acid Sequences of FIT107-1-7b-3 Component Chains

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
FIT107-1-7b-3 FIT-Ig Polypeptide Chain #1	217	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR** **VTITCRASQEISGYLSWLQQKPGGAIKRLIYAASTLDSGV** **PSRFSGSRSGSDYTLTISSLQPEDFADYYCLQYASYPLTF** **GQGTKLELK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**EVQL** **VESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQAPGKG**

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
		LEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLYLQMN **SLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVSS**AST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS LSLSPGK
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of mAb HumAb747V-73 (VL underlined)	218	DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKP GGAIKRLIYAASTLDSGVPSRFSGSRSGSDYTLTISSLQP EDFADYYCLQYASYPLTFGQGTKLELKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
VH-CH1 of mAb HumAb713-7 (VH underlined)	219	EVQLVESGGGLVQPGGSLRLSCAASGFTSSDYGMHWVRQA PGKGLEWVSYISSGSYTIYYADTVKGRFTISRDNAKNSLY LQMNSLRDEDTAVYYCAKRGGSSHVNVMDYWGQGTTVTVS SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSC
hinge-CH2-CH3 of human IgG1	82	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK
FIT107-1-7b-3 FIT-Ig Polypeptide Chain #2	220	MEFGLSWLFLVAILKGVQC**EVQLVQSGAEVKKPGATVKLS** **CTASGFNIKDDYMHWVKQRPEQGLDWIGWIVPRNANTVY** **ASKFQGKATITADTSTNTAYLELSSLRSEDTAVYYCTVYG** **DYWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGC LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

Polypeptide	SEQ ID NO:	Amino Acid Sequence 1234567890123456789012345678901234567890
signal sequence	84	MEFGLSWLFLVAILKGVQC
VH-CH1 of HumAb747V-73 (VH underlined)	221	EVQLVQSGAEVKKPGATVKLSCTASGFNIKDDYMHWVKQR PEQGLDWIGWIVPRNANTVYASKFQGKATITADTSTNTAY LELSSLRSEDTAVYYCTVYGDYWGQGTTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH KPSNTKVDKKVEPKSC
FIT107-1-7b-3 FIT-Ig Polypeptide Chain #3	222	MDMRVPAQLLGLLLLWFPGSRC**DIQMTQSPSSLSASVGDR VTITCKASDHINNWLAWYQQKPGKAPKLLIYGATSLETGV PSRFSGSGSGTDYTFTISSLQPEDIATYYCQQYWSPPYTF GGGTKVEIK**RTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
signal sequence	79	MDMRVPAQLLGLLLLWFPGSRC
VL-CL of HumAb713-7 (VL underlined)	223	DIQMTQSPSSLSASVGDRVTITCKASDHINNWLAWYQQKP GKAPKLLIYGATSLETGVPSRFSGSGSGTDYTFTISSLQP EDIATYYCQQYWSPPYTFGGGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC

Example 16: Characterization of New FIT-Ig Proteins

Example 16.1: Expression and SEC Analysis

The 12 FIT-Ig binding proteins described above (Tables 41-52) were expressed in the same manner as described in Example 10.5, *supra*, and purified by Protein A chromatography. The composition and purity of the purified FIT-Igs were analyzed by size exclusion chromatography (SEC). Purified FIT-Ig, in PBS, was applied on a TSKgel SuperSW3000, 300 × 4.6 mm column (TOSOH). A DIONEX™ UltiMate 3000 HPLC instrument (Thermo Scientific) was used for SEC using UV detection at 280 nm and 214 nm. See Table 53, below.

Table 53: Expression and SEC Analysis of PD-1/LAG-3 FIT-Ig Binding Proteins

FIT-Ig protein	DNA molar ratio: Chain #1 : #2 : #3	Expression level (mg/L)	% Peak Monomeric Fraction by SEC
FIT107-1-6a-1	1:2:1.5	6.21	78.9%
FIT107-1-6b-1	1:2:1.5	8.58	55.4%
FIT107-1-6a-2	1:2:1.5	6.11	90.9%
FIT107-1-6b-2	1:2:1.5	15.86	43.2%
FIT107-1-6a-3	1:2:1.5	11.37	43.0%
FIT107-1-6b-3	1:2:1.5	17.22	44.0%
FIT107-1-7a-1	1:2:1.5	14.47	80.0%
FIT107-1-7b-1	1:2:1.5	17.96	94.8%
FIT107-1-7a-2	1:2:1.5	19.25	88.3%
FIT107-1-7b-2	1:2:1.5	22.12	98.5%
FIT107-1-7a-3	1:2:1.5	14.31	80.9%
FIT107-1-7b-3	1:2:1.5	29.20	98.7%

The FIT-Ig proteins that had lower monomeric fraction contents (<80%) were excluded in further characterization.

Example 16.2: Functional Assays

The PD-1/LAG-3 FIT-Ig activity was tested in a PBMC activation assay using Staphylococcal enterotoxin B (SEB) as a superantigen as described in Example 12. Results are shown in Figure 11. The results showed that all the tested FIT-Ig variants can enhance IL-2 secretion from SEB-stimulated PBMC. The enhancement was somehow reversed in the highest doses of FIT107-1-7b-2 and FIT107-1-7b-3, therefore these two FIT-Ig proteins were not prioritized as lead molecules.

Example 16.3: Binding Activity

The kinetics of FIT-Ig binding to PD-1 and LAG-3 targets was determined by biolayer interferometry using the Octet® RED96 system (Pall FortéBio LLC). Binding affinities for both target antigens PD-1 and LAG-3 are shown in Table 54, below. All FIT-Ig proteins retained affinity for both huPD-1 and huLAG-3. All the FIT-Ig proteins that were tested against cynomolgus antigens also showed cross-reactivity with cynomolgus antigens.

Table 54: Binding Affinities for PD-1/LAG-3 FIT-Ig Binding Proteins

FIT-Ig captured on sensor chip	Analyte	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
FIT107-1-7b-1	Human PD-1-His	8.96×10^{4}	9.35×10^{-4}	1.04×10^{-8}
	Cyno PD-1-his	2.15×10^{5}	7.75×10^{-4}	3.61×10^{-9}
	Human LAG-3-His	2.00×10^{5}	2.43×10^{-4}	1.22×10^{-9}
	Cyno LAG-3-His	5.19×10^{5}	6.36×10^{-5}	1.23×10^{-10}
FIT107-1-7b-3	Human PD-1-His	1.16×10^{5}	1.03×10^{-3}	8.86×10^{-9}
	Cyno PD-1-His	2.48×10^{5}	9.19×10^{-4}	3.71×10^{-9}
	Human LAG-3-His	1.44×10^{5}	3.28×10^{-4}	2.28×10^{-9}
	Cyno LAG-3-His	2.81×10^{5}	4.70×10^{-5}	1.67×10^{-10}
FIT107-1-6a-2	Human PD-1-His	1.87×10^{5}	2.39×10^{-4}	1.28×10^{-9}
	Cyno PD-1-His	3.06×10^{5}	1.07×10^{-3}	3.49×10^{-9}
	Human LAG-3-His	1.15×10^{5}	1.17×10^{-4}	1.02×10^{-9}
	Cyno LAG-3-His	1.91×10^{5}	8.17×10^{-5}	4.28×10^{-10}
FIT107-1-7a-1	Human PD-1-His	1.77×10^{5}	5.38×10^{-4}	3.04×10^{-9}
	Human LAG-3-His	1.69×10^{5}	1.24×10^{-4}	7.32×10^{-10}
FIT107-1-7a-2	Human PD-1-His	1.66×10^{5}	5.26×10^{-4}	3.17×10^{-9}
	Human LAG-3-His	1.05×10^{5}	1.28×10^{-4}	1.22×10^{-9}
FIT107-1-7a-3	Human PD-1-His	2.08×10^{5}	6.28×10^{-4}	3.01×10^{-9}
	Human LAG-3-His	9.10×10^{4}	1.34×10^{-4}	1.47×10^{-9}
FIT107-1-7b-2	Human PD-1-His	1.05×10^{5}	8.27×10^{-4}	7.90×10^{-9}
	Human LAG-3-His	1.74×10^{5}	2.46×10^{-4}	1.41×10^{-9}

Example 16.4: Rat Pharmacokinetic Data

Based on the purity after one-step purification, expression titer in transient transfection, the binding affinity retained, as well as the functional activity in the PBMC-SEB assay, FIT107-1-7b-1 was selected as lead molecule. Pharmacokinetic properties of FIT107-1-7b-1 were assessed in male Sprague-Dawley (SD) rats. FIT-Ig protein was administered to male SD rats at a single intravenous dose of 5 mg/kg. Serum samples were collected at different time points over a period of 28 days with sampling at 0, 5, 15, and 30 minutes; 1, 2, 4, 8, and 24 hours; and 2, 4, 7, 10, 14, 21, and 28 days serial bleeding via tail vein, and analyzed by general ELISAs. Briefly, ELISA plates were coated with 125 ng/well of goat anti-human IgG Fc antibody (Rockland, Cat#: 609-101-017) at 4° C overnight, blocked with 1X PBS/1% BSA/0.05% Tween-

20/0.05% ProClin™ 300. All serum samples were diluted 20-fold in blocking buffer first. An additional dilution was made in 5% pooled rat serum and incubated on the plate for 60 minutes at 37° C. Detection was carried out with a goat Fab-specific anti-human IgG-peroxidase conjugated antibody (Sigma-Aldrich; Cat. No. A0293), and concentrations were determined with the help of standard curves using the four-parameter logistic fit. Values for the pharmacokinetic parameters were determined by non-compartmental model using WinNonlin software (Pharsight Corporation, Mountain View, Calif.). As demonstrated by these results shown in Table 55, the properties of FIT107-1-7b-1 are stable *in vivo*.

Table 55: Pharmacokinetic Properties of FIT107-1-7b-1

PK parameters	CL	Vss	Beta $t_{1/2}$	AUC	MRT
Antibody	mL/day/kg	mL/kg	day	day*μg/mL	day
FIT107-1-7b-1	9.17	114	8.82	436	12.4

Example 16.5: FGL1 receptor blocking assay (RBA)

It was reported recently that fibrinogen-like protein 1 (FGL1) is a major LAG-3 functional ligand independent from MHC Class II (Wang J. et al., *Cell*, 176(1):334-47 (2019)). Blockade of the FGL1/LAG-3 interaction by antibodies stimulates tumor immunity. To evaluate blocking activity of the anti-LAG-3 antibody or FIT-Ig, FGL1 (Wuhan USCN, Cat. No. RPD022Hu01) was diluted to 5 μg/ml with Dulbecco's phosphate buffered saline and 100 μl were added into a 96-well plate and incubated at 4° C overnight. The plate was washed three times with 300 μl/well PBS+TWEEN 20 (PBST). HumAb747V-67, FIT107-1-7b-1, hIgG (working concentration: 100nM, 10nM, 1nM and 0.1nM) and 1 μg/ml biotinylated LAG-3 (AcroBiosystem, Cat. No. H82E5) were added and incubated at room temperature for 2 hours. The plate was washed three times with 300 μl/well PBST, then read using a VARIOSKAN™ LUX microplate reader (Thermo Scientific) using the ELISA-Endpoint-TMB/HRP protocol. Results are shown in Figure 12. The results showed that both FIT107-1-7b-1 FIT-Ig and its parental anti-LAG-3 antibody HumAb747V-67 can block human LAG-3 binding to FGL1 protein.

Example 16.6: Primary cells binding activity of FIT107-1-7b-1 protein.

The foregoing assays demonstrated PD-1/LAG-3 FIT-Ig proteins can bind recombinant antigen proteins. To further evaluate the cell surface binding ability of FIT107-1-7b-1, the parental antibodies HumAb713-7, HumAb747V-67, and the bispecific FIT-Ig FIT107-1-7b-1 were biotinylated with biotin reagent (Sigma, Cat. No. S3259). For PBMC without stimulation, PBMCs were re-suspended at 5×10^6 cells/ml. For PD-1 antibody (HumAb713-7) or LAG-3 antibody (HumAb747V-67) testing, 100 μg/ml of antibody were added and the reaction mixture allowed to incubate at 37° C for 40 minutes separately, followed by two washes with FACS buffer. Then 100 μl, 5×10^5 PBMC/well (untreated group, anti-PD-1 antibody treated group and anti-LAG-3 antibody treated group) were seeded into wells of 96-well plate. Biotinylated HumAb713-7, HumAb747V-67 and FIT107-1-7b-1 were added and incubated at 37° C for 40 minutes (final working concentration starting from 100nM with 3-fold serial dilution) followed by washing with FACS buffer twice. FITC-streptavidin and BV421-anti human-CD3 antibody were added and the assay plate incubated at 4° C for 30 minutes, followed by washing with FACS buffer twice. The plate was analyzed with a Beckman Coulter CytoFlex flow cytometer. PBMC stimulation groups were stimulated with anti-CD3 plus anti-CD28 antibody for 72 hours, to induce PD-1 and LAG-3 expression on T cells. HumAb713-7, HumAb747V-67 and FIT107-1-7b-1 binding were tested on stimulated PBMC with the same grouping strategy (untreated, anti-PD-1 antibody treated and anti-LAG-3 antibody treated group) as in the unstimulated PBMC experiments. The binding of test antibodies was investigated on CD3-T cells subset by FACS. The results are shown in Figure 13. Results showed that FIT107-7b-1 exhibited a unique binding pattern indicating binding to both PD-1 and LAG-3 targets on T cells.

The contents of all references (including literature references, patents, patent applications, and websites) that are cited throughout this application are hereby expressly incorporated by reference in their entirety. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology and cell biology, which are well known in the art.

The invention may be embodied in other specific forms without departing from the essential characteristics of the invention described above. The foregoing embodiments are therefore to be considered illustrative rather than limiting of the invention described herein. The scope of the invention is indicated by the appended claims.

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CLAIMS

What is claimed is:

1. An antibody, or an antigen-binding portion thereof, capable of binding human PD-1, wherein the antigen-binding portion of the antibody comprises a set of six CDRs, CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2, and CDR-L3, selected from the group of CDR sets as follows:

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
1	CDR-H1	SYMMS	residues 31-35 of SEQ ID NO:4
	CDR-H2	SMSGGGRDTYYPDSVKG	residues 50-66 of SEQ ID NO:4
	CDR-H3	RGTYAMDY	residues 99-106 of SEQ ID NO:4
	CDR-L1	LASQTIGTWLT	residues 24-34 of SEQ ID NO:5
	CDR-L2	AATSLAD	residues 50-56 of SEQ ID NO:5
	CDR-L3	QQLYSTPWT	residues 89-97 of SEQ ID NO:5
2	CDR-H1	TGYYWN	residues 31-36 of SEQ ID NO:6
	CDR-H2	YMSYDGNNNYNPSLKN	residues 51-66 of SEQ ID NO:6
	CDR-H3	DRGTTILGGTMDY	residues 99-111 of SEQ ID NO:6
	CDR-L1	KASQSVSNDVA	residues 24-34 of SEQ ID NO:7
	CDR-L2	YAFYRYT	residues 50-56 of SEQ ID NO:7
	CDR-L3	QQDYSSPWT	residues 89-97 of SEQ ID NO:7
3	CDR-H1	FYTMS	residues 31-35 of SEQ ID NO:8
	CDR-H2	TISGGGRDTYYPDSVKG	residues 50-66 of SEQ ID NO:8
	CDR-H3	QGGNYLFAY	residues 99-107 of SEQ ID NO:8
	CDR-L1	KASQDVNTVVA	residues 24-34 of SEQ ID NO:9
	CDR-L2	WASTRHT	residues 50-56 of SEQ ID NO:9
	CDR-L3	QQHYTTPYT	residues 89-97 of SEQ ID NO:9
4	CDR-H1	DYGMH	residues 31-35 of SEQ ID NO:10

	CDR-H2	YISSGSYTIYYADTVKG	residues 50-66 of SEQ ID NO:10
	CDR-H3	RGGSSHVNVMDY	residues 99-110 of SEQ ID NO:10
	CDR-L1	KASDHINNWLA	residues 24-34 of SEQ ID NO:11
	CDR-L2	GATSLET	residues 50-56 of SEQ ID NO:11
	CDR-L3	QQYWSPPYT	residues 89-97 of SEQ ID NO:11
5	CDR-H1	DNNVE	residues 31-35 of SEQ ID NO:12
	CDR-H2	DINPNNGDTLYSQYFKD	residues 50-66 of SEQ ID NO:12
	CDR-H3	GKSDQFDY	residues 99-106 of SEQ ID NO:12
	CDR-L1	LASQTIGTWLA	residues 24-34 of SEQ ID NO:13
	CDR-L2	AATSLAD	residues 50-56 of SEQ ID NO:13
	CDR-L3	QQLYSSPWT	residues 89-97 of SEQ ID NO:13
6	CDR-H1	SYAMS	residues 31-35 of SEQ ID NO:14
	CDR-H2	TISGGGRDTYYPDSVKG	residues 50-66 of SEQ ID NO:14
	CDR-H3	QGGTYLFAS	residues 99-107 of SEQ ID NO:14
	CDR-L1	KASQDVNTAVA	residues 24-34 of SEQ ID NO:15
	CDR-L2	WASTRHT	residues 50-56 of SEQ ID NO:15
	CDR-L3	QQHYTTPYT	residues 89-97 of SEQ ID NO:15
7	CDR-H1	DYEMH	residues 31-35 of SEQ ID NO:16
	CDR-H2	VIEPESGGTVYNQKFKG	residues 51-66 of SEQ ID NO:16
	CDR-H3	EGFNSDHYFDY	residues 99-109 of SEQ ID NO:16
	CDR-L1	RSSQNIVHSNGNTYLE	residues 24-39 of SEQ ID NO:17
	CDR-L2	KVFNRFS	residues 55-61 of SEQ ID NO:17
	CDR-L3	FQGSHVPYT	residues 94-102 of SEQ ID NO:17
8	CDR-H1	SHLMS	residues 31-35 of SEQ ID NO:18
	CDR-H2	AISGGGADTYYPDSVKG	residues 50-66 of SEQ ID NO:18
	CDR-H3	QILAFDS	residues 99-105 of SEQ ID NO:18
	CDR-L1	HASQNIYVWLN	residues 24-34 of SEQ ID NO:19

	CDR-L2	KASNLHT	residues 50-56 of SEQ ID NO:19
	CDR-L3	QQGQSYPWT	residues 89-97 of SEQ ID NO:19
9	CDR-H1	SHLMS	residues 31-35 of SEQ ID NO:53
	CDR-H2	AISGGGADTYYPASVKG	residues 50-66 of SEQ ID NO:53
	CDR-H3	QILAFDA	residues 99-105 of SEQ ID NO:53
	CDR-L1	HASQNIYVWLN	residues 24-34 of SEQ ID NO:19
	CDR-L2	KASNLHT	residues 50-56 of SEQ ID NO:19
	CDR-L3	QQGQSYPWT	residues 89-97 of SEQ ID NO:19

2. An anti-PD-1 antibody comprising VH and VL domains, wherein the two variable domains comprise amino acid sequences selected from the group consisting of:

SEQ ID NO:4 and SEQ ID NO:5	SEQ ID NO:6 and SEQ ID NO:7
SEQ ID NO:8 and SEQ ID NO:9	SEQ ID NO:10 and SEQ ID NO:11
SEQ ID NO:12 and SEQ ID NO:13	SEQ ID NO:14 and SEQ ID NO:15
SEQ ID NO:16 and SEQ ID NO:17	SEQ ID NO:18 and SEQ ID NO:19
SEQ ID NO:20 and SEQ ID NO:23	SEQ ID NO:21 and SEQ ID NO:23
SEQ ID NO:22 and SEQ ID NO:23	SEQ ID NO:20 and SEQ ID NO:24
SEQ ID NO:21 and SEQ ID NO:24	SEQ ID NO:22 and SEQ ID NO:24
SEQ ID NO:20 and SEQ ID NO:25	SEQ ID NO:21 and SEQ ID NO:25
SEQ ID NO:22 and SEQ ID NO:25	SEQ ID NO:20 and SEQ ID NO:26
SEQ ID NO:21 and SEQ ID NO:26	SEQ ID NO:22 and SEQ ID NO:26
SEQ ID NO:20 and SEQ ID NO:27	SEQ ID NO:21 and SEQ ID NO:27
SEQ ID NO:22 and SEQ ID NO:27	SEQ ID NO:30 and SEQ ID NO:34
SEQ ID NO:31 and SEQ ID NO:34	SEQ ID NO:32 and SEQ ID NO:34
SEQ ID NO:33 and SEQ ID NO:34	SEQ ID NO:30 and SEQ ID NO:35
SEQ ID NO:31 and SEQ ID NO:35	SEQ ID NO:32 and SEQ ID NO:35
SEQ ID NO:33 and SEQ ID NO:35	SEQ ID NO:30 and SEQ ID NO:36
SEQ ID NO:31 and SEQ ID NO:36	SEQ ID NO:32 and SEQ ID NO:36

SEQ ID NO:33 and SEQ ID NO:36	SEQ ID NO:30 and SEQ ID NO:37
SEQ ID NO:31 and SEQ ID NO:37	SEQ ID NO:32 and SEQ ID NO:37
SEQ ID NO:33 and SEQ ID NO:37	SEQ ID NO:38 and SEQ ID NO:43
SEQ ID NO:39 and SEQ ID NO:43	SEQ ID NO:40 and SEQ ID NO:43
SEQ ID NO:41 and SEQ ID NO:43	SEQ ID NO:42 and SEQ ID NO:43
SEQ ID NO:38 and SEQ ID NO:44	SEQ ID NO:39 and SEQ ID NO:44
SEQ ID NO:40 and SEQ ID NO:44	SEQ ID NO:41 and SEQ ID NO:44
SEQ ID NO:42 and SEQ ID NO:44	SEQ ID NO:38 and SEQ ID NO:45
SEQ ID NO:39 and SEQ ID NO:45	SEQ ID NO:40 and SEQ ID NO:45
SEQ ID NO:41 and SEQ ID NO:45	SEQ ID NO:42 and SEQ ID NO:45
SEQ ID NO:38 and SEQ ID NO:46	SEQ ID NO:39 and SEQ ID NO:46
SEQ ID NO:40 and SEQ ID NO:46	SEQ ID NO:41 and SEQ ID NO:46
SEQ ID NO:42 and SEQ ID NO:46	SEQ ID NO:38 and SEQ ID NO:47
SEQ ID NO:39 and SEQ ID NO:47	SEQ ID NO:40 and SEQ ID NO:47
SEQ ID NO:41 and SEQ ID NO:47	SEQ ID NO:42 and SEQ ID NO:47
SEQ ID NO:48 and SEQ ID NO:55	SEQ ID NO:49 and SEQ ID NO:55
SEQ ID NO:50 and SEQ ID NO:55	SEQ ID NO:51 and SEQ ID NO:55
SEQ ID NO:52 and SEQ ID NO:55	SEQ ID NO:53 and SEQ ID NO:55
SEQ ID NO:54 and SEQ ID NO:55	SEQ ID NO:48 and SEQ ID NO:56
SEQ ID NO:49 and SEQ ID NO:56	SEQ ID NO:50 and SEQ ID NO:56
SEQ ID NO:51 and SEQ ID NO:56	SEQ ID NO:52 and SEQ ID NO:56
SEQ ID NO:53 and SEQ ID NO:56	SEQ ID NO:54 and SEQ ID NO:56
SEQ ID NO:48 and SEQ ID NO:57	SEQ ID NO:49 and SEQ ID NO:57
SEQ ID NO:50 and SEQ ID NO:57	SEQ ID NO:51 and SEQ ID NO:57
SEQ ID NO:52 and SEQ ID NO:57	SEQ ID NO:53 and SEQ ID NO:57
SEQ ID NO:54 and SEQ ID NO:57.	

3. An antibody, or an antigen-binding portion thereof, capable of binding human LAG-3, wherein the antigen-binding portion of the antibody comprises a set of six CDRs, CDR-H1,

CDR-H2, CDR-H3, CDR-L1, CDR-L2, and CDR-L3, selected from the group of CDR sets as follows:

CDR Set No.	CDR	CDR Amino Acid Sequence	SEQ ID NO:
10	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:60
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:60
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:60
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:61
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:61
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:61
11	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:60
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:60
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:60
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:62
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:62
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:62
12	CDR-H1	DYEMH	residues 31-35 of SEQ ID NO:63
	CDR-H2	AIDPETGGTAYNQKFKG	residues 50-66 of SEQ ID NO:63
	CDR-H3	WGSTVFPY	residues 101-108 of SEQ ID NO:63
	CDR-L1	KSTKSLLNSDGFTYLD	residues 24-39 of SEQ ID NO:64
	CDR-L2	LVSNRFS	residues 55-61 of SEQ ID NO:64
	CDR-L3	FQSNYLPWT	residues 94-102 of SEQ ID NO:64
13	CDR-H1	DYEMH	residues 31-35 of SEQ ID NO:65
	CDR-H2	AIDPATGGTAYNQKFKG	residues 50-66 of SEQ ID NO:65
	CDR-H3	WGTTVFPY	residues 99-106 of SEQ ID NO:65
	CDR-L1	KSTKSLLNSDGFTYLD	residues 24-39 of SEQ ID NO:66
	CDR-L2	LVSNRFS	residues 55-61 of SEQ ID NO:66

	CDR-L3	FQSNYLPWT	residues 94-102 of SEQ ID NO:66
14	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:67
	CDR-H2	WIDPENGDTEYASKFQG	residues 50-66 of SEQ ID NO:67
	CDR-H3	FDY	residues 99-101 of SEQ ID NO:67
	CDR-L1	KSSQSLLDSDGKTYLN	residues 24-39 of SEQ ID NO:68
	CDR-L2	LVSKLDS	residues 55-61 of SEQ ID NO:68
	CDR-L3	WQGSHFPQT	residues 94-102 of SEQ ID NO:68
15	CDR-H1	DDYVH	residues 31-35 of SEQ ID NO:69
	CDR-H2	WIDPENGDTEYASKFQG	residues 50-66 of SEQ ID NO:69
	CDR-H3	WDAEENY	residues 99-105 of SEQ ID NO:69
	CDR-L1	RSSKSLLHSNGNTYLY	residues 24-39 of SEQ ID NO:70
	CDR-L2	RMSNLAS	residues 55-61 of SEQ ID NO:70
	CDR-L3	MQHLEYPFT	residues 94-102 of SEQ ID NO:70
16	CDR-H1	DDYIH	residues 31-35 of SEQ ID NO:71
	CDR-H2	WIDPENGDTEYASKFQG	residues 50-66 of SEQ ID NO:71
	CDR-H3	DYRNWY	residues 100-105 of SEQ ID NO:71
	CDR-L1	KSSQSLLDSDGKTYLN	residues 24-39 of SEQ ID NO:68
	CDR-L2	LVSKLDS	residues 55-61 of SEQ ID NO:68
	CDR-L3	WQGSHFPQT	residues 94-102 of SEQ ID NO:68
17	CDR-H1	DFNIKDDYMH	residues 26-35 of SEQ ID NO:114
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:114
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:114
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:117
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:117
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:117
18	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:72
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:72

	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:72
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:77
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:77
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:77
19	CDR-H1	DDYMH	residues 30-34 of SEQ ID NO:119
	CDR-H2	WIVPENGNTVYASKFQG	residues 48-64 of SEQ ID NO:119
	CDR-H3	YGDY	residues 95-98 of SEQ ID NO:119
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:120
	CDR-L2	AASALDS	residues 50-56 of SEQ ID NO:120
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:120
20	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:121
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:121
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:121
	CDR-L1	RAMQEISGYLS	residues 24-34 of SEQ ID NO:122
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:122
	CDR-L3	LQYAYYPLT	residues 89-97 of SEQ ID NO:122
21	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:123
	CDR-H2	WIVPENGNTEYASKFQG	residues 50-66 of SEQ ID NO:123
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:123
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:124
	CDR-L2	AASHLDS	residues 50-56 of SEQ ID NO:124
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:124
22	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:125
	CDR-H2	WIVPENGLTEYASKFQG	residues 50-66 of SEQ ID NO:125
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:125
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:126
	CDR-L2	ATSTLDS	residues 50-56 of SEQ ID NO:126

	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:126
23	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:127
	CDR-H2	WIVPENGKTEYASKFQG	residues 50-66 of SEQ ID NO:127
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:127
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:128
	CDR-L2	AAMTLDS	residues 50-56 of SEQ ID NO:128
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:128
24	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:129
	CDR-H2	WIVPENGNTHYASKFQG	residues 50-66 of SEQ ID NO:129
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:129
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:130
	CDR-L2	EASTLDS	residues 50-56 of SEQ ID NO:130
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:130
25	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:131
	CDR-H2	WIVPRNGNTMYASKFQG	residues 50-66 of SEQ ID NO:131
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:131
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:132
	CDR-L2	AASTLDL	residues 50-56 of SEQ ID NO:132
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:132
26	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:135
	CDR-H2	WIVPENANTVYASKFQG	SEQ ID NO:224
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:135
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:138
	CDR-L2	AASALDS	residues 50-56 of SEQ ID NO:138
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:138
27	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:136
	CDR-H2	WIVPRNANTVYASKFQG	SEQ ID NO:225

	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:136
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:139
	CDR-L2	AASALDL	residues 50-56 of SEQ ID NO:139
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:139
28	CDR-H1	DDYMH	residues 31-35 of SEQ ID NO:136
	CDR-H2	WIVPRNANTVYASKFQG	SEQ ID NO:225
	CDR-H3	YGDY	residues 99-102 of SEQ ID NO:136
	CDR-L1	RASQEISGYLS	residues 24-34 of SEQ ID NO:117
	CDR-L2	AASTLDS	residues 50-56 of SEQ ID NO:117
	CDR-L3	LQYASYPLT	residues 89-97 of SEQ ID NO:117

4. An anti-LAG-3 antibody comprising VH and VL domains, wherein the two variable domains comprise amino acid sequences selected from the group consisting of:

SEQ ID NO:60 and SEQ ID NO:61	SEQ ID NO:60 and SEQ ID NO:62
SEQ ID NO:63 and SEQ ID NO:64	SEQ ID NO:65 and SEQ ID NO:66
SEQ ID NO:67 and SEQ ID NO:68	SEQ ID NO:69 and SEQ ID NO:70
SEQ ID NO:71 and SEQ ID NO:68	SEQ ID NO:74 and SEQ ID NO:75
SEQ ID NO:74 and SEQ ID NO:76	SEQ ID NO:74 and SEQ ID NO:77
SEQ ID NO:72 and SEQ ID NO:75	SEQ ID NO:72 and SEQ ID NO:76
SEQ ID NO:72 and SEQ ID NO:77	SEQ ID NO:73 and SEQ ID NO:75
SEQ ID NO:73 and SEQ ID NO:76	SEQ ID NO:73 and SEQ ID NO:77
SEQ ID NO:121 and SEQ ID NO:122	SEQ ID NO:123 and SEQ ID NO:124
SEQ ID NO:125 and SEQ ID NO:126	SEQ ID NO:127 and SEQ ID NO:128
SEQ ID NO:129 and SEQ ID NO:130	SEQ ID NO:131 and SEQ ID NO:132
SEQ ID NO:135 and SEQ ID NO:138	SEQ ID NO:136 and SEQ ID NO:139
SEQ ID NO:136 and SEQ ID NO:117	SEQ ID NO:226 and SEQ ID NO:138
SEQ ID NO:227 and SEQ ID NO:139	SEQ ID NO:227 and SEQ ID NO:117.

5. A bispecific, multivalent binding protein comprising first, second and third polypeptide chains, wherein

said first polypeptide chain comprises, from amino to carboxyl terminus, (i) VL_A-CL-VH_B-CH1-Fc wherein CL is directly fused to VH_B, or (ii) VH_B-CH1-VL_A-CL-Fc wherein CH1 is directly fused to VL_A;

said second polypeptide chain comprises, from amino to carboxyl terminus, VH_A-CH1; and

said third polypeptide chain comprises, from amino to carboxyl terminus, VL_B-CL; wherein VL is a light chain variable domain, CL is a light chain constant domain, VH is a heavy chain variable domain, CH1 is a heavy chain constant domain, Fc is an immunoglobulin Fc region, A is an epitope of PD-1 or LAG-3 and B is an epitope of PD-1 or LAG-3, with the proviso that A and B are different, said binding protein being capable of binding to both PD-1 and LAG-3.

6. The binding protein of Claim 5, wherein the VL_A-CL and VH_A-CH1 domains are from a parental antibody binding to one of the antigen targets PD-1 or LAG-3, and the VL_B-CL and VH_B-CH1 domains are from a different parental antibody binding to the other of the antigen targets PD-1 or LAG-3.

7. The binding protein of Claim 6, comprising first, second and third polypeptide chains, wherein said first polypeptide chain comprises, from amino to carboxyl terminus, VL_{PD-1}-CL-VH_{LAG-3}-CH1-Fc wherein CL is directly fused to VH_{LAG-3},

wherein said second polypeptide chain comprises, from amino to carboxyl terminus, VH_{PD-1}-CH1; and

wherein said third polypeptide chain comprises, from amino to carboxyl terminus, VL_{LAG-3}-CL;

wherein VL_{PD-1} is a light chain variable domain of an anti-PD-1 antibody, CL is a light chain constant domain, VH_{PD-1} is a heavy chain variable domain of an anti-PD-1 antibody, CH1 is a heavy chain constant domain, VL_{LAG-3} is a light chain variable domain of an anti-LAG-3

antibody, VH_{LAG-3} is a heavy chain variable domain of an anti-LAG-3 antibody, and Fc is an immunoglobulin Fc region.

8. The binding protein of Claim 7, wherein, in the first polypeptide chain, the domains VL_{PD-1}-CL are the same as the light chain of an anti-PD-1 parental antibody, the domains VH_{PD-1}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-PD-1 parental antibody, the domains VL_{LAG-3}-CL are the same as the light chain of an anti-LAG-3 parental antibody, and the domains VH_{LAG-3}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-LAG-3 parental antibody.

9. The binding protein of Claim 6, comprising first, second and third polypeptide chains, wherein said first polypeptide chain comprises, from amino to carboxyl terminus, VL_{LAG-3}-CL-VH_{PD-1}-CH1-Fc wherein CL is directly fused to VH_{PD-1},
wherein said second polypeptide chain comprises, from amino to carboxyl terminus, VH_{LAG-3}-CH1; and
wherein said third polypeptide chain comprises, from amino to carboxyl terminus, VL_{PD-1}-CL; wherein VL_{PD-1} is a light chain variable domain of an anti-PD-1 antibody, CL is a light chain constant domain, VH_{PD-1} is a heavy chain variable domain of an anti-PD-1 antibody, CH1 is a heavy chain constant domain, VL_{LAG-3} is a light chain variable domain of an anti-LAG-3 antibody, VH_{LAG-3} is a heavy chain variable domain of an anti-LAG-3 antibody, and Fc is an immunoglobulin Fc region.

10. The binding protein of Claim 9, wherein, in the first polypeptide chain, the domains VL_{LAG-3}-CL are the same as the light chain of an anti-LAG-3 parental antibody, the domains VH_{LAG-3}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-LAG-3 parental antibody, the domains VL_{PD-1}-CL are the same as the light chain of an anti-PD-1 parental antibody, and the domains VH_{PD-1}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-PD-1 parental antibody.

11. The binding protein of Claim 6, comprising first, second and third polypeptide chains, wherein said first polypeptide chain comprises, from amino to carboxyl terminus, VH_{LAG-3}-CH1-VL_{PD-1}-CL-Fc wherein CH1 is directly fused to VL_{PD-1},
wherein said second polypeptide chain comprises, from amino to carboxyl terminus, VL_{LAG-3}-CL; and
wherein said third polypeptide chain comprises, from amino to carboxyl terminus, VH_{PD-1}-CH1; wherein VL_{PD-1} is a light chain variable domain of an anti-PD-1 antibody, CL is a light chain constant domain, VH_{PD-1} is a heavy chain variable domain of an anti-PD-1 antibody, CH1 is a heavy chain constant domain, VL_{LAG-3} is a light chain variable domain of an anti-LAG-3 antibody, VH_{LAG-3} is a heavy chain variable domain of an anti-LAG-3 antibody, and Fc is an immunoglobulin Fc region.

12. The binding protein of Claim 11, wherein, in the first polypeptide chain, the domains VL_{LAG-3}-CL are the same as the light chain of an anti-LAG-3 parental antibody, the domains VH_{LAG-3}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-LAG-3 parental antibody, the domains VL_{PD-1}-CL are the same as the light chain of an anti-PD-1 parental antibody, and the domains VH_{PD-1}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-PD-1 parental antibody.

13. The binding protein of Claim 6, comprising first, second and third polypeptide chains, wherein said first polypeptide chain comprises, from amino to carboxyl terminus, VH_{PD-1}-CH1-VL_{LAG-3}-CL-Fc wherein CH1 is directly fused to VL_{LAG-3},
wherein said second polypeptide chain comprises, from amino to carboxyl terminus, VL_{PD-1}-CL; and
wherein said third polypeptide chain comprises, from amino to carboxyl terminus, VH_{LAG-3}-CH1; wherein VL_{PD-1} is a light chain variable domain of an anti-PD-1 antibody, CL is a light chain constant domain, VH_{PD-1} is a heavy chain variable domain of an anti-PD-1 antibody, CH1 is a heavy chain constant domain, VL_{LAG-3} is a light chain variable domain of an anti-

LAG-3 antibody, VH_{LAG-3} is a heavy chain variable domain of an anti-LAG-3 antibody, and Fc is an immunoglobulin Fc region.

14. The binding protein of Claim 13, wherein, in the first polypeptide chain, the domains VL_{LAG-3}-CL are the same as the light chain of an anti-LAG-3 parental antibody, the domains VH_{LAG-3}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-LAG-3 parental antibody, the domains VL_{PD-1}-CL are the same as the light chain of an anti-PD-1 parental antibody, and the domains VH_{PD-1}-CH1 are the same as the heavy chain variable and heavy chain constant domains of an anti-PD-1 parental antibody.

15. The antibody or binding protein according to any one of Claims 1-14, further comprising an Fc region comprising SEQ ID NO:28.

16. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-679 of SEQ ID NO:78, said second polypeptide chain comprises an amino acid sequence of amino acids 20-240 of SEQ ID NO:83, and said third polypeptide chain comprises an amino acid sequence of amino acids 23-236 of SEQ ID NO:86.

17. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-684 of SEQ ID NO:88, said second polypeptide chain comprises an amino acid sequence of amino acids 20-235 of SEQ ID NO:91, and said third polypeptide chain comprises an amino acid sequence of amino acids 23-236 of SEQ ID NO:93.

18. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-679 of SEQ ID NO:95; said second polypeptide chain comprises an amino acid sequence of amino acids 20-242 of SEQ ID NO:98; and said third polypeptide chain comprises an amino acid sequence of amino acids 23-236 of SEQ ID NO:100.

19. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-684 of SEQ ID NO:102; said second polypeptide chain comprises an amino acid sequence of amino acids 20-235 of SEQ ID NO:105; and said third polypeptide chain comprises an amino acid sequence of amino acids 23-236 of SEQ ID NO:107.

20. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-679 of SEQ ID NO:140; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:144; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:146.

21. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-684 of SEQ ID NO:147; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:151; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:153.

22. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-679 of SEQ ID NO:154; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:158; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:160.

23. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-684 of SEQ ID NO:161; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:165; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:167.

24. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-679 of SEQ ID NO:168; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:172; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:174.

25. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-684 of SEQ ID NO:175; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:179; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:181.

26. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-679 of SEQ ID NO:182; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:186; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:188.

27. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-687 of SEQ ID NO:189; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:193; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:195.

28. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-679 of SEQ ID NO:196; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:200; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:202.

29. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-687 of SEQ ID NO:203; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:207; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:209.

30. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-679 of SEQ ID NO:210; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:214; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:216.

31. The binding protein of Claim 5, wherein said first polypeptide chain comprises an amino acid sequence of amino acids 23-687 of SEQ ID NO:217; said second polypeptide chain comprises the amino acid sequence of SEQ ID NO:221; and said third polypeptide chain comprises the amino acid sequence of SEQ ID NO:223.

32. A pharmaceutical composition comprising at least one anti-PD-1 antibody or antigen-binding fragment thereof according to Claim 1, and a pharmaceutically acceptable carrier.

33. A pharmaceutical composition comprising at least one anti-LAG-3 antibody or antigen-binding fragment thereof according to Claim 3, and a pharmaceutically acceptable carrier.

34. A pharmaceutical composition comprising a combination of anti-PD-1 antibody or antigen-binding portion thereof according to Claim 1 and anti-LAG-3 antibody or antigen-binding portion thereof according to Claim 3, and a pharmaceutically acceptable carrier.

35. A pharmaceutical composition comprising at least one bispecific binding protein according to Claim 5 and a pharmaceutically acceptable carrier.

36. Use of a bispecific binding protein according to Claim 5 to treat a disease or disorder in which PD-1-mediated activity and/or LAG-3-mediated activity is detrimental.

37. The use according to Claim 36 wherein said disease is cancer.

38. The use according to Claim 37, wherein the cancer is: a melanoma (e.g., metastatic malignant melanoma), a renal cancer (e.g., clear cell carcinoma), a prostate cancer (e.g., hormone refractory prostate adenocarcinoma), a pancreatic adenocarcinoma, a breast cancer, a colon cancer, a lung cancer (e.g., non-small cell lung cancer), an esophageal cancer, a squamous cell carcinoma of the head and neck, a liver cancer, an ovarian cancer, a cervical cancer, a thyroid cancer, a glioblastoma, a glioma, a leukemia, a lymphoma, or a primary bone cancer (e.g., osteosarcoma, Ewing sarcoma, malignant fibrous histiocytoma, or chondrosarcoma).

39. A method for making a medicament for use in treating a disease or disorder in which PD-1-mediated activity and/or LAG-3-mediated activity is detrimental comprising formulating at least one bispecific binding protein according to Claim 5 with a pharmaceutically acceptable carrier.

40. The method according to Claim 39 wherein said disease is cancer.

41. The method according to Claim 40, wherein the cancer is: a melanoma (e.g., metastatic malignant melanoma), a renal cancer (e.g., clear cell carcinoma), a prostate cancer (e.g., hormone refractory prostate adenocarcinoma), a pancreatic adenocarcinoma, a breast cancer, a colon cancer, a lung cancer (e.g., non-small cell lung cancer), an esophageal cancer, a squamous cell carcinoma of the head and neck, a liver cancer, an ovarian cancer, a cervical cancer, a thyroid cancer, a glioblastoma, a glioma, a leukemia, a lymphoma, or a primary bone cancer (e.g., osteosarcoma, Ewing sarcoma, malignant fibrous histiocytoma, or chondrosarcoma).

42. A method of treating a disorder wherein PD-1-mediated and/or LAG-3-mediated activity is detrimental, comprising administering to a subject in need thereof an effective amount of a pharmaceutical composition according to any one of Claims 32-35, or a combination thereof.

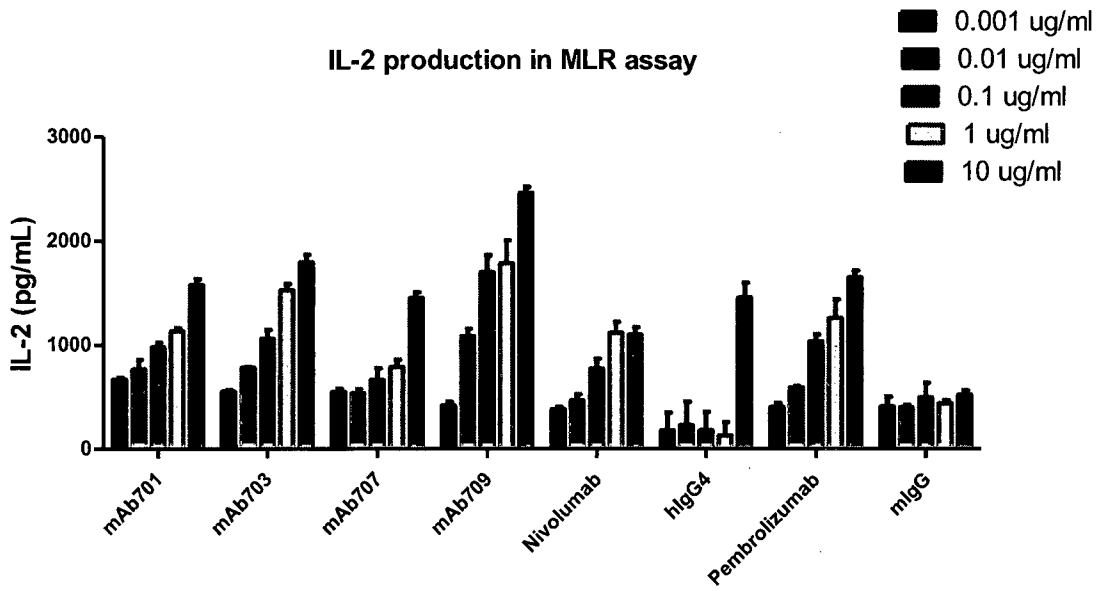
43. The method according to Claim 42 wherein said disease is cancer.

44. The method according to Claim 43, wherein the cancer is: a melanoma (e.g., metastatic malignant melanoma), a renal cancer (e.g., clear cell carcinoma), a prostate cancer (e.g., hormone refractory prostate adenocarcinoma), a pancreatic adenocarcinoma, a breast cancer, a colon cancer, a lung cancer (e.g., non-small cell lung cancer), an esophageal cancer, a squamous cell carcinoma of the head and neck, a liver cancer, an ovarian cancer, a cervical cancer, a thyroid cancer, a glioblastoma, a glioma, a leukemia, a lymphoma, or a primary bone cancer (e.g., osteosarcoma, Ewing sarcoma, malignant fibrous histiocytoma, or chondrosarcoma).

45. The method according to Claim 42, wherein said subject is a human.

Fig. 1

A


IL-2 production in MLR assay
0.001 ug/ml
0.01 ug/ml
0.1 ug/ml
1 ug/ml
10 ug/ml
IL-2 (pg/mL)
3000
2000
1000
0
mAb701
mAb703
mAb707
mAb709
Nivolumab
hIgG4
Pembrolizumab
mIgG

B

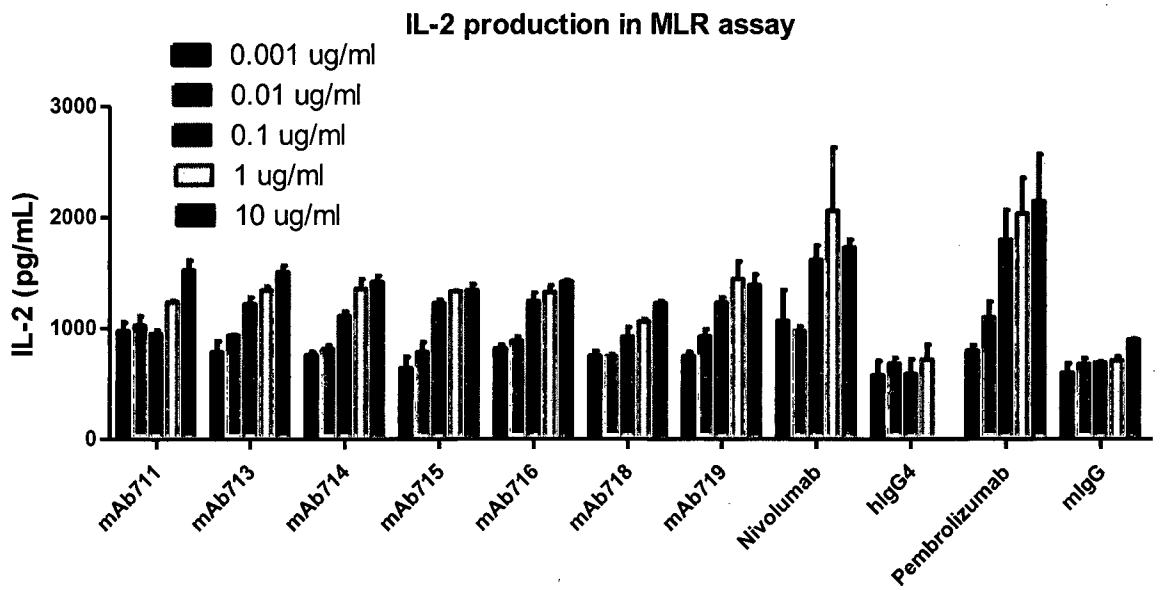
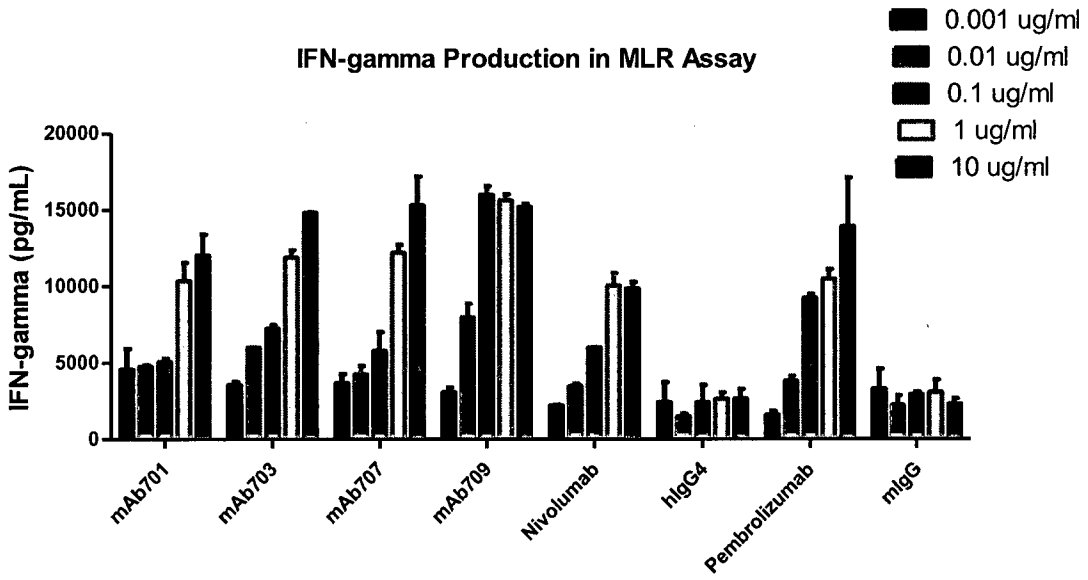

IL-2 production in MLR assay
0.001 ug/ml
0.01 ug/ml
0.1 ug/ml
1 ug/ml
10 ug/ml
IL-2 (pg/mL)
3000
2000
1000
0
mAb711
mAb713
mAb714
mAb715
mAb716
mAb718
mAb719
Nivolumab
hIgG4
Pembrolizumab
mIgG

Fig. 2

A


IFN-gamma Production in MLR Assay
0.001 ug/ml
0.01 ug/ml
0.1 ug/ml
1 ug/ml
10 ug/ml
IFN-gamma (pg/mL)
20000
15000
10000
5000
0
mAb701
mAb703
mAb707
mAb709
Nivolumab
hIgG4
Pembrolizumab
mIgG

B

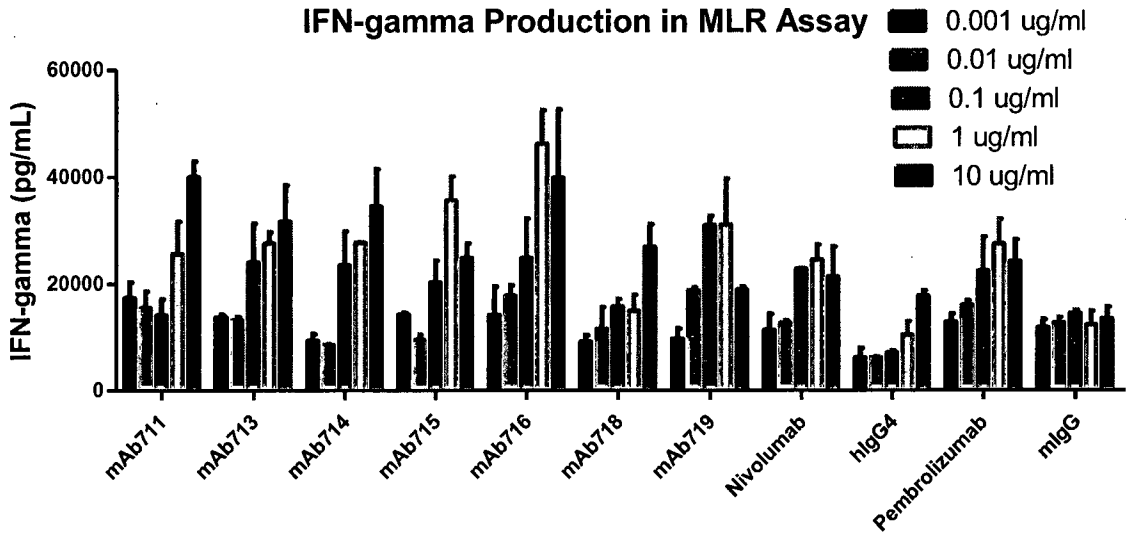

IFN-gamma Production in MLR Assay
0.001 ug/ml
0.01 ug/ml
0.1 ug/ml
1 ug/ml
10 ug/ml
IFN-gamma (pg/mL)
60000
40000
20000
0
mAb711
mAb713
mAb714
mAb715
mAb716
mAb718
mAb719
Nivolumab
hIgG4
Pembrolizumab
mIgG

Fig. 3

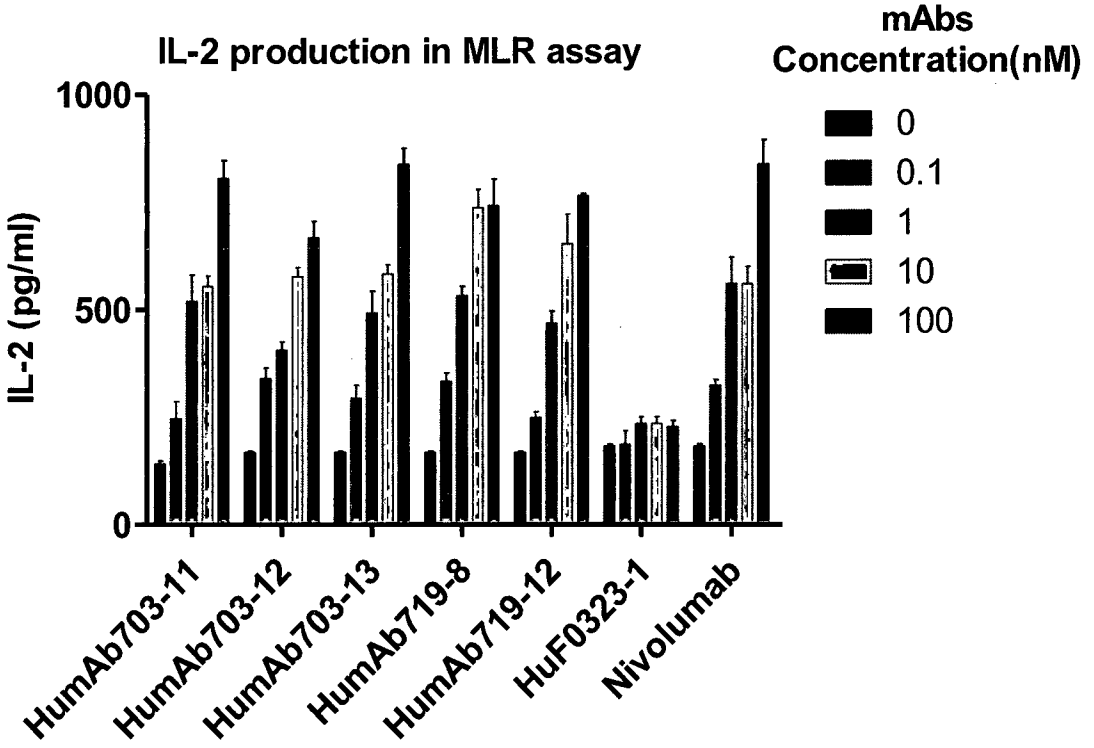

IL-2 production in MLR assay
mAbs
Concentration(nM)
0
0.1
1
10
100
IL-2 (pg/ml)
1000
500
0
HumAb703-11
HumAb703-12
HumAb703-13
HumAb719-8
HumAb719-12
HuF0323-1
Nivolumab

Fig. 4

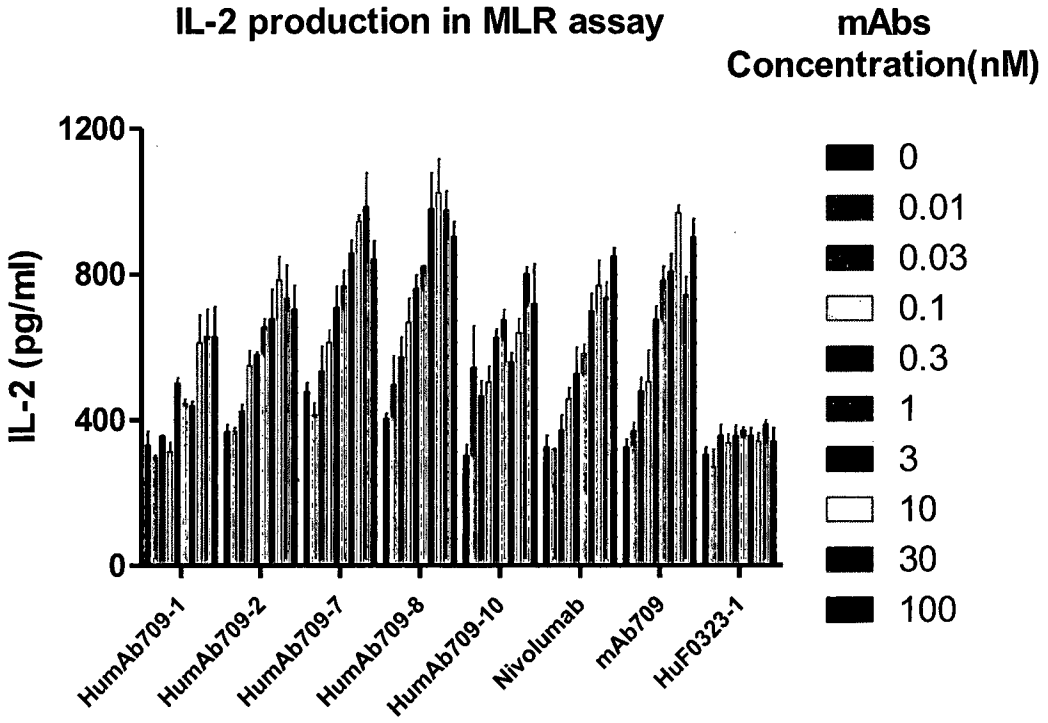

IL-2 production in MLR assay
mAbs Concentration(nM)
IL-2 (pg/ml)
1200
800
400
0
0
0.01
0.03
0.1
0.3
1
3
10
30
100
HumAb709-1
HumAb709-2
HumAb709-7
HumAb709-8
HumAb709-10
Nivolumab
mAb709
HuF0323-1

Fig. 5

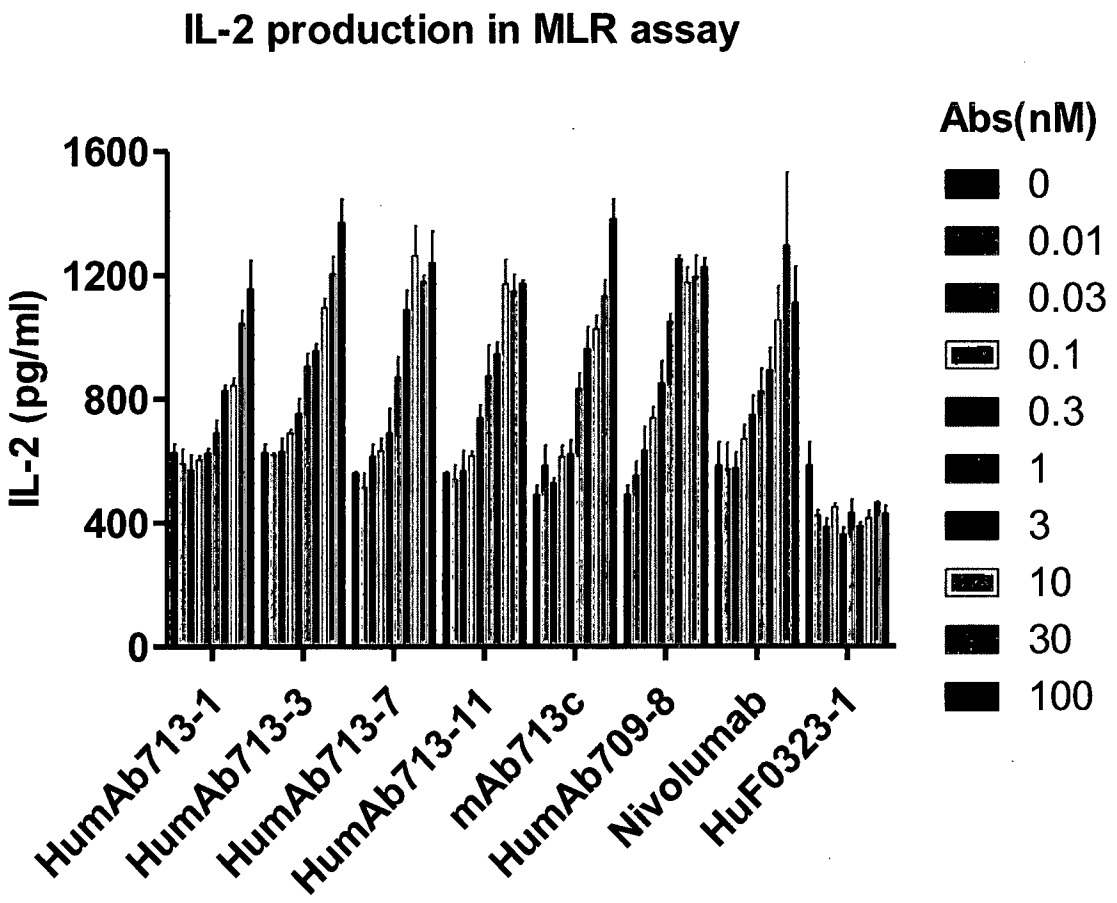

IL-2 production in MLR assay
IL-2 (pg/ml)
1600
1200
800
400
0
HumAb713-1
HumAb713-3
HumAb713-7
HumAb713-11
mAb713c
HumAb709-8
Nivolumab
HuF0323-1
Abs(nM)
0
0.01
0.03
0.1
0.3
1
3
10
30
100

Fig. 6

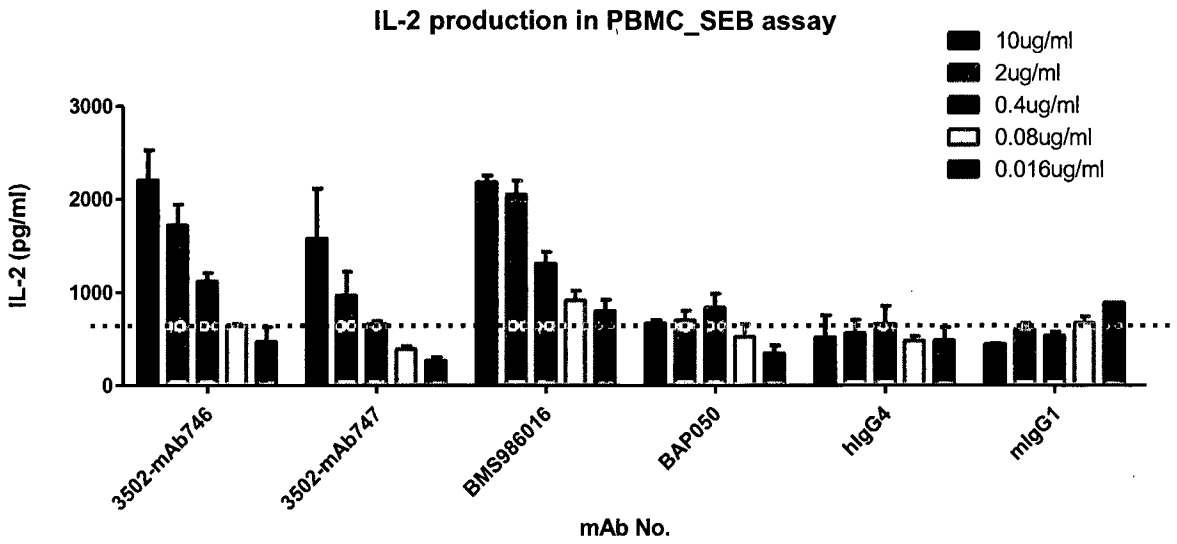

IL-2 production in PBMC_SEB assay
10ug/ml
2ug/ml
0.4ug/ml
0.08ug/ml
0.016ug/ml
IL-2 (pg/ml)
3000
2000
1000
0
3502-mAb746
3502-mAb747
BMS986016
BAP050
hIgG4
mIgG1
mAb No.

Fig. 7

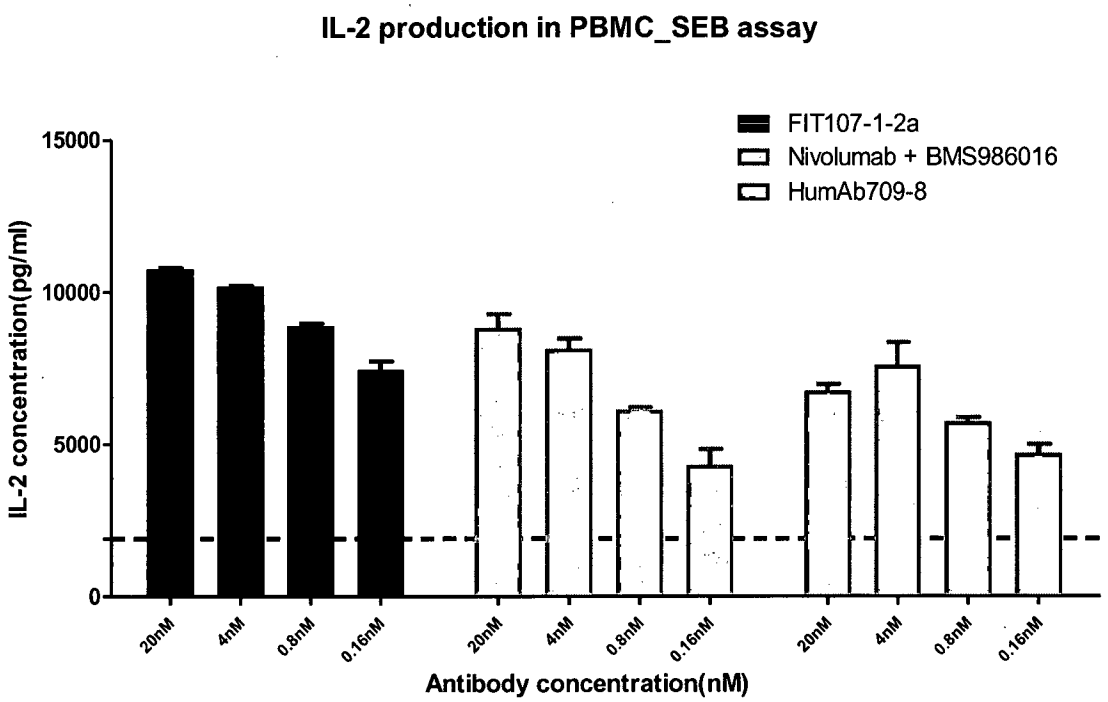
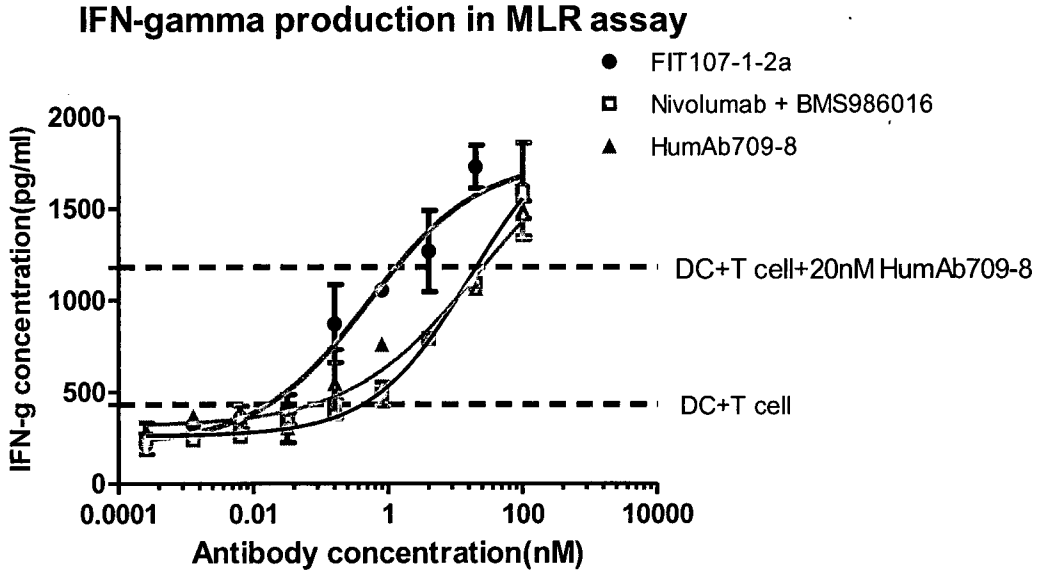

IL-2 production in PBMC_SEB assay
FIT107-1-2a
Nivolumab + BMS986016
HumAb709-8
IL-2 concentration(pg/ml)
15000
10000
5000
0
20nM
4nM
0.8nM
0.16nM
20nM
4nM
0.8nM
0.16nM
20nM
4nM
0.8nM
0.16nM
Antibody concentration(nM)

Fig. 8



	FIT107-1-2a	Nivolumab + BMS986016	HumAb709-8
EC50	0.5084	16.84	22.62

Fig. 9

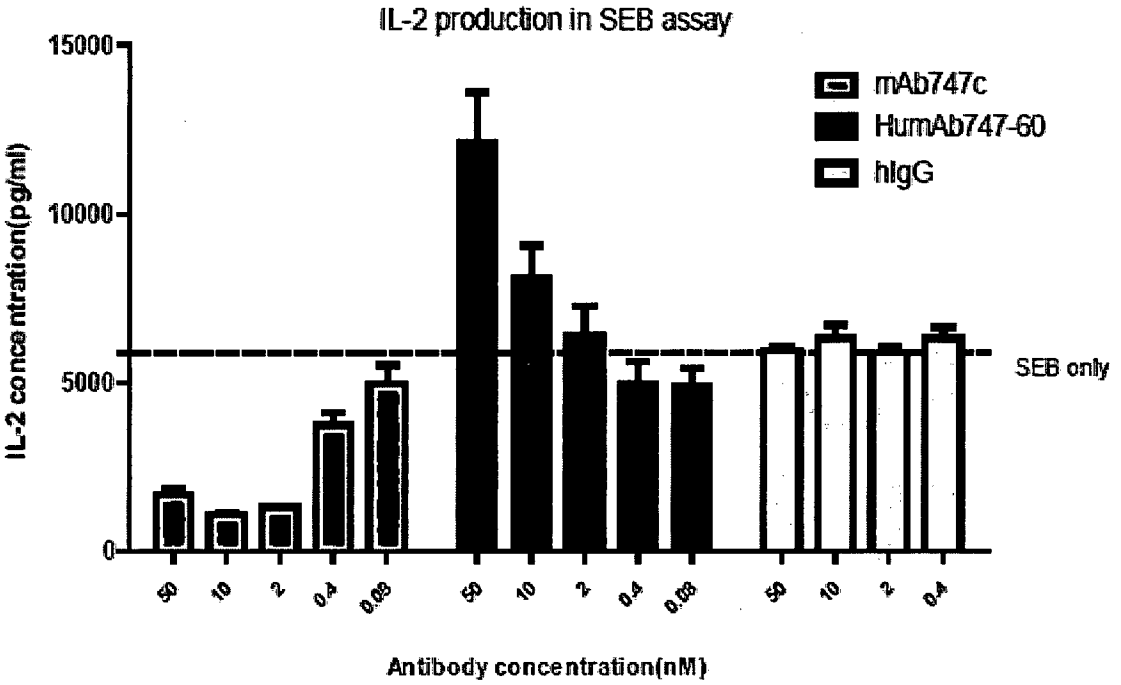

IL-2 production in SEB assay
15000
10000
5000
0
IL-2 concentration(pg/ml)
mAb747c
HumAb747-60
hIgG
SEB only
50
10
2
0.4
0.08
50
10
2
0.4
0.08
50
10
2
0.4
Antibody concentration(nM)

Fig. 10

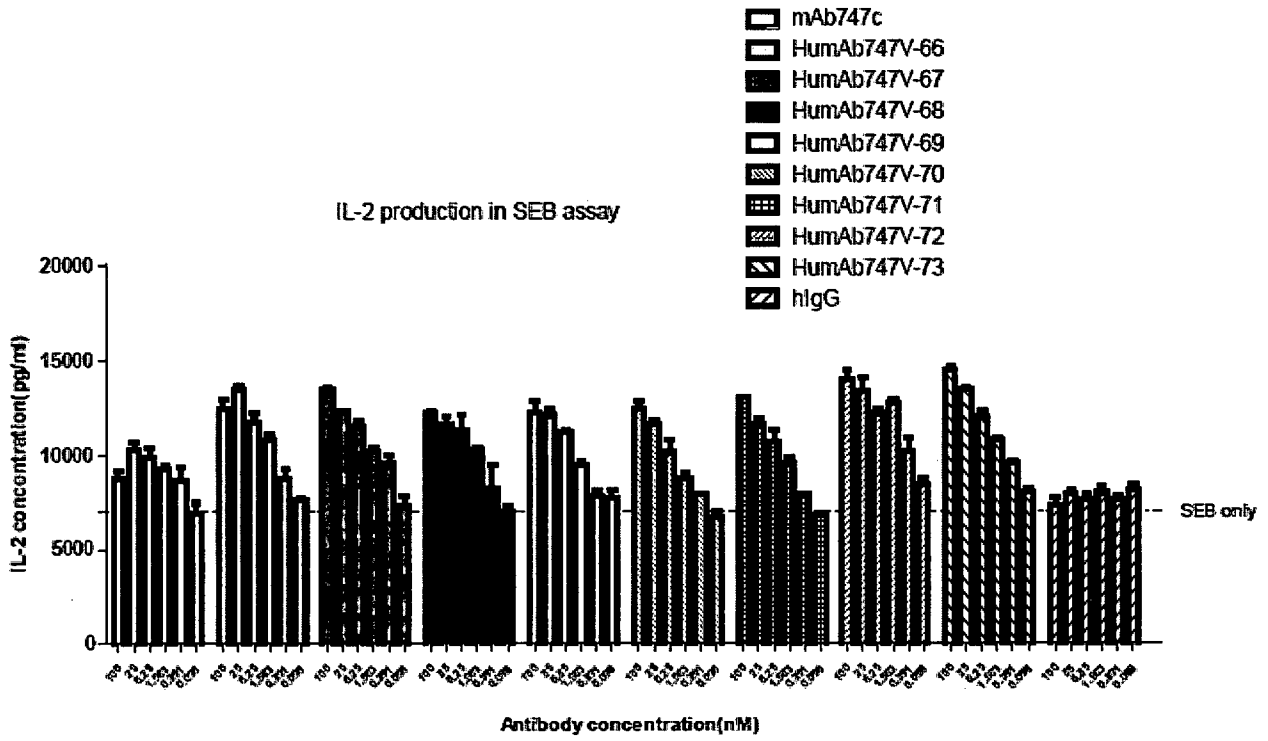

mAb747c
HumAb747V-66
HumAb747V-67
HumAb747V-68
HumAb747V-69
HumAb747V-70
HumAb747V-71
HumAb747V-72
HumAb747V-73
hIgG
IL-2 production in SEB assay
IL-2 concentration(pg/ml)
20000
15000
10000
5000
0
SEB only
Antibody concentration(nM)

Fig. 11

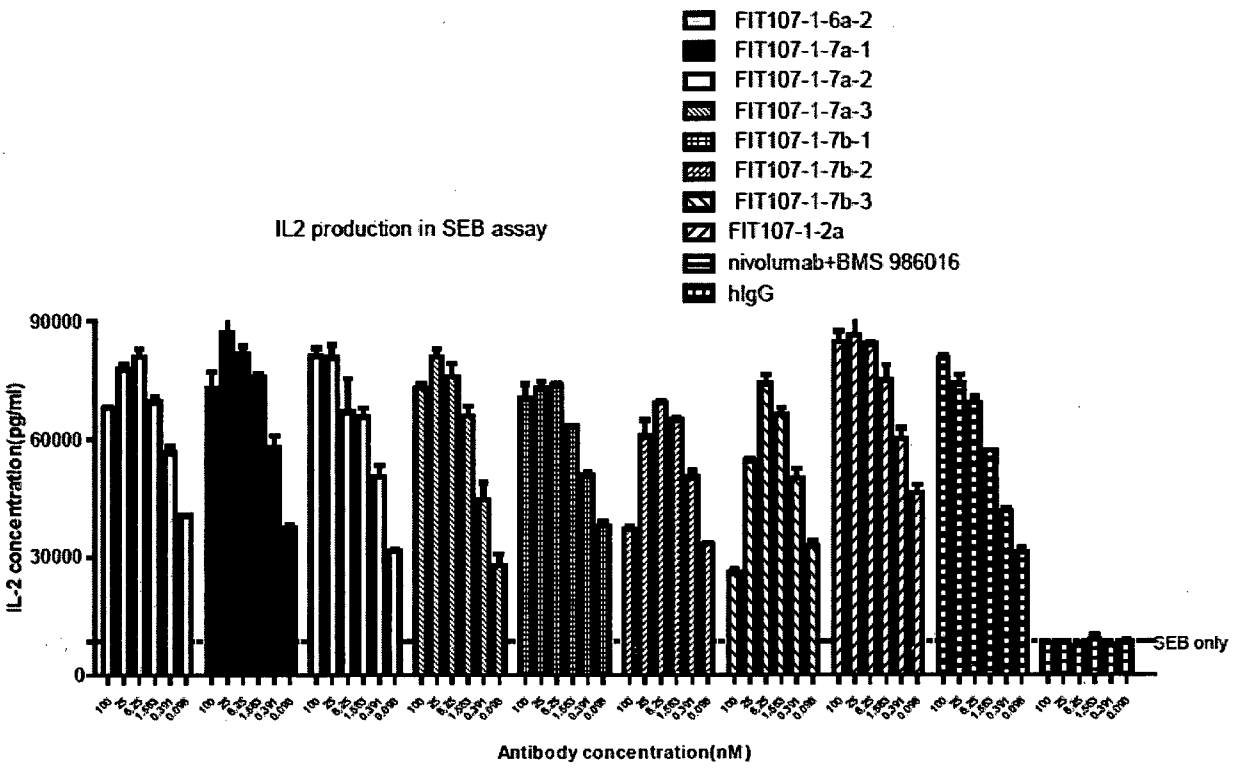

FIT107-1-6a-2
FIT107-1-7a-1
FIT107-1-7a-2
FIT107-1-7a-3
FIT107-1-7b-1
FIT107-1-7b-2
FIT107-1-7b-3
FIT107-1-2a
nivolumab+BMS 986016
hIgG
IL2 production in SEB assay
IL-2 concentration(pg/ml)
90000
60000
30000
0
SEB only
Antibody concentration(nM)

Fig. 12

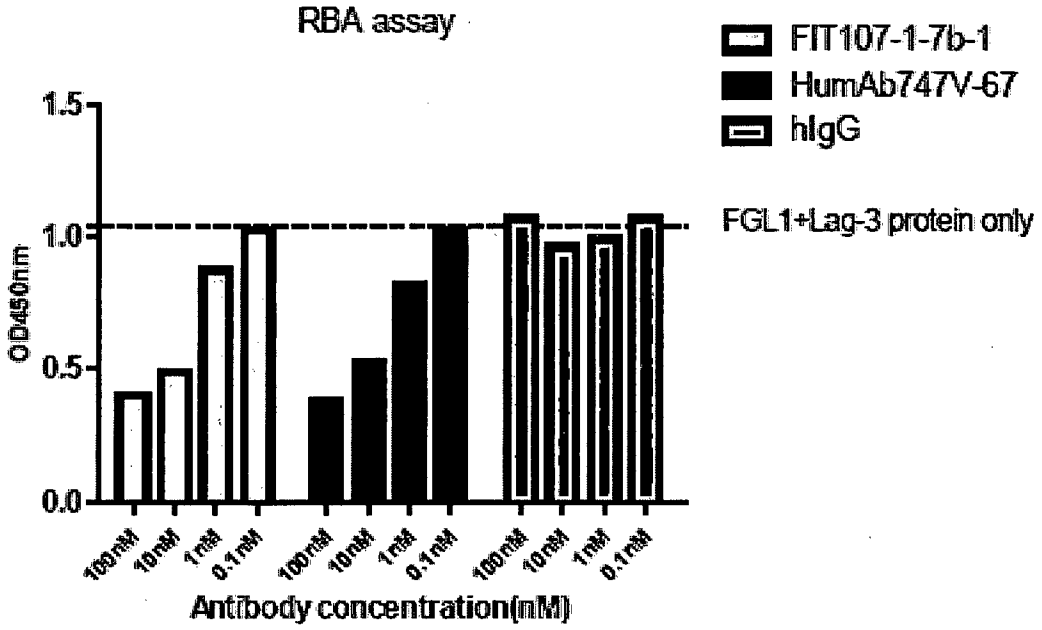
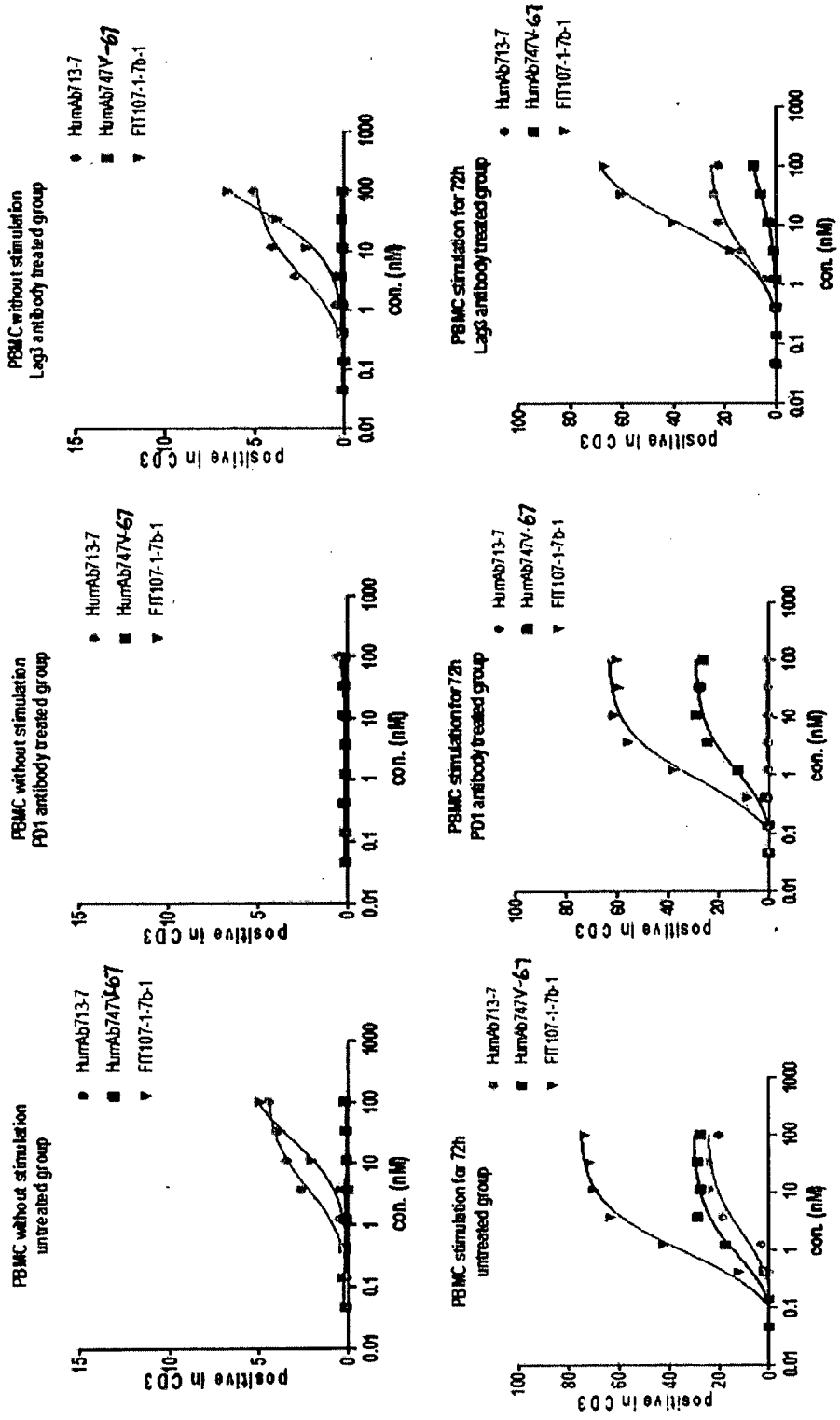

RBA assay
FIT107-1-7b-1
HumAb747V-67
hIgG
FGL1+Lag-3 protein only
OD450nm
1.5
1.0
0.5
0.0
100nM
10nM
1nM
0.1nM
Antibody concentration(nM)

Fig. 13


PBMC without stimulation
Lag3 antibody treated group
PBMC stimulation for 72h
Lag3 antibody treated group
PBMC without stimulation
PD1 antibody treated group
PBMC stimulation for 72h
PD1 antibody treated group
PBMC without stimulation
untreated group
PBMC stimulation for 72h
untreated group
HumAb713-7
HumAb747V-67
FIT107-1-7b-1
positive in CD3
con. (nM)